open-field activity and running wheel activity in offspring after perinatal NP exposure. Latendresse et al. (2001) reported that the intake of a sodium solution was increased in offspring perinatally treated with NP (2,000 ppm in diet; > 200 mg/kg/day), but instead of focusing on CNS alterations, the authors' focus was the relationship between this increased intake and the renal toxicity of NP. However, the possibility remains that NP at a much lower (≤ 10 mg/kg/day) dose alters certain cognitive functions and/or fine behavioral characteristics, including the response to fear-provoking stimuli, but without being associated with general motor dysfunction.

In the present study, we examined the adverse effects of low-dose (0.1 mg/kg/day) perinatal BPA or NP exposure on behavioral characteristics. To this end, we performed a series of behavioral tests: an open-field test, a measurement of spontaneous activity during a dark phase, a step-through passive avoidance test, an elevated plus-maze test, and a two-way shuttle-box avoidance test. In addition, in order to evaluate suspected alterations in the monoaminergic system, we investigated behavioral responses to tranylcypromine (Tcy), a monoamine oxidase inhibitor.

Materials and Methods

Animals and treatments. Male and female F344/N rats were purchased from SLC (Sizuoka, Japan). The animals were maintained under controlled temperature (24 ± 1°C) and humidity (55 ± 5%), on a 12-hr light (09:00–21:00 hr):12-hr dark (21:00–09:00 hr) cycle. Food and water were freely available. After acclimatization for 1 week, female rats were placed with males. Vaginal smears were examined daily: a sperm-positive smear determined gestational day (GD)0. After detection, the pregnant dams were housed individually and were randomly assigned to an exposure condition (n = 10–11/condition). The dams were orally exposed to BPA (0.1 mg/kg/day; Tokyo Kasei Kogyo, Tokyo, Japan) or NP (0.1 or 10 mg/kg/day; Tokyo Kasei Kogyo) dissolved in corn oil, or to corn oil alone (vehicle control; 2 mL/kg/day) from GD3 until postnatal day (PND)20. Oral administrations of BPA and NP were performed by gavage. Because animals were trained to receive the feeding needle before mating, this procedure was not stressful. The dams were examined for clinical signs of toxicity and were weighed daily before dosing. After parturition (PND0), the pups were counted, weighed, and assigned to groups of six pups per litter, maintaining equivalent sex distributions when possible. Pups remained with their biological mother. Offspring were weighed and the body weights recorded on PND0, 3, 7, 10, 14, and 21 and again at 8 and 13 weeks of age. We included the mean weight of each littermate in the statistical analysis. Male pups were marked with ink for identification; On PND21, the marked male pups were gathered from different litters and housed together according to treatment group (7–8/cage). The dams were anesthetized with diethyl ether and then sacrificed by exsanguination; the body weights and organ weights (liver, kidney, spleen, and thymus) were then recorded.

We randomly selected one male pup per litter to undergo a series of behavioral tests (n = 9–10/group). The remaining male pups in the litter were subjected to the measurement of organ weights (liver, kidney, spleen, thymus, brain, and testis) at weaning (PND21) or at 8 weeks of age. Although male rats were usually housed in groups according to experimental treatment, rats were housed individually for some behavioral tests. At the end of each behavioral test, rats were again housed in a group according to treatment. In this study, we excluded female pups from behavioral tests because the estrous cycle in mature females affects various behavioral characteristics. When using female animals, it is important to consider the estrous cycle in evaluating the results of behavioral tests that require several consecutive days. This study was approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, University of Tokyo.

Open-field behavior test. At 8 weeks of age, animals were subjected to an open-field test. Each subject was housed individually for 24 hr before the test. The open-field apparatus was a rectangular field (56 × 39 cm); none of the animals was familiar with this apparatus. Open-field behavior was recorded for 5 min by a video camera positioned above the apparatus during the dark phase (21:30–23:00 hr) under a low white light; responses were automatically analyzed by a computer-assisted system, which classified observed behavior as locomotion, rearing, or "other" behaviors.

Spontaneous motor activity. We measured spontaneous motor activity in 12-week-old male offspring using a Supermex (Muromachi Kikai, Tokyo, Japan) (Masuo et al. 1997). The Supermex consisted of a sensor monitor, which was mounted above the cage to detect changes in heat across multiple zones of the cage through an array of Fresnel lenses. The body heat radiated by the animal was detected with the sensor head of the monitor, which contained paired infrared pyroelectric detectors. In this manner, the system allowed the monitoring and counting of all spontaneous movements. Each animal was housed individually in the experimental cage—a differently arranged housing cage with food and water freely available—for 24 hr before the measurement to become accustomed to this experimental condition; spontaneous activity was then measured for about 12 hr (starting at 21:00 hr). All counts were automatically totaled and recorded in 2-min intervals. We defined "immobile time" as 2 min with no signal (count = 0).

Passive avoidance test. We conducted the step-through passive avoidance test when the animals were 13 weeks of age. The test was carried out during the light phase (13:00–17:00 hr), and each animal was housed individually during the test. The passive avoidance apparatus consisted of light and dark compartments. The first time each animal was placed in this apparatus, an electric foot shock (0.25 mA, 3 sec) was delivered to the animal through the grid floor just after the animal had completely left the light compartment for the dark compartment. We recorded the latency period required before each animal entered the dark compartment after having been placed in the light compartment. Twenty-four hours later, a retention trial (with no shock) was performed, and the latency period before entering the dark compartment was recorded. In addition to the traditional measure, we recorded the frequency and percentage of duration of poking into the dark compartment until the animal completely entered the dark compartment in the retention trial. If an animal failed to enter the dark compartment within 20 min, the test was terminated.

Elevated plus-maze test. The elevated plus-maze apparatus consisted of two open arms (50 × 10 cm) and two closed arms (50 × 10 cm, with 50-cm high walls) extending from a central square platform (10 × 10 cm); arms were arranged so that those of the same type were opposite each other. The apparatus was elevated 60 cm above the floor. At 14 weeks of age, each animal was placed in the central square facing an open arm during the dark phase (21:30–23:00 hr). We then recorded standard spatiotemporal factors for 5 min (i.e., the frequency of entries into the open arms and the closed arms was recorded, whereby "arm entry" was defined as moving the head into an open arm).

Active avoidance test. At 15 weeks of age, animals were subjected to an active avoidance test in a two-way shuttle-box (Muromachi Kikai, Tokyo, Japan) consisting of two compartments connected to each other by a hole in the wall; this test was carried out during the light phase (13:00–17:00 hr). Each animal was housed individually through the active avoidance test. Each animal was allowed to become accustomed to the shuttle-box apparatus for 5 min before every session; the animal was then subjected to 25 daily trials/session of avoidance conditioning in four consecutive sessions (acquisition test). For each trial, a 5-sec conditioned stimulus (CS), consisting of a buzzer and light, was followed by a 5-sec unconditioned stimulus (UCS), which included a scrambled shock of 0.2 mA delivered through the floor grid. In addition, on the day after the

fourth session, each rat performed the extinction test, which is basically the avoidance text without the UCS. Each trial was separated by variable intertrial intervals (10–90 sec between trials; total of 1,250 sec/session). During the acquisition tests in sessions 1–4 and the extinction test, we recorded the percentage of correct avoidance responses, in which the animals moved to the other compartment of the shuttle box within a 5-sec CS in each block of 25 trials. To evaluate further behavioral characteristics in this procedure, we recorded the percentage of failures to avoid the stimulus within 5-sec UCS and the latency periods associated with both the CS and UCS throughout the four acquisition sessions.

Monoamine-disruption test. Disturbances of the monoaminergic system in the CNS were induced by a single intraperitoneal (i.p.) injection of Tcy (*trans*-2-phenylcyclopropyl-amine hydrochloride; Sigma-Aldrich, St. Louis, MO, USA). At 22–24 weeks of age, BPA- or NP-treated male offspring were subjected to the monoamine-disruption test. Before the monoamine-disruption tests, we determined the optimal dose of Tcy for the monoamine-disruption test in a different set of male F344 rats (n = 15) at 9 weeks of age. We injected (i.p.) Tcy solution in 0.9% saline at 0, 2, 5, and 10 mg/mL (1 mL/kg) at 16:00 hr (n = 4, 4, 4, and 3, respectively) and then measured spontaneous motor activity as described above. We confirmed that animals treated with 5 mg/kg Tcy showed a high increment of activity at 21:30 hr, when open-field behavior was recorded.

Saline challenge. On the first day of the monoamine-disruption test, we injected 0.9% saline (1 mL/kg; i.p.) as a vehicle into each rat; 5.5 hr after the injection, we observed and recorded the behavior of the animals in the open-field apparatus for 4 min.

Tcy challenge. On the day after the saline challenge, we injected 5 mg/kg Tcy (i.p.) into the same animal and recorded open-field behavior for 4 min, as described for the saline challenge. Behavioral analyses in the monoamine-disruption test were performed as described for the open-field test.

Statistical analyses. We conducted statistical analyses using StatView, Version 5.0 (SAS Institute, Cary, NC, USA). We analyzed the effects of perinatal BPA or NP exposure on maternal body weight increase and the body weight of male offspring by analysis of variance (ANOVA) with one between-subject factor (treatment) and one repeated-measures factor (days). The number of total, male, and female pups;, the organ weights of dams at weaning; and the organ weights of male offspring at PND21 and at 8 weeks of age were analyzed by one-way ANOVA. Behavioral measurements were analyzed by one-way ANOVA, except for the percentages of correct avoidance in the shuttle-box avoidance test, which were assessed by repeated measures of ANOVA over days (sessions). In the analysis of latency in the passive avoidance test, data processed through logarithmic transformations were used for the ANOVA because of their significantly inappropriate distributions with respect to the normal distributions. In each statistical analysis, the effects of BPA and NP exposure were analyzed with respect to the control in the same ANOVA. When the ANOVA produced significant results, we then performed the post hoc Fisher's protected least-significant difference test for comparisons between groups. The significance level for all tests was set at $p < 0.05$.

Results

Maternal toxicity and reproductive results. Oral exposure to BPA or NP showed no statistically significant effect on maternal body weight increase during pregnancy and lactation or on the number of total, male, and female pups (data not shown). All dams in this study delivered their offspring on GD22. There was no significant effect of 40-day exposure to BPA or NP on either body weight or organ weights (data not shown).

Development of male offspring. Perinatal exposure to BPA or NP had no significant effect on either body weight gain or organ weights of male offspring on PND21 or at 8 weeks of age (data not shown). No male offspring died during the course of the study (> 25 weeks of age).

Open-field test. In the open-field test, neither BPA nor NP exposure significantly affected the percentage of locomotion [$F_{(3,32)}$ = 0.271, $p > 0.5$] or the number of rearings [$F_{(3,32)}$ = 0.189, $p > 0.5$; data not shown].

Spontaneous motor activity. To assess general motor activity under nonstress conditions, we recorded spontaneous motor activity of male offspring. Neither BPA nor NP exposure had any effect on the rhythm of activity, the total counts of activity [$F_{(3,32)}$ = 0.554, $p > 0.5$], or the immobile time [$F_{(3,35)}$ = 0.078, $p > 0.5$] during the 12-hr dark phase (data not shown).

Passive avoidance test. On shock-presenting day of the passive avoidance test, the subjects readily entered the dark compartment (< 30 sec). During the retention trial 24 hr after shock presentation, ANOVA [$F_{(7,63)}$ = 12.174, $p < 0.001$] and multiple comparisons revealed that the subjects showed significant hesitation ($p < 0.01$) to enter the dark compartment compared with the short latency during shock presentation in all of the experimental groups (Figure 1A). Although both BPA- and NP-treated groups tended to remain in the light compartment longer than the control offspring, there was no significant difference in latency periods during the retention trial among the experimental groups. Neither the frequency of poking into the dark [$F_{(3,28)}$ = 1.166, $p > 0.1$; Figure 1B] nor the percentage of duration of poking into the dark [$F_{(3,28)}$ = 1.919, $p > 0.1$; Figure 1C] during the retention trial was affected by chemical exposure.

