each object and the total time spent exploring both objects were recorded. To analyse cognitive performance, a discrimination index was calculated as the difference in time exploring the novel and familiar object, expressed as the ratio of the total time spent exploring both objects.

Offspring's circadian rhythm of locomotor activity

On PND 42–44 and PND 84–86, the male offspring's circadian rhythm for locomotor activity was measured and calculated as described above for the dam.

Immobilization stress and plasma corticosterone measurement

On PND 84, to study the HPA response to stress in adult offspring, a single immobilization stress experiment was performed between 21:00 and 23:00 h with blood sampling from the tail vein at 0, 30 and 120 min after the beginning of immobilization. Immobilization stress was applied as described previously (Morinobu *et al.*, 2003). Animals were immobilized in clear plastic cone bags, sized so that animals were equally immobilized. After centrifugation with prechilled ethylenediamine tetraacetic acid (EDTA; 500 *g* at 4 °C for 30 min), plasma samples were frozen and stored at −70 °C until the day of analysis. The plasma corticosterone level was determined using the rat corticosterone [^{125}I] assay system (Amersham).

Real-time-quantitative polymerase chain reaction

To collect tissue for the assessment of NR1, NR2A and NR2B mRNA expression by RT-PCR, rats were decapitated less than 30 s after removal from the home cage. To collect tissue for the assessment of GR, rats were decapitated immediately after immobilization stress for 2 h. The entire hippocampus was dissected, frozen on dry ice and stored at −80 °C until the time of assay. RT-PCR was conducted as described previously (Suenaga *et al.*, 2004). Total RNA was extracted using the RNAqueous™ Total RNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. After treatment with RNase-free DNase I (Takara, Shiga, Japan), a single-stranded cDNA was synthesized using reverse transcriptase (Toyobo, Osaka, Japan). RT-PCR was performed with an ABI7700 sequence detection system (Applied Biosystems) to quantify relative mRNA levels in samples. RT-PCR was performed to amplify the mRNA of NR1, NR2A, NR2B and GR. The primers and TaqMan hybridization probes were designed using PRIMER EXPRESS software (Applied Biosystems). Table 1 shows the sequences and fluorescent dyes of the PCR primers and TaqMan probes. The TaqMan probe, which was designed to hybridize to the PCR products, was labeled with a fluorescent reporter dye at the 5′-end and a quenching dye at the 3′-end. PCR was carried out with TaqMan Universal PCR Master Mix (Applied Biosystems). All standards and samples were assayed in triplicate. Thermal cycling was initiated with an initial denaturation at 50 °C for 2 min and 95 °C for 10 min. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95 °C for 15 s for melting and at 60 °C for 1 min for annealing and extension. The PCR assay for glyceraldehyde-3-phosphate dehydrogenase was performed using the TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase Control Reagents kit (Applied Biosystems). The mRNA levels of NR1, NR2A, NR2B and GR were detected by RT-PCR and the ratio of the concentration of the target molecule to that of glyceraldehyde-3-phosphate dehydrogenase (target molecule : glyceraldehyde-3-phosphate dehydrogenase) in unknown samples was calculated.

TABLE 1. Primers and TaqMan probe for each gene

Primers and TaqMan probes	Sequences and fluorescent dyes
NR1	
Forward primer	5'-GTTCTTCCGCTCAGGCTTTG-3'
Reverse primer	5'-AGGGAAACGTTCTGCTTCCA-3'
TaqMan probe	5'-FAM-CGGCATGGGCAAGGACAGC-CTAMRA-3'
NR2A	
Forward primer	5'-AGCCCCCTTCGTCATCGTA-3'
Reverse primer	5'-GACAGGGCACCGTGTTCCT-3'
TaqMan probe	5'-FAM-AGGACATAGACCCCCTGACT-GAGACCTGTG-TAMRA-3'
NR2B	
Forward primer	5'-CCCCCAAGTTCTGGTTGGT-3'
Reverse primer	5'-TTTTGGGAACGAGCTTTGCT-3'
TaqMan probe	5'-FAM-TTGGCCGTCTTGGCCGTATC-AGGTAMRA-3'
GR	
Forward primer	5'-TTCGAAGGAAAAACTGCCCAG-3'
Reverse primer	5'-CGAGCTTCAAGGTTCATTCCA-3'
TaqMan probe	5'-FAM-TGCCGCTATCGGAAATGTCT-TCAGG-TAMRA-3'

The nucleotide positions range from 2521 to 2586 (66 bp) from the sequence of *N*-methyl-D-aspartate receptor (NR) subunit NR1 cDNA (GeneBank no. x63255), from 1254 to 1324 (70 bp) from the sequence of NR2A cDNA (GeneBank no. D13211), from 376 to 439 (63 bp) from the sequence of NR2B cDNA (GeneBank no. NM_012574) and from 1482 to 1551 (69 bp) from the sequence of glucocorticoid receptor (GR) cDNA (GeneBank no. Y12264).