Elevated plus-maze test. Neither BPA nor NP exposure significantly altered the frequency of entering the open arms [$F_{(3,27)}$ = 0.571, $p > 0.5$] or the closed arms [$F_{(3,27)}$ = 0.139, $p > 0.5$] of the elevated plus-maze test, although the frequency of entering the open arms was slightly higher in the BPA-treated group than in the controls (data not shown).

Active avoidance test. BPA and low-dose NP exposure significantly affected the avoidance responses of the male offspring in the active avoidance test. Repeated-measures one-way ANOVA showed a significant effect of chemical exposure [$F_{(3,35)}$ = 5.724, $p < 0.01$] and number of sessions [$F_{(3,105)}$ = 107.322, $p < 0.0001$], as well as a significant interaction between chemical exposure and the number of sessions [$F_{(9,105)}$ = 3.536, $p < 0.001$]. One-way ANOVAs and post hoc multiple comparisons for each session indicated significantly fewer avoidance responses in BPA-treated offspring at the first, second, and third sessions than in the control offspring (Figure 2A). Low-dose NP-treated offspring showed a lower avoidance

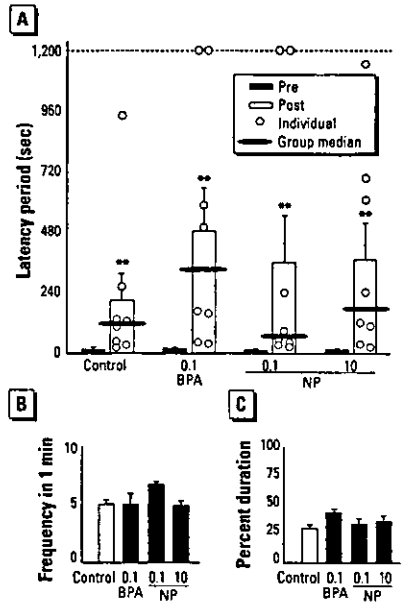


Figure 1. Effect of perinatal exposure (mean ± SE) to BPA or NP (mg/kg/day) on the behavioral characteristics in a passive avoidance test (n = 8/group). Abbreviations: Post, latency during the retention trial; Pre, latency on shock-presenting day. (*A*) The latency period until the animals completely entered the dark compartment. (*B*) The frequency of poking into a dark box until complete entrance. (*C*) The percent duration of poking into a dark box until complete entrance.
**$p < 0.01$ compared with Pre for same treatment.

rate than the control offspring, but only in the first session (Figure 2B). In the fifth extinction session (i.e., without electrical shocks as the UCS), the BPA- and NP-treated offspring showed slightly less correct avoidance behavior than the control offspring, although the effect of chemical exposure, as determined by one-way ANOVA, was not statistically significant [$F_{(3,35)} = 0.571$, $p = 0.104$]. One-way ANOVA indicated a significant effect of chemical exposure and that the frequency of failure of avoidance within 5 sec of shock presentation—in which one-way ANOVA indicated a significant effect of chemical exposure [$F_{(3,35)} = 3.700$, $p < 0.05$]—in BPA-treated offspring was significantly higher ($p < 0.001$) than that in the control offspring; low-dose NP-treated offspring showed a similar tendency (Figure 2C). We found no significant effect of chemical exposure on the mean of the latency periods associated with CS [$F_{(3,35)} = 0.722$, $p > 0.5$; Figure 2D] and UCS [$F_{(3,35)} = 1.186$, $p > 0.1$; Figure 2E] in 100 trials of four sessions.

Monoamine-disruption test. Tcy injection led to a large, slow increase in motor activity at 5 and 10 mg/kg compared with the saline control, although 2 mg/kg Tcy did not induce an increase (Figure 3A). We confirmed that the animals administered 5 mg/kg Tcy showed a high increase of activity 5.5 hr after injection, which corresponded to the schedule for the monoamine-disruption test. One-way ANOVA and post hoc tests about locomotion behavior [$F_{(7,58)} = 2.498$, $p < 0.05$] and the number of rearing behaviors [$F_{(7,58)} = 9.629$, $p < 0.01$] yielded the following results. In the monoamine-disruption test, control and high-dose NP-treated offspring showed a significant increase in locomotion behavior resulting from Tcy injection ($p < 0.01$; Figure 3B). However, BPA-treated or low-dose NP-treated offspring failed to show a clear increase in locomotion ($p > 0.1$). Tcy also caused significant decreases in the number of rearing behaviors in all experimental groups (Figure 3C) in the monoamine-disruption test. When only saline was administered, low-dose NP-treated offspring showed a significantly increased number of rearing behaviors, compared with those of the control offspring in the saline challenge; in addition, the BPA-treated offspring also appeared to have a similar tendency ($p < 0.1$), which was abolished by monoamine disruption by Tcy (Figure 3C).

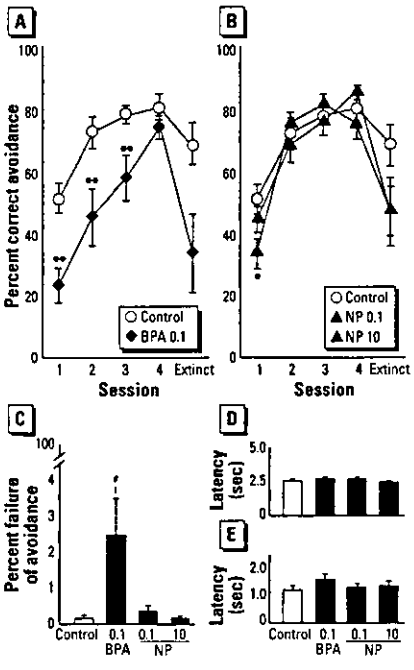


Figure 2. Effects of perinatal exposure to BPA or NP (mg/kg/day) on behavioral characteristics in a shuttle-box avoidance test (mean ± SE; n = 9–10/group). Avoidance learning curves of male offspring perinatally exposed to BPA (*A*) or NP (*B*). (*C*) Percentage of failure of avoidance when an electrical shock was presented for 5 sec among 100 trials of four sessions. Length of latency period associated with a CS (*D*) and a UCS (*E*) in four sessions.
*$p < 0.05$; **$p < 0.01$; and $^{\#}p < 0.001$ compared with control.

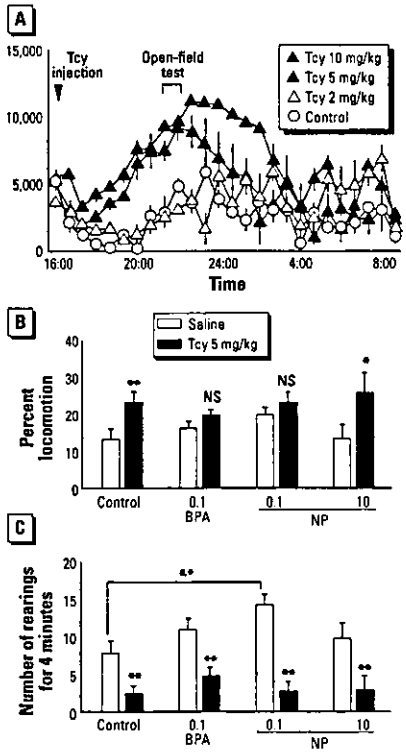


Figure 3. Effects of perinatal exposure to BPA or NP (mg/kg/day) on behavior in the monoamine-disruption test 5.5 hr after Tcy treatment. NS, not significant. (*A*) Locomotor activity (mean ± SE) after a single injection with 2, 5, or 10 mg/kg/day Tcy (n = 3–4/group). (*B*) Effect of perinatal BPA and NP on Tcy (5 mg/kg)-induced increases in locomotion behavior in an open-field apparatus (mean ± SE; n = 7–9/group). (*C*) Effects of perinatal BPA and NP on Tcy (5 mg/kg)-induced suppression of rearing in an open-field apparatus (mean ± SE; n = 7–9/group).
$^{\#}$The bracket indicates the comparison of offspring exposed to BPA and low-dose NP in the saline challenge. *$p < 0.05$. **$p < 0.01$ compared with saline control.

Discussion

In the present study, we carried out a series of behavioral tests and demonstrated the subtle and complex functional effects of perinatal exposure to BPA and NP on the behavior of male rat offspring. Behavioral alterations by perinatal exposure to BPA and NP were detected only in specific challenges involving fear-provoking stimuli and pharmacologic disruption of monoaminergic system, whereas spontaneous explorative behavior and responses to novelty were not affected by the exposure to these chemicals.

Evaluation of the toxicity of BPA as well as that of NP on maternal body weight, parturition, maternal organ weights at weaning (PND21), and general development (body weight and organ weights) of the offspring confirmed that there were no adverse effects of BPA at 0.1 mg/kg/day or of NP at 0.1 and 10 mg/kg/day, which was consistent with the findings of previous reports (Ferguson et al. 2000; Kwon et al. 2000).

In the present study, we detected no statistically significant alterations by BPA or NP in the spontaneous activity and behaviors of rats in the open-field test at 8 weeks of age and in the elevated plus-maze test at 14 weeks of age. This suggests that these chemical exposures induce no severe abnormalities in general behavior.

In the passive avoidance test, offspring perinatally exposed to BPA or NP seemed to be more sensitive to fear-inducing shock than were the control offspring, which might have led to the somewhat stronger retention in the chemical-treated groups; however, such changes were statistically ambiguous because of the large individual differences in the experimental conditions used in this study.

In the active avoidance test, BPA and low-dose NP showed clear or partial adverse effects on behavior, respectively. In particular, BPA-treated offspring may have been less able to learn than the control offspring in terms of causality. Low-dose NP-treated offspring were also affected to some extent. Although the possibility remains that BPA-treated offspring were more insensitive to electrical shock than were the control offspring, the slight elongation of the latency period in the passive avoidance test would have excluded this possibility. If BPA-treated offspring had found the electrical stimuli less fear-provoking and/or painful, they would have entered the dark compartment more quickly than the control offspring in the passive avoidance test. It would also be unlikely that less learning took place as a result of motor dysfunction or sensory abnormality

because there was no alteration in the spontaneous activity during the dark phase and in the duration of locomotion in the open-field test, as well as in the latency periods associated with CS and UCS in the active avoidance test. When electrical shock was presented as a UCS, BPA-treated offspring failed to enter the opposite compartment within 5 sec more frequently than the control offspring, and NP-treated offspring showed a similar tendency. BPA-treated offspring tended to stiffen in the corner of the box during the UCS, and these animals appeared to stop avoiding the UCS more often than did the control offspring, as determined by direct observation (data not shown). It is possible that excessive fear of the UCS would interfere with the smooth progression of avoidance learning. Perinatal BPA exposure may render male offspring exceedingly vulnerable to intolerable levels of fear. Interestingly, this hypothesis may be supported by a previous study (Aloisi et al. 2002), which indicated that perinatal BPA exposure increased the sensitivity of the central neural pathways for nociception in male offspring. Farabollini and colleagues reported the details of various behavioral changes observed due to perinatal exposure to BPA at 0.04 or 0.4 mg/kg/day in rats (Adriani et al. 2003; Aloisi et al. 2002; Dessi-Fulgheri et al. 2002; Farabollini et al. 1999, 2002). In the present study we also provided new evidence of the behavioral adverse effects of perinatal exposure to BPA at a low dose of 0.1 mg/kg/day on electrical UCS-related responses in an active avoidance test.

In the monoamine-disruption test, Tcy-induced increases in locomotion were significantly less marked in BPA-treated and low-dose NP-treated offspring, but not in the high-dose NP-treated offspring, compared with the control offspring. This is the first study reporting behavioral alterations due to perinatal BPA and NP exposure shown by responses to the disruption of monoaminergic systems, with Tcy having clear and straightforward pharmacologic effects as a monoamine oxidase inhibitor. Previous studies have reported changes after BPA exposure in a mouse model of psychostimulant abuse (methamphetamine) (Suzuki et al. 2003) and in a rat model of an increment in activity by amphetamine (Adriani et al. 2003), which have complicated pharmacologic effects on CNS function. It is possible that BPA-treated and low-dose NP-treated offspring might be insensitive to monoamines overflowing in excess into the extrasynaptic space. Such animals might show abnormal expressions of each type of monoamine receptor (dopamine, serotonin, and noradrenaline receptors, including the subtypes of each receptor class) or monoamine oxidase in certain region(s) of the CNS. Further investigations considering each of the monoamergic systems (dopaminergic, serotoninergic, and noradrenalinergic) are likely to produce more insight into the mechanism of traces induced by perinatal BPA and NP exposure in the CNS. Although monoamine disruption by Tcy significantly reduced the number of rearing behaviors, the response to Tcy was not influenced by perinatal chemical exposure. There was a discrepancy between the results of behavior in the open-field apparatus at 8 weeks of age and > 22 weeks of age. When the animals were > 22 weeks of age, the observed significant increase in the number of rearing behaviors suggested that low-dose NP-treated offspring might have experienced less anxiety than the control offspring in the open-field apparatus. The results furthermore suggested that these behavioral alterations caused by perinatal low-dose NP exposure might appear only at a stage of advanced age, that is, at a time when rats are relatively slow in their movements and rarely show rearing compared with juveniles. Further investigations will be required in this regard. In any case, the effective dose of NP from a neurobehavioral standpoint is much lower than the dose associated with general physical toxicity, as observed in the case of BPA in our previous study of that substance (Negishi et al. 2003b).

We cannot address differences in sexes in the present study because we limited this behavioral study to the male offspring; our primary goal was to detect behavioral alteration by perinatal BPA exposure in male offspring. However, studies in rats by Kubo et al. (2001, 2003) have demonstrated that perinatal exposure to BPA removed the differences between the sexes in the volume of locus ceruleus and in behavioral characteristics. In addition, some studies have reported sex differences in the effects of BPA (Dessi-Fulgheri et al. 2002; Farabollini et al. 1999, 2002). Further experiments using the active avoidance test and the monoamine-disruption test on both male and female offspring would be informative.

In the present study, one animal per litter sequentially underwent all of the behavioral tests. It is possible that an experience in an earlier behavioral test influenced the results of the subsequent behavioral tests. For example, a painful experience immediately after exploratory behavior in the passive avoidance test might interfere with the behavioral propensity in the elevated plus-maze test. However, we believed that using sequential behavioral tests in the same animal would not obstruct the evaluation of effects of perinatal exposure to chemicals because all of the animals in the four treatment groups experienced the stimuli.

In summary, perinatal exposure to BPA and NP, both at 0.1 mg/kg/day, affected the extent of shock-related behavior and affected responses to the disruption of the monoaminergic system, although the direct mechanisms of these alterations remain unclear at present. Moreover, the neurobehavioral toxicity of both BPA and NP may be out of proportion with the *in vitro* and *in vivo* estrogenic potency of these compounds determined by certain simple assay systems. It may be useful to consider other potencies and/or metabolites of BPA and NP (Moriyama et al. 2002; Yoshihara et al. 2001) in addition to their weak estrogenic activity. We suggest that there may be a causal relationship between behavioral alterations in response to fear-provoking stimuli and abnormality in the monoaminergic system because both dopamine and serotonin play important roles in the processing of fear-provoking and/or stressful stimuli in the CNS (Gingrich 2002; Giorgi et al. 2003; Inoue et al. 1994). In our recent study using primary cultured neurons (Negishi et al. 2003a), BPA and NP inhibited staurosporine-induced neuronal cell death, interfering with caspase-3 activation. BPA and NP may, in this manner, disrupt programmed neuronal cell death during development, which would irreversibly lead to an abnormal neural network—including the monoaminergic system—and cause behavioral abnormalities in adulthood.

Conclusion

We conclude that perinatal BPA and NP exposure, even at slightly higher doses than those associated with environmental exposure in humans, had adverse behavioral effects on rats, especially when the animals were forced to avoid fear-provoking stimuli such as electrical shocks. Perinatal exposure to BPA and NP disrupted the reception of intolerable stress, which may be due to the alterations in monoaminergic system.

References

Adriani W, Seta DD, Dess-Fulgheri F, Farabollini F, Laviola G. 2003. Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to d-amphetamine in rats perinatally exposed to bisphenol A. Environ Health Perspect 111:395–401.

Aloisi AM, Della Seta D, Rendo C, Ceccarelli I, Scaramuzzino A, Farabollini F. 2002. Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats. Brain Res 937:1–7.

Dessi-Fulgheri F, Porrini S, Farabollini F. 2002. Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environ Health Perspect 110(suppl 3):403–407.

Farabollini F, Porrini S, Della Seta D, Bianchi F, Dessi-Fulgheri F. 2002. Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ Health Perspect 110(suppl 3):409–414.

Farabollini F, Porrini S, Dessi-Fulgherit F. 1999. Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol Biochem Behav 64:687–694.

Ferguson SA, Flynn KM, Delclos KB, Newbold RR. 2000. Maternal and offspring toxicity but few sexually dimorphic behavioral alterations result from nonylphenol exposure. Neurotoxicol Teratol 22:583–591.

Gaido KW, Leonard LS, Lovell S, Gould JC, Babai D, Portier CJ, et al. 1997. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Toxicol Appl Pharmacol 143:205–212.

Gingrich JA. 2002. Mutational analysis of the serotonergic system:

recent findings using knockout mice. Curr Drug Target CNS Neurol Disord 1:449–465.

Giorgi O, Piras G, Lecca D, Hansson S, Driscoll P, Corda MG. 2003. Differential neurochemical properties of central serotonergic transmission in Roman high- and low-avoidance rats. J Neurochem 86:422–431.

Inoue T, Tsuchiya K, Koyama T. 1994. Regional changes in dopamine and serotonin activation with various intensity of physical and psychological stress in the rat brain. Pharmacol Biochem Behav 49:911–920.

Kubo K, Arai O, Ogata R, Omura M, Hori T, Aou S. 2001. Exposure to bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behavior in the rat. Neurosci Lett 304:73–76.

Kubo K, Arai O, Omura M, Watanabe R, Ogata R, Aou S. 2003. Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci Res 45:345–356.

Kwon S, Stedman DB, Elswick BA, Cattley RC, Welsch F. 2000. Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. Toxicol Sci 55:399–406.

Latendresse JR, Newbold RR, Weis CC, Delclos KB. 2001. Polycystic kidney disease induced in F(1) Sprague-Dawley rats fed *para*-nonylphenol in a soy-free, casein-containing diet. Toxicol Sci 62:140–147.

Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 54:154–167.

Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, Lee K. 2003. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. Toxicol Sci 75:40–46.

Masuo Y, Matsumoto Y, Morita S, Noguchi J. 1997. A novel method for counting spontaneous motor activity in the rat. Brain Res Brain Res Protoc 1:321–326.

Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, et al. 2002. Thyroid hormone action is disrupted by bisphenol A as an antagonist. J Clin Endocrinol Metab 87:5185–5190.

Negishi T, Ishii Y, Kyuwa S, Kuroda Y, Yoshikawa Y. 2003a. Inhibition of staurosporine-induced neuronal cell death by bisphenol A and nonylphenol in primary cultured rat hippocampal and cortical neurons. Neurosci Lett 353:99–102.

Negishi T, Kawasaki K, Takatori A, Ishii Y, Kyuwa S, Kuroda Y, et al. 2003b. Effects of perinatal exposure to bisphenol A on the behavior of offspring in F344 rats. Environ Toxicol Pharmacol 14:99–108.

Palanza PL, Howdeshell KL, Parmigiani S, vom Saal FS. 2002. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ Health Perspect 110(suppl 3):415–422.

Schantz SL, Widholm JJ. 2001. Cognitive effects of endocrine-disrupting chemicals in animals. Environ Health Perspect 109:1197–1206.

Sohoni P, Sumpter JP. 1998. Several environmental oestrogens are also anti-androgens. J Endocrinol 158:327–339.

Staples CA, Dorn PB, Klecka GM, O'Block ST, Harris LR. 1998. A review of the environmental fate, effects, and exposures of bisphenol A. Chemosphere 36:2149–2173.

Suzuki T, Mizuo K, Nakazawa H, Funae Y, Fushiki S, Fukushima S, et al. 2003. Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state. Neuroscience 117:639–644.

White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. 1994. Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182.

Yoshihara S, Makishima M, Suzuki N, Ohta S. 2001. Metabolic activation of bisphenol A by rat liver S9 fraction. Toxicol Sci 62:221–227.

Neuropathology and Applied Neurobiology (2005), **31**, 170–180 doi: 10.1111/j.1365-2990.2004.00624.x

Age-related changes of intracellular Aβ in cynomolgus monkey brains

N. Kimura*‡, K. Yanagisawa†, K. Terao‡, F. Ono‡, I. Sakakibara‡, Y. Ishii*, S. Kyuwa* and Y. Yoshikawa*

**Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, †Department of Alzheimer's Disease Research, National Institute for Longevity Sciences National Center for Geriatrics and Gerontology, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, and ‡The Tsukuba Primate Center, National Institute of Infectious Diseases, 1 Hachimandai, Tsukuba-shi, Ibaraki 305-0843, Japan*

N. Kimura, K. Yanagisawa, K. Terao, F. Ono, I. Sakakibara, Y. Ishii, S. Kyuwa and Y. Yoshikawa (2005) *Neuropathology and Applied Neurobiology* 31, 170–180

Age-related changes of intracellular Aβ in cynomolgus monkey brains

To confirm the intracellular accumulation of amyloid β-protein (Aβ), we carefully performed immunohistochemistry using brains of cynomolgus monkeys of various ages. Cortical neurones and their large neurites were immunostained with antibodies against Aβ in young monkey brains. In aged monkey brains, intracellular Aβ localized within cortical neurones; no clear association was found between the presence of intracellular Aβ and senile plaques (SPs). Interestingly, we did not observe Aβ-immunoreactive cortical neurones in brains fixed with neutral buffered formalin. Western blot analyses of microsomal and nerve ending fractions derived from the brains of young to aged monkeys revealed that intracellular Aβ generation changed with age. In the microsomal fraction, the amount of Aβ42 significantly increased in brains from older monkeys (> 30 years of age), and the amount of Aβ43 significantly decreased with age in the microsomal fraction. The amount of Aβ40 remained the same regardless of age. Biochemical analyses also showed that intracellular levels of each of these Aβ molecules significantly increased with age in nerve ending fractions. As we previously observed that a similar accumulation of presenilin1, β-amyloid precursor protein (APP) and APP C-terminal fragment cleaved by β-secretase in the nerve ending fractions obtained from brains with SPs, the accumulation of intracellular Aβ in this fraction may be closely related to formation of spontaneous SPs with age. Taken together, these results suggest that intensive investigation of age-related changes in the nerve ending will contribute to a better understanding of the pathogenesis of age-related neurodegenerative disorders such as sporadic Alzheimer's disease.

Keywords: ageing, cynomolgus monkeys, intracellular Aβ, nerve ending fraction, paraformaldehyde, proteinase-K pretreatment

Introduction

Amyloid beta (Aβ) peptide consists of 40–43 amino acids and is derived from β-amyloid precursor protein (APP) [1]. Aβ is the major protein component of senile plaques (SPs), a characteristic feature of Alzheimer's disease (AD) [2]. Mutation of the APP gene is one causative factor underlying early onset familial AD; such mutations cause increased accumulation of Aβ in the brain [3–5]. APP is sensitive to proteolysis by α-, β- and γ-secretases, and cleavage of APP by β- and γ-secretases leads to the generation of Aβ [6–8].

Recent studies show that intracellular accumulation of Aβ in neuronal cells, rather than extracellular accumulation of Aβ aggregates (e.g. SPs), plays a very important role in promoting Aβ pathogenesis [9–17]. In brains from aged cynomolgus monkeys, particularly in those from monkeys over 20 years old, SPs spontaneously form in the

Correspondence: Nobuyuki Kimura, The Tsukuba Primate Center, National Institute of Infectious Diseases, 1 Hachimandai, Tsukuba-shi, Ibaraki 305-0843, Japan. Tel: +81 0298 37 2121; Fax: +81 0298 37 0218; E-mail: kimura@nih.go.jp

neocortex; thus, the cynomolgus monkey is a useful animal model to investigate Aβ pathology [18]. We previously described in detail the age-related changes of the subcellular distribution of AD-related proteins, including presenilin1 (PS1), APP and APP C-terminal fragment (βCTF), a cleavage by-product of APP and β-secretase, in the brains of cynomolgus monkeys [19,20]. These AD-related proteins mainly accumulate in the nerve ending fraction. In the present study, we investigated the localization of intracellular Aβ in brains from young (4–8 years of age), adult (11–21 years of age) and aged (24–36 years of age) cynomolgus monkeys. Age-related changes in the subcellular distribution of intracellular Aβ were also biochemically assessed.