Data analysis

SPSS was used for all analyses. Data were analysed by repeated-measures ANOVA for split-plot designs, following a statistical method by Macri *et al.* (2004). For analysis of 24-h maternal behavior, the general model was 13 days × 8 h × three treatments. Treatment was a between-litter factor whereas all other variables were within-litter factors. Additionally, for the analysis of diurnal pattern, one-way ANOVA with Bonferroni test was performed at each time point during the day. Data on 24-h maternal behavior were transformed to the arc sine of the square root of the relative frequencies of behavioral scores. For reasons of clarity, all figures are based on non-transformed values.

Differences in 1-h maternal behavior and the locomotor activities of the dam at each time point, body weight and day of eye opening of pups, and the subsequent behavioral and molecular variables of offspring in adulthood among the three lighting conditions were determined by one-way ANOVA with the Bonferroni test.

The general model for analysis of plasma levels of corticosterone was three time × three treatments. Treatment was a between-litter effect whereas time points were within-litter factors. When appropriate, the Bonferroni test was performed. Values of $P < 0.05$ were considered significant.

Results

Growth, survival and neurodevelopmental milestones among offspring of mothers from normal lighting conditions, prolonged dark phase conditions and prolonged light phase conditions

The mean body weights of male offspring of NLC, PDC and PLC mothers did not differ at any time-point (Table 2). The eye-opening day was similar among the three groups (Table 2). At weaning (PND 22), survival rates among these three groups were almost equal (99%). These findings suggest that variations of lighting conditions do not affect gross development.

TABLE 2. Reproductive parameters

	NLC	PDC	PLC
Pup body weight (g)			
PND10	14.35 ± 0.67	13.39 ± 0.45	14.26 ± 0.26
PND22	63.10 ± 0.90	61.95 ± 1.77	62.65 ± 1.24
Eye opening day (PND)	13.78 ± 0.10	13.56 ± 0.12	13.69 ± 0.08
1-h focal observation			
Duration of LG bout (s)	54.50 ± 3.13	18.26 ± 4.13*†	76.13 ± 5.43*
Numbers of LG bout (s)	6.45 ± 0.74	2.80 ± 0.59*†	7.64 ± 0.64

Mean values ± SEM of pup body weight (n = 17–19 pups, 12 litters/group), eye-opening day (n = 49–54, 24 litters/group) and variables measuring maternal behavior for normal lighting conditions (NLC), prolonged dark phase conditions (PDC) and prolonged light phase conditions (PLC) mothers. PND, post natal day; LG, licking/grooming. *$P < 0.05$ compared with NLC; †$P < 0.05$ compared with PLC.

Different mothering behaviors among mothers from normal lighting conditions, prolonged dark phase conditions and prolonged light phase conditions

24-h intermittent observation of maternal behavior

Figure 2A depicts mean daily levels of active nursing from PND 2 to PND 14 for NLC, PDC and PLC mothers. Active nursing gradually decreased across days (days, $F_{12,396} = 34.94$, $P < 0.001$). However, ELC significantly affected levels of active nursing (treatment, $F_{2,33} = 13.18$, $P < 0.001$). Both NLC and PLC mothers exhibited significantly higher levels of active nursing than PDC dams (posthoc test; NLC vs. PDC, $P < 0.001$; PLC vs. PDC, $P < 0.001$). This difference between PDC and the other two conditions emerged on PND 3 and remained stable throughout the remaining period except on PND 10. Analysis of the diurnal pattern (Fig. 2C) revealed that active nursing was elevated almost throughout the day in NLC and PLC dams compared with PDC dams (one-way ANOVA with Bonferroni test at each time point; NLC vs. PDC, $P < 0.05$ at 21:00–22:00, 24:00–01:00 and 06:00–07:00 h; $P < 0.10$ at 09:00–10:00, 12:00–13:00 and 15:00–16:00 h; PLC vs. PDC, $P < 0.05$ at 15:00–16:00, 21:00–22:00, 24:00–01:00, 03:00–04:00 and 06:00–07:00 h; $P < 0.10$ at 09:00–10:00 and 18:00–19:00 h). Differences in active nursing time were not consistent with contact time with pups (Fig. 2B and D). Contact time with pups was not affected by neonatal lighting manipulations, indicating that differences in active nursing were not simply due to individual differences in the active interacting time of dams. Analysis of the diurnal pattern (Fig. 2D) revealed that, whereas contact time with pups in PDC mothers was higher at 12:00–13:00 h ($P = 0.048$) and also tended to be higher at 15:00–16:00 h ($P = 0.053$), contact in PLC mothers was

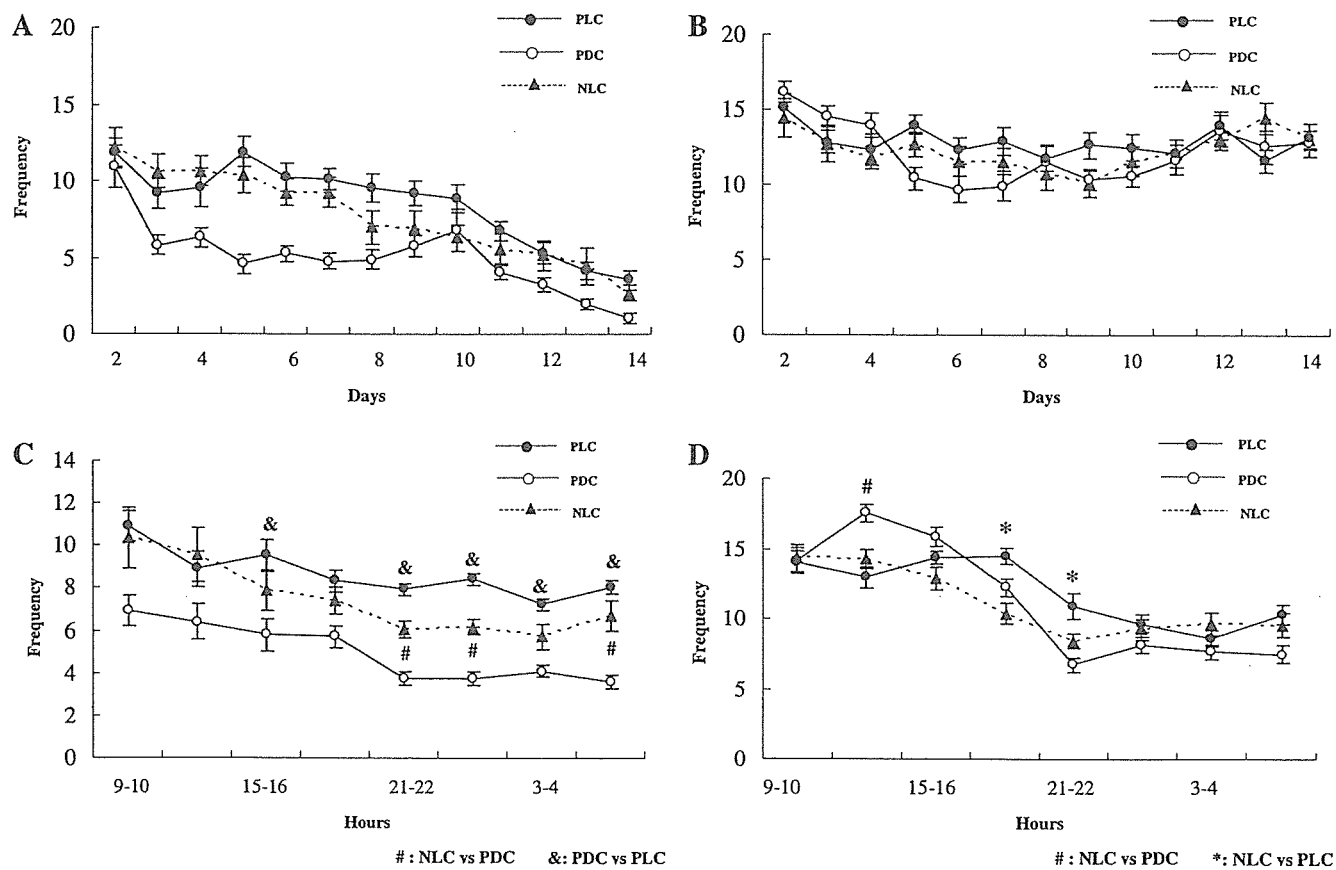

FIG. 2. Effects of early lighting conditions on maternal behavior. Daily frequency (mean/h ± SEM) of (A) active nursing and (B) contact time with pups by dams subjected daily to normal lighting conditions (NLC) (n = 12), prolonged dark phase conditions (PDC) n = 12) or prolonged light phase conditions (PLC) (n = 12). Daily scores are based on eight daily 1-h sampling sessions. Diurnal patterns of (C) active nursing and (D) contact time scores (mean ± SEM) by NLC (n = 12), PDC (n = 12) and PLC (n = 12) dams. Active nursing was elevated almost throughout the day in NLC and PLC dams compared with PDC dams (NLC vs. PDC, $P < 0.05$ at 21–22, 24–1 and 6–7 h; $P < 0.10$ at 9–10, 12–13 and 15–16 h; PLC vs. PDC, $P < 0.05$ at 15–16, 21–22, 24–1, 3–4 and 6–7 h; $P < 0.10$ at 9–10 and 18–19 h). Although contact time with pups in PDC mothers was higher at 12–13 h ($P < 0.05$) and tended to be higher at 15–16 h ($P < 0.10$), contact time in PLC mothers was higher at 18–19 and 21–22 h ($P < 0.05$) compared with NLC mothers. Scores from each of eight daily 1-h sampling sessions were averaged across postnatal day 2–14. #$P < 0.05$, NLC vs. PDC; &$P < 0.05$, PDC vs. PLC, *$P < 0.05$, NLC vs. PLC.

higher at 18:00–19:00 h ($P = 0.003$) and 21:00–22:00 h ($P = 0.027$) compared with NLC mothers.

1-h focal observation of maternal behavior

Overall, the length of LG bouts ranged from 3 s to over 309.1 s. As shown in Table 2, there were significant group differences in mean duration ($F_{2,29} = 44.0$, $P < 0.001$). Although the duration of LG bouts was reduced under PDC relative to NLC and PLC (posthoc test; PDC vs. NLC, $P < 0.001$; PDC vs. PLC, $P < 0.001$), the duration of bouts under PLC was increased relative to NLC (posthoc test; $P = 0.004$). In addition, PDC mothers licked less frequently than NLC and PLC mothers (posthoc test; PDC vs. NLC, $P = 0.002$; PDC vs. PLC, $P < 0.001$).