Materials and Methods

Animals

Twenty-seven cynomolgus monkey (*Macaca fascicularis*) brains were used in this study. Of these, 8 brains were from young monkeys (age: 4–8 years), 13 were from adult monkeys (age: 11–21 years), and 6 were from aged monkeys (age: 24–36 years). With the exception of two cases (cases 19, 20), the frontal, temporal and occipital lobes were used for immunohistochemical studies of intracellular Aβ in the neocortex. Occipital lobes of 10 cases (cases 1–4, 17–20, 25, 27) were used for Western blot analyses.

All brains were obtained from The Tsukuba Primate Center, National Institute of Infectious Diseases, Japan. All animals were housed in individual cages and maintained according to the National Institute of Infectious Disease rules and guidelines for experimental animal welfare. Only one monkey (case 27) died naturally. The remaining animals were deeply anaesthetized with pentobarbital. Tissue blocks were fixed (see below) and embedded in paraffin for sectioning. Table 1 summarizes the age and sex of the animals and indicates which cases were used for immunohistochemical or biochemical analyses.

Table 1. Cynomolgus monkeys used in the present study

Case	*Age (years)*	*Sex*	*SP*	*NFT*	*Experimental purpose*
1	4	M	ND	ND	IH and WB
2	4	M	ND	ND	IH and WB
3	4	M	ND	ND	IH and WB
4	4	F	ND	ND	IH and WB
5	7	M	ND	ND	IH
6	7	M	ND	ND	IH
7	8	F	ND	ND	IH
8	8	M	ND	ND	IH
9	11	F	ND	ND	IH
10	11	M	ND	ND	IH
11	17	F	ND	ND	IH
12	17	F	ND	ND	IH
13	17	M	ND	ND	IH
14	17	M	ND	ND	IH
15	20	F	ND	ND	IH
16	20	F	+	ND	IH
17	20	F	+	ND	IH and WB
18	21	M	+	ND	IH and WB
19	21	M	NT	NT	WB
20	21	M	NT	NT	WB
21	21	F	+	ND	IH
22	24	M	+	ND	IH
23	29	M	+	ND	IH
24	30	M	+	ND	IH
25	30	F	+	ND	IH and WB
26	35	F	+	ND	IH
27	36	M	+	ND	IH and WB

SP, senile plaques; NFT, neurofibrillary tangles; IH, immunohistochemistry; WB, Western blotting; M, male; F, female; +, detected; ND, not detected; NT, not tested.

Antibodies

For immunohistochemical investigations, a mouse monoclonal antibody against Aβ (4G8; Signet, Dedham, MA, USA) was used for detecting total Aβ. For double immunohistochemistry, a rabbit polyclonal antibody against Aβ40 (Ab40; IBL, Gunma, Japan) was used for detecting Aβ40; a rabbit polyclonal antibody against Aβ42 (Ab42; IBL, Gunma, Japan) was used for detecting Aβ42; and a rabbit polyclonal antibody against Aβ43 (Ab43; IBL, Gunma, Japan) was used for detecting Aβ43. As Ab40, Ab42 and Ab43 specifically react with the C-terminal amino acids of Aβ40, Aβ42 and Aβ43, respectively, we concluded that these three antibodies would be appropriate for detecting complete Aβ40, Aβ42 and Aβ43. A mouse monoclonal antibody against NeuN (Chemicon, Temecula, CA, USA) was used for specifically staining neurones. For Western blotting, 4G8, Ab40, Ab42 and Ab43 were used to assess age-related changes in the subcellular localization of total Aβ, Aβ40, Aβ42 and Aβ43, respectively.

Immunohistochemistry

Brain samples were fixed with 10% neutral buffered formalin or 4% paraformaldehyde and embedded in paraffin.

Sections were deparaffinized by pretreating with 0.5% periodic acid, followed by autoclaving for 5 min at 121°C. Sections were then incubated free floating in primary antibody solution overnight at 4°C. Primary antibody dilutions were: 4G8 (1:150). It is noteworthy to emphasize that we did not pretreat these sections with 99% formic acid. This point will be elaborated later in the Results section. After brief washes with buffer, the sections were sequentially incubated with either biotinylated goat antimouse, followed by streptavidin–biotin–horseradish peroxidase complex (sABC kit; DAKO, Denmark). Immunoreactive elements were visualized by treating the sections with 3-3′ diaminobenzidine tetroxide (Dojin Kagaku, Japan). The sections were then counterstained with haematoxylin.

For double immunohistochemistry, sections were deparaffinized by using the autoclaving method for 5 min at 121°C, then incubated free floating in primary antibody solutions containing NeuN (1:300) and Ab40 (1:200), Ab42 (1:200) or Ab43 (1:150) overnight at 4°C. Sections were then incubated with Alexa 488-conjugated goat antimouse IgG (1:500; Molecular Probes, Eugene, OR, USA) or Alexa 568-conjugated goat antirabbit IgG (1:500; Molecular Probes, Eugene, OR, USA) for 2 h at room temperature.

Subcellular fractionation of monkey brains

Subcellular fractions were prepared at 0–4°C from occipital lobes of 10 monkeys (cases 1–4, 17–20, 25, 27), as described by Tamai *et al.* [21]. Sucrose solutions were prepared in a buffer containing 10 mM Tris-HCl (pH 7.6), 0.25 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM endoplasmic reticulum (EDTA). Brain tissue (~1.0 g) was homogenized in a glass homogenizer with 10 ml of 0.32 M sucrose solution and then centrifuged at 1000 g for 10 min. The pellet (P1) was resuspended in 0.32 M sucrose, and this solution was layered over a discontinuous density gradient consisting of 1.2 M and 0.85 M sucrose solutions. The suspension was centrifuged at 75 000 g for 30 min in a Hitachi RPS-27 swing rotor (Hitachi, Tokyo, Japan) to separate out P1 myelin and nuclei fractions. The supernatant (S1) from the P1 fraction was centrifuged at 13 000 g for 15 min to yield the P2 fraction. The supernatant (S2) from the P2 fraction was centrifuged at 10 5000 g for 60 min to obtain the microsomal (pellet) and cytosol (supernatant) fractions. P2 was resuspended in 0.32 M sucrose, layered over a sucrose density gradient consisting of 1.2 M and 0.85 M sucrose solutions, and then centrifuged at 10 5000 g for 60 min to isolate the mitochondrial fraction. P2 myelin and synaptosome fractions separated as interface bands between the 0.32 M and 0.85 M sucrose layers and the 0.85 M and 1.2 M sucrose layers, respectively. The P1 and P2 myelin fractions were combined, diluted with ice-cold water, homogenized, and then centrifuged at 13 000 g for 15 min to sediment the myelin fraction. The P2 synaptosomal fraction was diluted in sucrose solution (final concentration: 0.85 M), layered over a solution of 1.2 M sucrose, and centrifuged at 75 000 g for 30 min. The purified nerve ending (synaptosome and synaptic plasma membrane) fraction separated as the interface between these two sucrose solutions.

Proteinase-K treatment

As the nerve ending fraction contained both synaptosomes and synaptic plasma membranes, some peptides or proteins associated with the exterior synaptic plasma membrane were included in this fraction. To rigorously investigate intracellular Aβ, each nerve ending fraction was pretreated with 20 μl/ml Proteinase-K (Pro-K, tritirachium alkaline proteinase; Wako, Osaka, Japan) to obtain pure synaptosomes. This treatment was aimed at excluding from our analysis contamination of extracellular peptides and proteins.

Immunoblotting

To normalize the loading variance and confirm the purity of the three subcellular fractions, we immunoblotted the fractions as described previously [19]. Immunoblots for calnexin and synaptophysin served as controls. Endoplasmic reticulum (ER) and vesicles in the microsomal fraction were examined with a rat monoclonal antibody against calnexin (Transformation Research Inc., Framingham, MA, USA). The nerve ending fraction pretreated with Pro-K was examined with a mouse monoclonal antibody against synaptophysin (DAKO, Denmark).

Western blot analyses

Western blot analyses were performed to assess age-related changes in the subcellular distribution of each Aβ

molecule. In cases 1–4, 17–20, 25 and 27, brain homogenates from the occipital lobes were fractionated by sucrose gradient centrifugation into microsomal and nerve ending fractions. The nerve ending fraction was pretreated with Pro-K as described above (see Proteinase-K Treatment). The proteins in each fraction were adjusted to 30 μg, and then each fraction was analysed by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE using 12.5% acrylamide gels). Separated proteins were blotted onto polyvinylidense fluoride membranes (Immobilon P; Millipore, Bedford, MA, USA).

The membranes were blocked with 5% nonfat dried milk in 20 mM PBS (pH 7.0) and 0.1% Tween-20 (Wako, Japan) overnight at 4°C, and then incubated with primary antibodies (4G8, 1:1000; Ab40, 1:1000; Ab42, 1:1000; Ab43, 1:1000) for 1 h at room temperature. They were then incubated with either horseradish peroxidase-conjugated goat antimouse IgG or mouse antirabbit IgG (1:6000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h at room temperature. Immunoreactive elements were visualized by using enhanced chemiluminescence (ECLplus, Amersham, UK).

Data analyses

To confirm the reproducibility of age-related changes in intracellular Aβ, immunoreactive bands obtained from the Western blots were quantified by using commercially available software (Quantity One; PDI, Inc., Upper Saddle River, NJ, USA). Data are shown as means ± SD. For statistical analyses, one-way ANOVAs were performed, followed by the Bonferroni/Dunn *post hoc* test.

Results

Immunohistochemistry

In brain sections fixed with paraformaldehyde, 4G8 immunostained intracellular Aβ in a granular pattern in the neocortex of cynomolgus monkeys (Figure 1a–d). 4G8 immunoreactivity was observed in neurones, regardless of the age of the animal (Figure 1a–d). In addition, there were no regional differences in the pattern of 4G8 immunostaining (i.e. intracellular Aβ). Moreover, besides staining cell bodies, 4G8 also immunostained large neurites of large pyramidal neurones, even in brains from young monkeys (Figure 1a,b).

Although 4G8 immunoreactivity was observed in some cortical neurones of aged monkeys (Figure 1c,d), this immunoreactivity apparently was not associated with the formation of SPs.

In contrast to brain sections fixed with paraformaldehyde, those fixed with neutral buffered formalin failed to show 4G8 immunoreactivity in cortical neurones of a young monkey. In a brain from an aged monkey, although 4G8 immunostained several cortical neurones, immunoreactivity was much weaker than that observed in sections from a brain fixed with paraformaldehyde (data not shown).

Preabsorption of 4G8 with a specific antigen peptide completely abolished 4G8 immunoreactivity (Figure 1e), thereby confirming the high specificity of 4G8 for Aβ. These preabsorption experiments also served as controls.

To confirm whether Aβ specifically localizes only within neurones (not glial cells), brain sections from young and aged monkeys were double-immunostained with the neurone-specific antibody, anti-NeuN, and with one of the anti-Aβ antibodies Ab40, Ab42, or Ab43. Sections used for double immunohistochemistry were from brain samples fixed with paraformaldehyde. Ab40, Ab42 and Ab43 immunostained NeuN-immunoreactive cells, even in sections from the brains of young monkeys (Figure 2a–i). Ab40 immunostained cortical neurones and large neurites of large pyramidal neurones, regardless of age (Figure 2a–c). Similarly, both Ab42 and Ab43 also immunostained cortical neurones and large neurites of monkeys regardless of age (Figure 2d–i).

Immunoblotting

In immunoblots of proteins from microsomal fractions, anticalnexin labelled a 95 kDa band representing ER and vesicles; immunostaining intensity was unchanged with age of the animal (data not shown). In immunoblots of proteins from nerve ending fractions treated with Pro-K (Pro-NE fraction), antisynaptophysin labelled a 38 kDa band likely to represent synaptic vesicles. The intensity of antisynaptophysin immunostaining remained constant across all age groups (data not shown).

Western blotting and data analysis

As 4G8 reacts with all forms of Aβ, we concluded that 4G8-immunoreactive bands represent the total amount of Aβ present in any given sample. We investigated age-

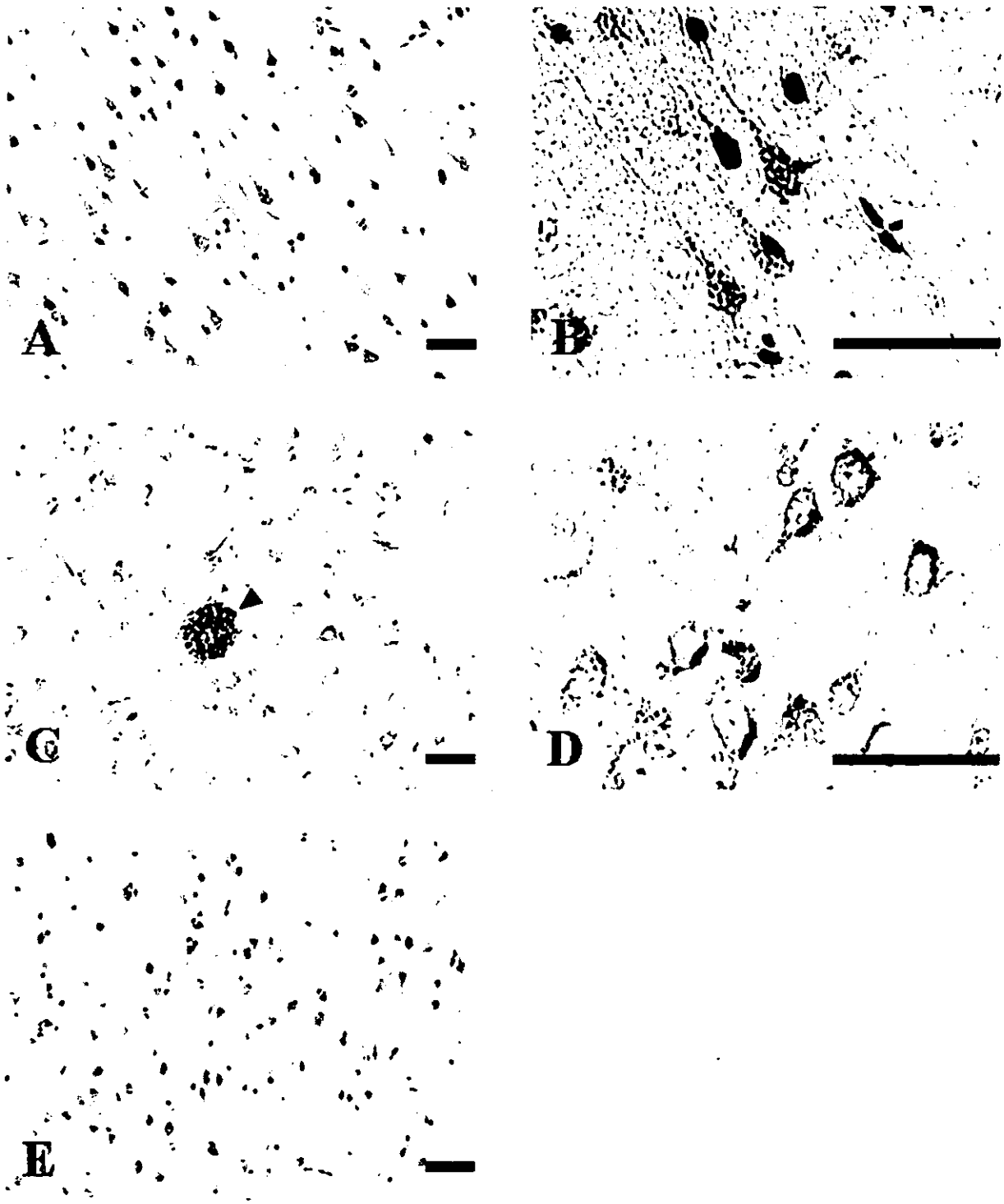


Figure 1. Photomicrographs of temporal lobe sections from a 4-year-old cynomolgus monkey (case 1; a, b, e) and a 36-year-old cynomolgus monkey (case 27; c, d). Sections were immunostained with the Aβ-specific antibody 4G8 and counterstained with haematoxylin. (a) In sections from the young monkey, 4G8 immunostained cortical neurones in a granular fashion. (b) Higher magnification view of section shown in Figure 1a. In the neocortex, 4G8 also immunostained large neurites of pyramidal neurones. (c) In sections from the aged monkey, 4G8 immunostained cortical neurones. Arrowhead: senile plaque. (d) Higher magnification view of the section shown in Figure 1c. Immunoreactive granules primarily localized to cortical neurones. (e) Preabsorption control. The section adjacent to the one shown in Figure 1a was incubated in primary antibody solution preabsorbed with Aβ. Scale bars = 50 μm.

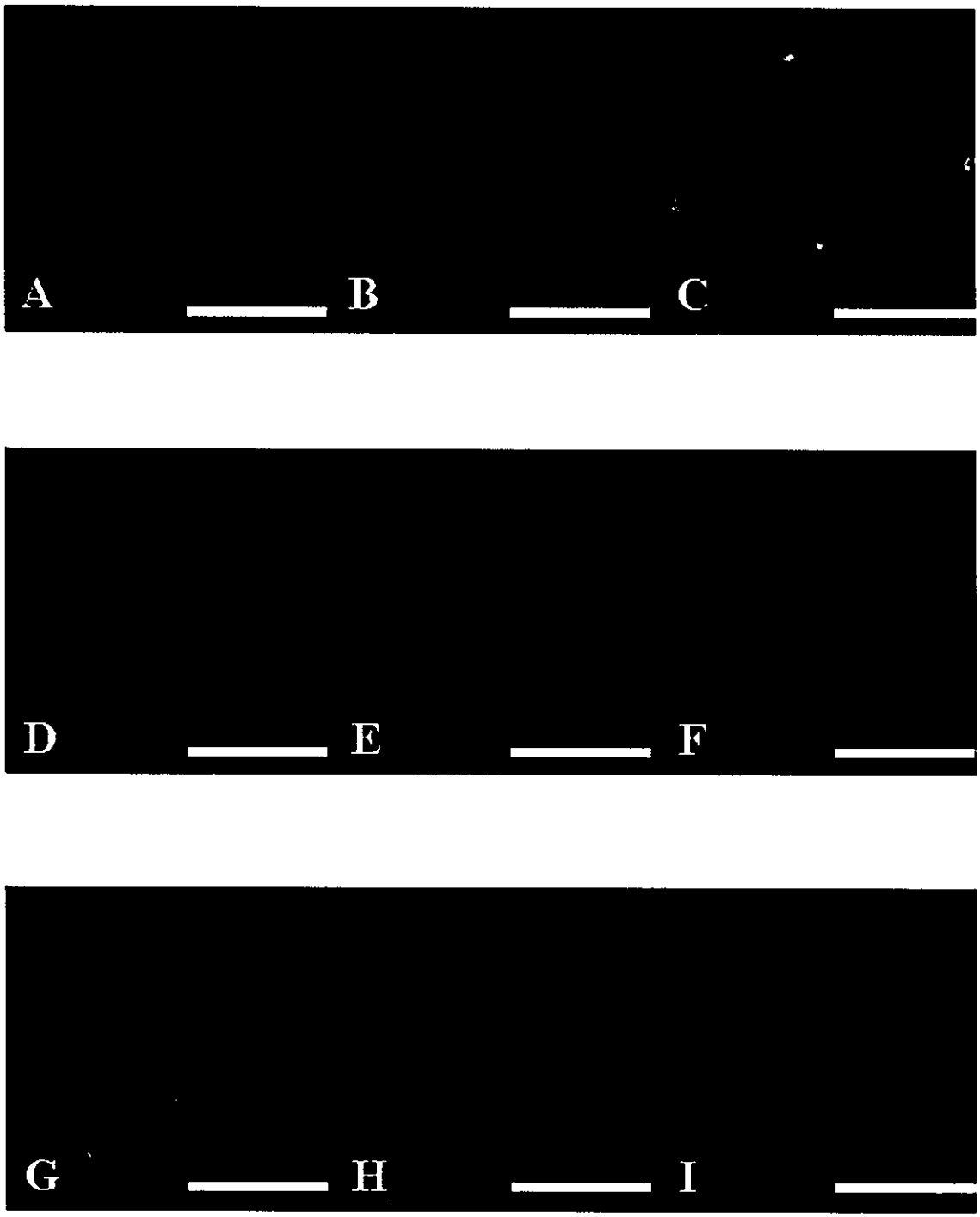


Figure 2. Photomicrographs of occipital lobe sections from a different 4-year-old monkey (case 4). Sections were double immunostained with NeuN and Ab40, Ab42 or Ab43. (**a–c**). Double immunostaining with NeuN (**a**) and Ab40 (**b**) reveal that Aβ40 localizes to neurones in the neocortex. (cf. panel **c**, merged image of **a** and **b**). (**d–f**) Double immunostaining with NeuN (**d**) and Ab42 (**e**) indicates that Aβ42 also localizes to neurones in the neocortex. (cf. panel **f**, merged image of **d** and **e**). (**g–i**) Double immunostaining with NeuN (**g**) and Ab43 (**h**) shows that Aβ43 similarly localizes to neurones in the neocortex. (cf. panel **i**, merged image of **g** and **h**). Scale bars = 50 μm.

related changes in the subcellular distribution of monomeric Aβ in the brain, because Aβ is produced as a monomer during APP cleavage, not as an aggregate [1,6–8]. This prompted us to evaluate the amount of monomeric Aβ in cynomolgus monkey brains.

In microsomal fractions, 4G8 immunostained a 4 kDa band representing total Aβ (Figure 3). The amount of total Aβ in the microsomal fraction increased with age, although not significantly (Figure 4a). Ab40, Ab42 and Ab43 also immunostained a 4 kDa band, representing Aβ40, Aβ42 and Aβ43, respectively (Figure 3). The amount of Aβ40 did not change much with age (Figure 4a). On the other hand, we found age-related changes of Aβ42 and Aβ43 in this fraction. The amount of Aβ42 in the microsomal fraction significantly increased with age; in contrast, the amount of Aβ43 significantly decreased with age (Figure 4a).

In Pro-NE fractions, all anti-Aβ antibodies also immunostained a 4 kDa band (Figure 3). In this fraction, the amount of total intracellular Aβ significantly increased with age (Figure 4b), and the magnitude of this increase tended to be greater than that observed in microsomal fractions (Figure 4). Intracellular Aβ40, Aβ42 and Aβ43 levels significantly increased with age, although the magnitude of the increase of intracellular Aβ43 tended to be smaller than that observed for Aβ40 and Aβ42 (Figure 4b). Intracellular Aβ40 levels increased about 3.7 times across age groups; intracellular Aβ42 increased about 5.0 times; and intracellular Aβ43 increased about 2.3 times (Figure 4b).

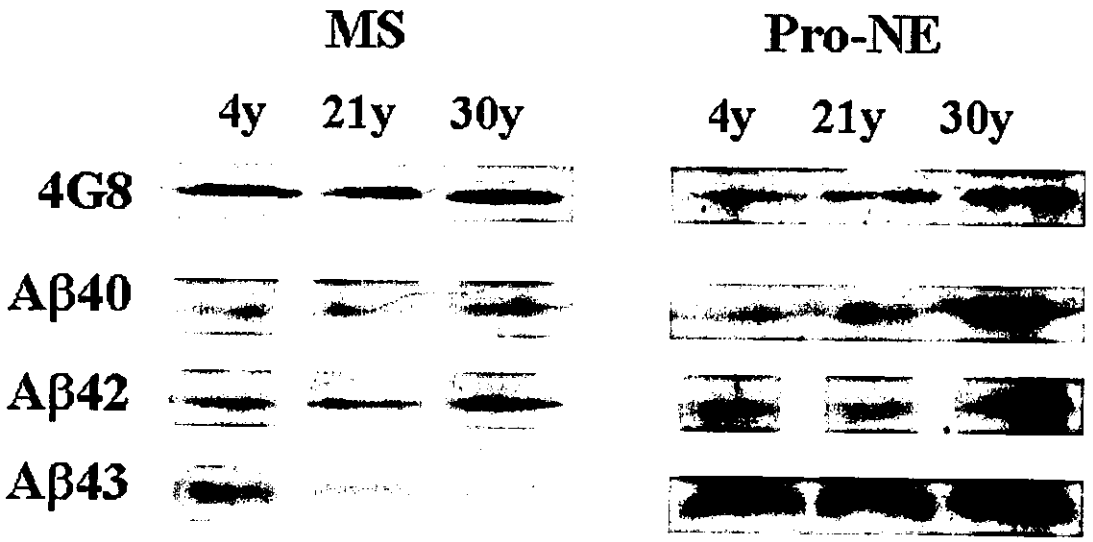
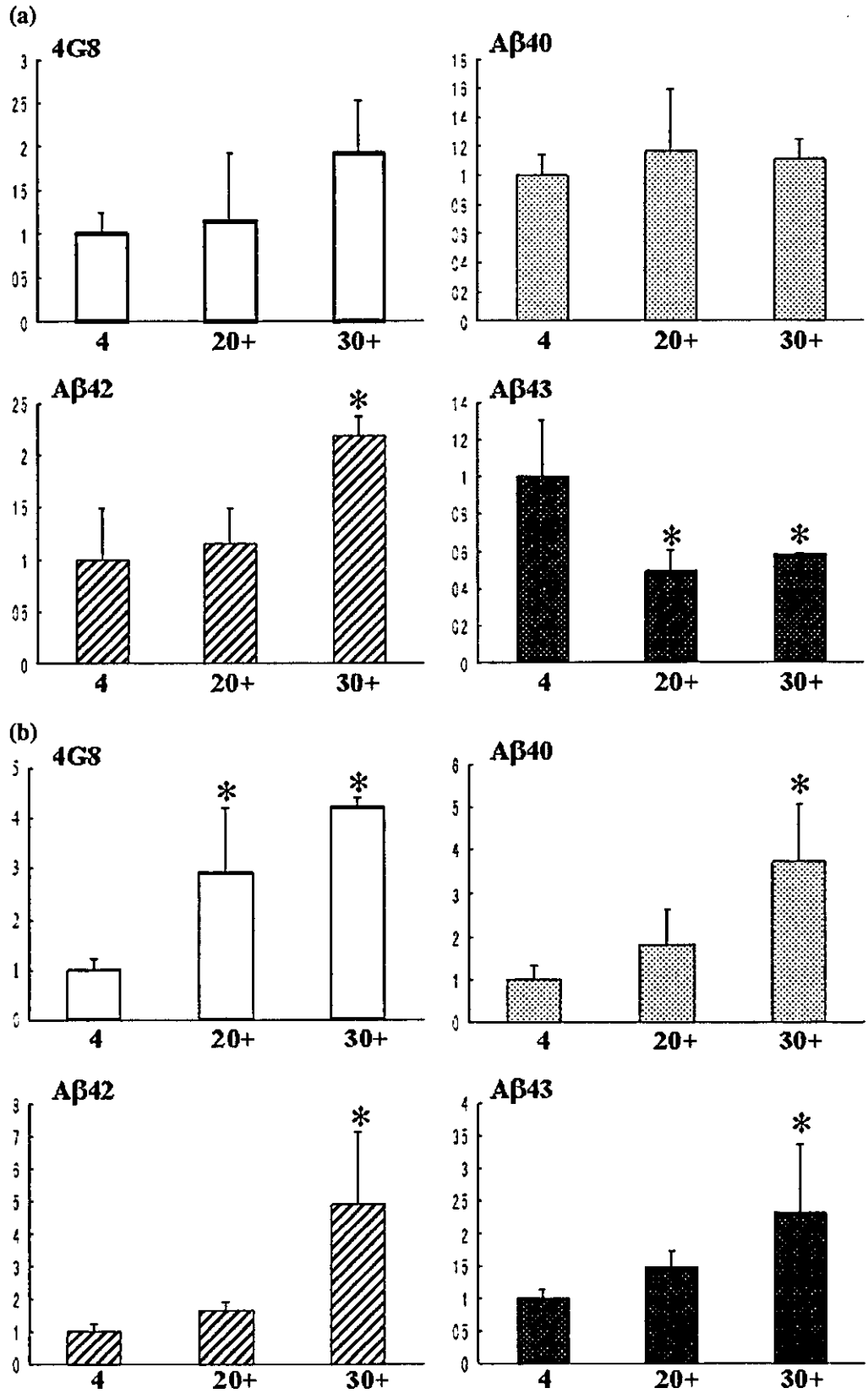


Figure 3. Western blots showing the expression of intracellular Aβ in microsomal and Pro-NE fractions from brains of various aged cynomolgus monkeys. The Aβ-specific antibody 4G8 immunostained a 4 kDa band representing total intracellular Aβ. Ab40, Ab42 and Ab43 immunostained 4 kDa bands representing intracellular Aβ40, Aβ42 and Aβ43, respectively. Lanes contained fractions derived from the brains of young, adult and aged monkeys, as follows: 4y, 4-year-old monkeys (case 1); 21y, 21-year-old (case 20); and 30y, 30-year-old (case 25). MS, microsome fraction; Pro-NE, nerve ending fraction pretreated with Pro-K.

Figure 4. Age-related changes in the amount of intracellular Aβ in microsomal and Pro-NE fractions from the brains of various aged cynomolgus monkeys. Proteins from each fraction were separated by SDS-PAGE and immunoblotted with appropriate antibodies. Immunoreactivity of the resulting bands was quantified, and data from the various fractions from old monkeys were compared to those from 4-year-old monkeys. $^*P < 0.02$. Y axes show the mean values of the quantified data, and those from 4-year-old monkeys were set as standards, respectively. (a) Analysis of intracellular Aβ in the microsomal fraction. The amount of total intracellular Aβ (4G8) in the microsomal fraction increased with advancing age. Although the amount of intracellular Aβ40 (Aβ40) in the microsomal fraction remained stable with age, the amount of intracellular Aβ42 (Aβ42) significantly increased and the amount of intracellular Aβ43 (Aβ43) significantly decreased with age. (b) Analysis of intracellular Aβ in the Pro-NE fraction. The amount of total intracellular Aβ (4G8) in the Pro-NE fraction significantly increased with age. The amounts of intracellular Aβ40, Aβ42 and Aβ43 (Aβ40, Aβ42 and Aβ43) also significantly increased with age. In the Pro-NE fraction, intracellular Aβ40 increased about 3.7 times with age, intracellular Aβ42 increased about 5 times, and intracellular Aβ43 increased about 2.3 times. Key: 4, 4-year-old monkey (cases 1–4, $n = 4$); 20+, 20- to 21-year-old (cases 17–20, $n = 4$); and 30+, 30- and 36-year-old monkeys (cases 25 and 27, $n = 2$).


(a)
4G8
Aβ40
Aβ42
Aβ43
(b)
4G8
Aβ40
Aβ42
Aβ43
4
20+
30+
*

Discussion

In the present study, immunohistochemical and biochemical methods were used to investigate the localization and age-related changes of intracellular Aβ in brains from young, adult and aged cynomolgus monkeys.

Recent studies have shown that intracellular Aβ is found only in the brains of aged individuals or brains of AD patients, but not in brains of nonhuman primates [9,10,12,16]. However, in the present study, we found intracellular Aβ in cortical neurones of monkeys of various ages (Figures 1 and 2). One possible technical explanation for this apparent discrepancy is that in previous studies, material came from brains fixed with neutral buffered formalin, whereas in the present study material was derived from brains fixed with paraformaldehyde [9,10,12,16]. For immunohistochemical studies we fixed brain samples, derived from the same monkeys, with either neutral buffered formalin or paraformaldehyde. Anti-Aβ antibodies failed to immunostain intracellular Aβ in cortical neurones from samples fixed with the formalin protocol (data not shown). On the other hand, all anti-Aβ antibodies successfully immunostained neurones from samples fixed with paraformaldehyde (Figures 1 and 2). It is widely known that the cross linkages in paraformaldehyde-fixed tissues are much weaker than those in tissues fixed with neutral buffered formalin, leading to a kind of fixation with the former that more effectively preserves tissues and cell surface antigens [22]. Thus, the antigens of intracellular Aβ would be preserved well and might be recognized by antibodies without formic acid treatment. It is also known that periodic acid pretreatment can improve immunoreactivity. These two factors may explain why we were able to detect intracellular Aβ even in brains from young monkeys.

In the brains of young monkeys, Aβ immunoreactivity was granular in nature. This diffuse pattern of immunoreactivity indicates that the intracellular Aβ in young monkeys may represent Aβ generated biologically in neurones, rather than accumulated Aβ that is typically found in neurones of aged animals (Figure 1a,b).

In the brains of aged monkeys, Aβ immunoreactivity in some cortical neurones tended to cluster together in clumps (Figure 1c,d). This clustering of immunoreactivity may represent Aβ accumulated in neurones and is consistent with previous hypotheses that Aβ accumulates in lipid rafts and that clustering of GM1 ganglioside within lipid raft-like membranes may facilitate the aggregation of Aβ (fibrils) in these rafts [23]. Although the pattern of Aβ immunostaining (i.e. intracellular Aβ) changed with age, we failed to find clear evidence that intracellular Aβ colocalizes with SPs.

In our previous studies, we described age-related changes of AD-related proteins in the cynomolgus monkey brain [19,20]. In the present study, we determined with biochemical methods whether intracellular Aβ accumulates with advancing age. In the microsomal fraction, the amount of total Aβ increased with age, whereas the amount of Aβ40 did not change with age (Figures 3 and 4a). Both Aβ42 and Aβ43, however, showed very interesting age-related changes. The amount of Aβ42 in the microsomal fraction increased significantly with age (Figures 3 and 4a). On the contrary, the amount of Aβ43 significantly decreased in this fraction (Figures 3 and 4a). This is the first study to show that the amounts of different Aβ molecules generated in the brain change with age of the individual. These results suggest that during ageing the cleavage of APP by γ-secretase may shift to generate more Aβ42 than Aβ43, which in turn may lead to increased accumulation of Aβ42 in the brain. Several studies have shown that Aβ42 is more closely associated with AD pathogenesis than is Aβ40 [24–27]. The age-related increase of Aβ42 observed in the present study supports these previous findings and is consistent with the hypothesis that increased Aβ42 generation or accumulation may promote, or even cause, sporadic AD pathogenesis in the aged brain.

As the nerve ending fraction prepared in the standard way contains both synaptic vesicles and synaptic plasma membranes, the amount of Aβ detected in this fraction may be extracellular Aβ associated with the outer part of the synaptic plasma membrane. To avoid potential contamination with extracellular Aβ, we pretreated each nerve ending fraction with Pro-K (this resulting fraction was termed the Pro-NE fraction). In the Pro-NE fraction, the amount of total intracellular Aβ significantly increased with age (Figures 3 and 4b). Our previous study showed that both full-length APP and βCTF accumulated in the nerve ending fraction with age [20]. It is noteworthy that AD-related proteins also significantly accumulate in the nerve ending fraction [19,20]. Thus, one possible explanation for this age-related accumulation of Aβ in the nerve ending fraction is that increased APP and βCTF in this fraction would subsequently cause intracellular Aβ to also accumulate in this fraction. Alternatively, the

age-related reduction of axonal transport to the soma for degradation may also cause the accumulation of these proteins, including Aβ, in the nerve ending fraction. Although the amounts of intracellular Aβ40, Aβ42 and Aβ43 significantly increased with age, the magnitude of increase varied for all three isoforms (Figures 3 and 4b). Of these three Aβ molecules, intracellular Aβ43 increased much less than did Aβ40 and Aβ42 (Figure 4b). This may explain why, in general, the generation of Aβ43 decreased with age (Figure 4a).

In summary, we found that intracellular Aβ exists in cortical neurones of monkeys as young as 4 years old, albeit in the form of diffuse granules. With advancing age (24–36 years old), however, intracellular Aβ tended to localize in neurones in the form of clusters or clumps (Figures 1 and 2). Our biochemical assays revealed that Aβ40 and Aβ43 were mainly generated in the brains of young monkeys. During ageing, the generation of intracellular Aβ shifted such that Aβ42 generation increased, while Aβ40 generation remained unchanged (Figures 3 and 4).

It is also noteworthy that the amount of intracellular Aβ increased in the nerve ending fraction with age (Figures 3 and 4), and this result closely parallels our findings with other AD-related proteins reported in our previous studies [19,20]. The accumulation of APP and βCTF in synaptosomes and/or synaptic plasma membranes may lead to Aβ overproduction in these compartments, and the accumulated intracellular Aβ, in turn, may be secreted extracellularly and accelerate SP formation. Thus, the nerve ending may be where SPs are initially formed or may be significantly associated with spontaneous SP formation that occurs with advancing age. Taken together, these results support the line of research that focuses in on age-related changes in the nerve ending. Findings from this line of research should reveal more details about the association between AD pathogenesis and other age-related neurodegenerative disorders.

Acknowledgements

The authors thank Y. Tamai and H. Kojima for their technical advice on processing brain samples for Western blotting. This study was supported by a grant-in-aid from Comprehensive Research on Ageing and Health, Ministry of Health and Welfare, Japan.

References

1 Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, Vigo-Pelfrey C, Lieberburg I, Selkoe DJ. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* 1992; **360**: 672–4
2 Glenner GG. Alzheimer's disease: its proteins and genes. *Cell* 1988; **52**: 307–8
3 Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, Goate A, Rossor M, Roques P, Hardy J, Mullan M. Early-onset Alzheimer's disease caused by mutations at codon 717 of the β-amyloid precursor protein gene. *Nature* 1991; **353**: 844–6
4 Murell J, Farlow M, Ghetti B, Benson MD. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 1991; **254**: 97–9
5 Scheuer D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe DJ, Younkin S. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nature Med* 1996; **2**: 864–70
6 De Strooper B, Saftig P, Craessaerts K, Vanderstichele H, Guhde G, Annaert W, Von Figura K, Van Leuven F. Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* 1998; **391**: 387–90
7 Wolfe MS, Xia W, Ostaszewski BL, Diehl TS, Taylor Kimberly W, Selkoe DJ. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. *Nature* 1999; **398**: 513–17
8 Xia X, Zhang J, Perez R, Koo EH, Selkoe DJ. Interactions between amyloid precursor protein and presenilins in mammalian cells. Implications for pathogenesis of Alzheimer's disease. *Proc Natl Acad Sci U S A* 1997; **94**: 8208–13
9 D'Andrea MR, Nagele RG, Wang H.-Y, Peterson PA, Lee DHS. Evidence that neurons accumulating amyloid can undergo lysis to form amyloid plaques in Alzheimer's disease. *Histopathology* 2001; **38**: 120–34
10 D'Andrea MR, Nagele RG, Wang H.-Y, Lee DHS. Consistent immunohistochemical detection of intracellular β-amyloid42 in pyramidal neurons of Alzheimer's disease entorhinal cortex. *Neurosci Lett* 2002; **333**: 163–6
11 Echeverria V, Cuello AC. Intracellular A-beta amyloid, a sign for worse things to come? *Mol Neurobiol* 2002; **26**: 299–316
12 Gouras GK, Tsai J, Naslund J, Vincent B, Edgar M, Checler F, Greenfield JP, Haroutunian V, Buxbaum JD, Xu H, Greengard P, Relkin NR. Intraneuronal Aβ42 accumulation in human brain. *Am J Pathol* 2000; **156**: 15–20
13 Klein WL, Krafft GA, Finch CE. Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? *Trends Neurosci* 2001; **24**: 219–24

14 Tabira T, Chui DH, Kuroda S. Significance of intracellular Abeta42 accumulation in Alzheimer's disease. *Front Biosci* 2002; 7: 44–9
15 Tabira T, de Chui H, Nakayama H, Kuroda S, Shibuya M. Alzheimer's disease with spastic paresis and cotton wool type plaques. *J Neurosci Res* 2002; 70: 367–72
16 Wang H.-Y, D'Andrea MR, Nagele RG. Cerebellar diffuse amyloid plaques are derived from dendritic Aβ42 accumulations in Purkinje cells. *Neurobiol Aging* 2002; 23: 213–23
17 Xu G, Gonzales V, Borchelt DR. Abeta deposition does not cause the aggregation of endogenous tau in transgenic mice. *Alzheimer Dis Assoc Disord* 2002; 16: 196–201
18 Nakamura S, Nakayama H, Goto N, Ono F, Sakakibara I, Yoshikawa Y. Histopathological studies of senile plaques and cerebral amyloidosis in cynomolgus monkeys. *J Med Primatol* 1998; 27: 244–52
19 Kimura N, Nakamura S, Honda T, Takashima A, Nakayama H, Ono, Sakakibara I, Doi K, Kawamura S, Yoshikawa Y. Age-related changes in the localization of presenilin-1 in cynomolgus monkey brain. *Brain Res* 2001; 922: 30–41
20 Kimura N, Tanemura K, Nakamura S, Takashima A, Ono F, Sakakibara I, Ishii Y, Kyuwa S, Yoshikawa Y. Age-related changes of Alzheimer's disease-associated proteins in cynomolgus monkey brains. *Biochem Biophys Res Comm* 2003; 310: 303–11
21 Tamai Y, Kojima H, Ohtani Y, Uchida K, Taguchi F, Kawaguchi T, Miura S, Tateishi J. Subcellular distribution of the transmissible agent in Creutzfeldt-Jakob disease mouse brain. *Microbiol Immunol* 1989; 33: 35–42
22 Smit JW, Meijer CJ, Decary F, Feltkamp-Vroom TM. Paraformaldehyde fixation in immunofluorescence and immunoelectron microscopy. Preservation of tissue and cell surface membrane antigens. *J Immunol Meth* 1974; 6: 93–8
23 Kakio A, Nishimoto SI, Yanagisawa K, Kozutsumi Y, Matsuzaka K. Cholesterol-dependent formation of GM1 ganglioside-bound amyloid-protein, an endogenous seed for Alzheimer amyloid. *J Biol Chem* 2001; 276: 24985–90
24 Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henshen A, Yates J, Cotman C, Glabe C. Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs. *J Biol Chem* 1992; 267: 546–54
25 Jarrett JT, Berger EP, Lansbury PT Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* 1993; 32: 4693–7
26 Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L Jr, Eckman C, Golde TE, Younkin SG. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science* 1994; 264: 1336–40
27 Younkin SG. The amyloid beta protein precursor mutations linked to familial Alzheimer's disease alter processing in a way that fosters amyloid deposition. *Tohoku J Exp Med* 1994; 174: 217–23

Received12 July 2004
Accepted after revision 2 August 2004

J Med Primatol 2005: 34: 73–85

JOURNAL OF MEDICAL PRIMATOLOGY

Ovarian follicular development stimulated by leuprorelin acetate plus human menopausal gonadotropin in chimpanzees

Yoshimoto N, Shimoda K, Mori Y, Honda R, Okamura H, Ide Y, Nakashima T, Nakagata N, Torii R, Yoshikawa Y, Hayasaka I. Ovarian follicular development stimulated by leuprorelin acetate plus human menopausal gonadotropin in chimpanzees. J Med Primatol 2005; 34:73–85. © Blackwell Munksgaard, 2005

Abstract: We attempted ovarian stimulation using gonadotropins in 14 chimpanzees. Subjects were given a single administration of leuprorelin acetate, followed by repeated administration of human menopausal gonadotropin (hMG) for 16–21 days. During the dosing period, the ovarian follicle diameter and count were measured by transvaginal ultrasonography. The hormone administration induced the development of multiple follicles, and multiple oocytes were subsequently retrieved. However, the follicle count was decreased, suggesting atresia, in some subjects. Statistically, the final follicle diameter was dependent on the dosing duration and the hMG dose in the late stage, while the maximum follicle count during hMG administration was dependent on age and the hMG dose in the early stage. Five subjects showed mild ovarian hyperstimulation syndrome (OHSS)-like symptoms with a high serum estradiol (E_2) concentration. These results suggest that leuprorelin acetate plus hMG administration successfully stimulates the development of multiple ovarian follicles for oocyte retrieval and that the serum E_2 concentration is predictive of OHSS-like symptoms in chimpanzees.

Nobuhiko Yoshimoto[1], Kohji Shimoda[1], Yusuke Mori[1], Ritsuo Honda[2], Hitoshi Okamura[2], Yukie Ide[3], Tatsuyuki Nakashima[3], Naomi Nakagata[3], Ryuzo Torii[4], Yasuhiko Yoshikawa[5], Ikuo Hayasaka[1]

[1]Kumamoto Primates Research Park, Sanwa Kagaku Kenkyusho Co., Ltd, Kumamoto, Japan, [2]Department of Obstetrics and Gynecology, School of Medicine, Kumamoto University, Kumamoto, Japan, [3]Division of Reproductive Engineering, Center for Animal Resources and Development, Kumamoto University, Kumamoto, Japan, [4]Research Center for Animal Life Science, Shiga University of Medical Science, Shiga, Japan, [5]Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

Key words: atresia – ovarian hyperstimulation syndrome – *Pan troglodytes* – superovulation – transvaginal ultrasonography

Accepted August 25, 2004

Kohji Shimoda, PhD, Kumamoto Primates Research Park, Sanwa Kagaku Kenkyusho Co., Ltd, 990 Nishikuroiwa, Ohtao, Misumi-cho, Uto-gun, Kumamoto 869-3201, Japan. Tel.: +81-(0)964-53-1398; fax: +81-(0)964-34-1530; e-mail: k_shimoda@skk-net.com

Funding: This work was supported in part by Grants-in-Aid for scientific research (no. H12-genome-014) from the Ministry of Health, Labor and Welfare, Japan.

Introduction

Chimpanzees (*Pan troglodytes*) are the closest evolutionary related species to humans. Approximately 95% of the chimpanzee genome seems to align directly with corresponding regions of the human genome, and sequence divergence is only 1.2% within these aligned segments [31]. Therefore, chimpanzees are expected to have similar physiological and pathological responses to humans and consequently are of great interest in various fields of research [6, 25, 37]. However, numbers living in the wild have decreased considerably due to extensive demolition of natural habitat, and the species has been classified as endangered. In addition, the use of chimpanzees as experimental animals is strictly limited by ethical constrains, and alternative approaches will be required. On the other hand, the number of captive births has gradually increased, and management problems such as costs and limited genetic diversity have become problems in most breeding colonies [16].

Recent progress in reproductive and developmental biology has enabled the artificial

manipulation of embryos, such as *in vitro* fertilization (IVF), cryopreservation and transfer, and these techniques may help to overcome the problems in chimpanzees. IVF and cryopreservation would be helpful for conserving genetic diversity, and embryo transfer would allow birth control for rational colony management. Moreover, embryo-derived cell lines such as embryonic stem (ES) and embryonal carcinoma (EC) cells have been attracting a great deal of attention for regenerative medicine, drug development and basic research in developmental biology [11, 18, 29, 30, 44]. The establishment of chimpanzee embryo-derived cell lines may provide a novel tool for research, and would be alternative to *in vivo* studies of chimpanzees.

As a pre-requisite for the realization of this idea, we need to obtain large numbers of oocytes from chimpanzees. Ovarian stimulation with exogenous gonadotropins for multiple follicular development has become a common technique of reproductive medicine in humans, and has also been reported in primates including rhesus monkeys [46], cynomolgus monkeys [13], squirrel monkeys [7], marmosets [22], lowland gorillas [15] and chimpanzees [14, 45].

In the present study, we attempted ovarian stimulation with leuprorelin acetate, a gonadotropin releasing hormone analog (GnRHa), plus human menopausal gonadotropin (hMG) in chimpanzees for efficient collection of multiple oocytes. The ultrasonographic, physical and endocrine data obtained were statistically analyzed to examine the factors leading to multiple follicular development. In addition, predictive parameters for ovarian hyperstimulation syndrome (OHSS)-like symptoms were examined to minimize suffering.

Materials and methods

Subjects

Fourteen adult female chimpanzees maintained at the Kumamoto Primates Research Park, Sanwa Kagaku Kenkyusho Co., Ltd (Kumamoto, Japan), were used for the present study. They consisted of six nulliparous and eight multiparous individuals, aged 11–32 years and weighing 42.1–76.2 kg (Table 1). They were housed in a facility with a large outside enclosure and indoor bed rooms, and temporarily moved to stainless-steel cages (W140 × D2125 × H1760 mm) in an air-conditioned room (temperature, 18–28°C; lighting, 7:00–18:00 hours) during the experiment. Appropriate amounts of vegetables, fruits and commercial monkey chow (PS, Oriental Yeast Co., Ltd, Tokyo, Japan; Monkey Bit, Nosan Corporation, Yokohama, Kanagawa, Japan) were given twice daily, and tap water was available *ad libitum*. Their appearance and behavior were checked daily for health and evidence of menstruation. All experimental procedures described below were approved by the Animal Ethical Committee of the Kumamoto Primates Research Park, Sanwa Kagaku Kenkyusho Co., Ltd, and were in accordance with the Guide for the Care and Use of Laboratory Animals (ILAR).

Table 1. Female chimpanzees used in the present study

Subject	Age (year)	Body weight (kg)[1]	Parity
Sachi	25	46.5	M
Nacky	27	47.1	M
Sango	26	60.8	M
Cookie	11	46.2	N
Tamae	23	57.4	M
Koiko	26	60.1	M
Yoko	14	53.7	N
Inko	14	45.8	N
Kanae	24	57.4	M
Suzu	20	45.0	M
Niko	29	76.2	M
Betty	32	53.2	N
Chiko	14	44.6	N
Yoshizu	12	42.1	N

N, nulliparous; M, multiparous.
[1]At commencement of the hormone administration.

Hormone treatment

The schedule of hormone administration is presented in Fig. 1. The dosing regimens were carefully determined for individuals by referring to ultrasonographic, physical and endocrine data so as to avoid adverse events, especially OHSS. Within a couple of days after commencement of menstruation, 3.75 mg/head of leuprorelin acetate (Leuplin® for Injection 3.75; Takeda Chemical Industries Ltd, Osaka, Japan), a sustained active suspension of GnRHa, was subcutaneously administered to the subjects (day 0). This administration induced a transient release and a subsequent continuous suppression of endogenous pituitary gonadotropins for 4 weeks in a preliminary study (data not shown). From day 1, the subjects were given repeated intramuscular administration of 75–300 IU/head of hMG (Humegon®; N.V. Organon, Oss, The Netherlands) once daily for 16–21 days. In most of the cases, hMG administration was terminated when follicles over 17 mm in diameter were found by transvaginal ultrasonography (see below), because the follicle diameter at LH surge was approximately 17 mm in the normal menstrual cycle in a preliminary study

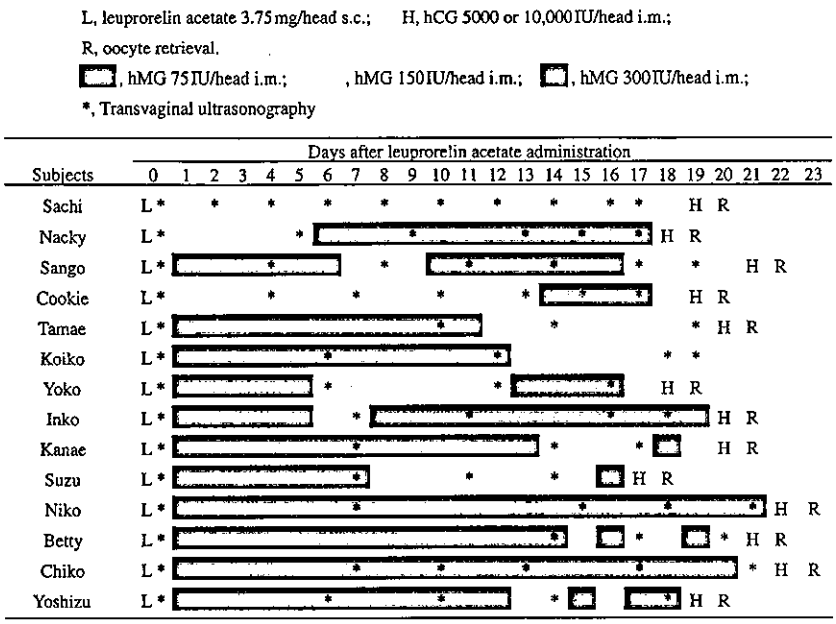


Fig. 1. Schedule of hormone administration, oocyte retrieval and transvaginal ultrasonographic examinations in female chimpanzees.

(data not shown). On the next day or a couple of days after the end of hMG administration, 5000 or 10,000 IU/head of human chorionic gonadotropin (hCG) (Pregnyl®; N.V. Organon) was intramuscularly administered once. One subject, Koiko, was not given hCG and excluded from the subsequent oocyte retrieval because she showed low food-intake and oliguria. She was thereafter under veterinary control and recovered in a week.

Transvaginal ultrasonography

During the hormone administration, time course changes of follicle diameter and count were measured by transvaginal ultrasonography using the Sonovista®-MSC (5.0 mHz probe; Mochida Pharmaceutical Co., Ltd, Tokyo, Japan) by the same observer (N.Y.) under ketamine anesthesia (Fig. 1). Meanwhile, the peripheral region of the ovary was checked to detect a sign of adverse events. For Sachi, Nacky, Sango and Cookie, transvaginal ultrasonography was performed frequently during hMG administration, but ascites was detected only at oocyte retrieval. Therefore, the frequency of the examination was decreased for the other subjects because repeated anesthetization was not preferable for health.

Oocyte retrieval

Follicular fluid was withdrawn between 30 and 35.5 h after hCG administration by ultrasound-guided transvaginal aspiration under ketamine anesthesia. The ascites around the ovary was also aspirated if detected. This method is considered to be much less invasive than laparoscopy, and no adverse events caused by this operation were observed in the present study. Meanwhile, ovarian enlargement and severity of ascites were checked. Cumulus-oocyte complexes (COCs) were collected from the aspirate, and oocytes were denuded with a 0.03% hyaluronidase solution. The oocytes were observed by light microscopy and used in other studies. After the retrieval, the subjects were treated with antibiotics and sent back to the bed rooms. Careful observation was continued until their complete recovery.

Hormone assay

While the subjects were under ketamine anesthesia for the ultrasonographic examinations and oocyte retrieval, blood was withdrawn via the median antebrachial vein. Sera were separated by centrifugation at 1600 *g* for 20 min and sent to SRL Inc. (Tokyo, Japan), where the serum concentrations of estradiol (E_2) and progesterone (P) were measured by the solid-phase ^{125}I radioimmunoassay method using a commercial kit (Coat-A-Count® Estradiol-6; Diagnostic Products Corporation, Los Angeles, CA, USA).

Statistical analysis

The dose levels of hMG were converted to values per body weight to minimize the effect of body

Table 2. Total dose of human menopausal gonadotropin (hMG) per body weight (∑hMG) in female chimpanzees

Subject	∑hMG (IU day/kg)			
	D1-final[1]	D1-D6[2]	D7-D12[3]	D13-final[4]
Sachi	54.3	19.3	19.1	15.9
Nacky	90.7	22.3	37.8	30.7
Sango	75.6	29.8	22	23.8
Cookie	47.5	19.4	18.8	9.3
Tamae	76.0	31.4	28.9	15.8
Koiko	73.8	29.8	29.2	14.8
Yoko	52.7	30.7	16.5	5.5
Inko	57.2	36.0	11.4	9.8
Kanae	77.7	31.4	31.1	15.2
Suzu	70.1	40.0	23.5	6.7
Niko	82.8	23.6	23.7	35.4
Betty	100.5	33.8	33.8	32.9
Chiko	132.9	40.4	38.6	53.9
Yoshizu	52.7	30.7	16.5	5.5

The day of leuprorelin acetate administration was designated day 0.
[1]From day 1 to the final ultrasonographic examination.
[2]From day 1 to day 6.
[3]From day 7 to day 12.
[4]From day 13 to the final ultrasonographic examination.

size [17]. To examine the relationship between hMG regimen and follicular development, the dosing period of hMG was divided into three stages: day 1 to day 6 as the early stage; day 7 to day 12 as the intermediate stage; and day 13 to the final ultrasonographic examination as the late stage. Then, the total hMG dose per body weight (ΣhMG) from day 1 to the final examination ($\Sigma hMG_{D1\text{-}final}$) and the doses in the respective stages ($\Sigma hMG_{D1\text{-}6}$, $\Sigma hMG_{D7\text{-}12}$ and $\Sigma hMG_{D13\text{-}final}$) were calculated (Table 2). The dependency of follicle diameter and count on age, dosing duration and each ΣhMG were estimated by correlation coefficient and univariate linear regression analysis to examine the factors leading multiple follicular development in subjects differently treated for individuals. For comparisons between subjects apparently in normal health and those showing OHSS-like symptoms, Student's *t*-test was applied when variances were found to be homogenous with the *F*-test, or Aspin–Welch's test was applied when variances were found to be heterogeneous with the *F*-test.

Results

Follicle diameter

Multiple ovarian follicles were formed by stimulation of leuprorelin acetate and hMG (Fig. 2). Time course changes in mean follicle diameter measured by transvaginal ultrasonography are shown in Fig. 3. There was no great difference in follicle growth rate among most of the subjects. At the final ultrasonographic examination between day 16 and day 21, the mean diameter was >10 mm, and multiple follicles reached >15 mm in diameter. On the other hand, Koiko showed slower follicle growth than the others. At the final examination on day 18, the mean diameter was only 6.7 mm, and no follicles reached 15 mm in diameter.

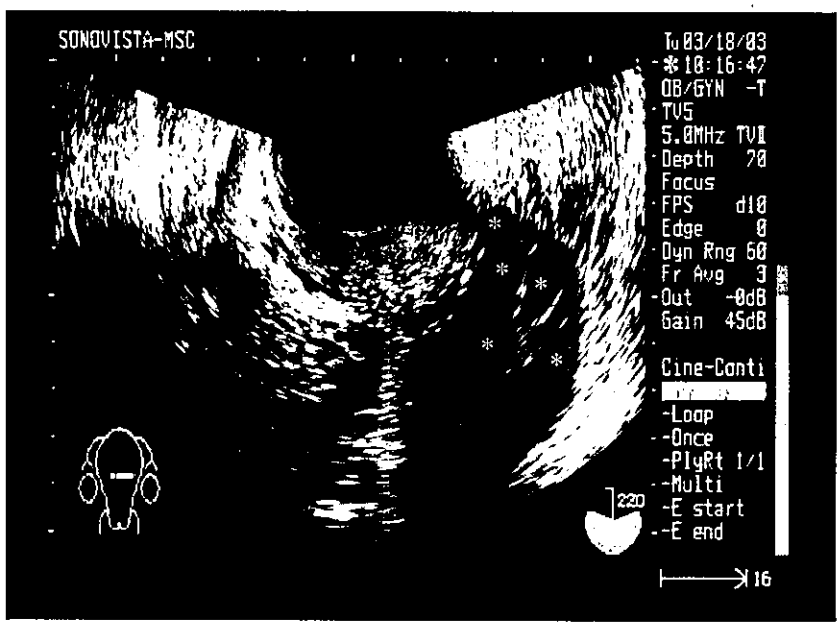


Fig. 2. A transvaginal ultrasonograph showing the ovary possessing multiple follicles. Asterisks indicate ovarian follicles.