Dams' circadian rhythm of locomotor activity

Figure 3A shows the locomotor activity of NLC, PDC and PLC mothers on PND 4–6. Locomotor activity in PDC mothers was higher at 10:00 h ($P = 0.006$) and lower at 21:00 h ($P = 0.005$). Compared with NLC mothers, in PDC mothers there was a 1.5–2.0-h phase delay at the onset of activity and a 2.5–3.0-h phase delay at the offset of activity. Locomotor activity in PLC mothers was lower at 21:00 h ($P = 0.001$). In PLC mothers, there was a 2.0–2.5-h phase delay at the onset of activity in comparison with NLC mothers. Figure 3B shows the locomotor activity of NLC, PDC and PLC mothers on PND 10–12. Locomotor activity in PDC mothers was higher from 09:00 to 11:00 h ($P < 0.001$, $P = 0.003$ and $P = 0.005$) and lower at 18:00 and 20:00 h ($P = 0.004$ and $P = 0.001$). Compared with NLC mothers, PDC mothers exhibited a 2.5–3.0-h phase delay at the onset of activity and a 3.0–3.5-h phase delay at the offset of activity. Locomotor activity in PLC mothers was higher at 09:00 and 11.00 h ($P < 0.001$ and $P = 0.006$) and lower at 20:00, 21:00 and 23:00 h ($P = 0.001$, $P = 0.001$ and $P = 0.003$). In comparison with NLC mothers, PLC mothers exhibited a 3.0–3.5-h phase delay at the onset of activity and a 1.0–1.5-h phase delay at the offset of activity.

ANOVA revealed that daily means (counts/min) of activity differed among the three groups (NLC, 67.5; PDC, 66.1; PLC, 38.9) on PND 10–12 but not on PND 4–6 (Fig. 3C). On PND 10–12, PLC mothers exhibited significantly less movement than NLC and PDC mothers ($P < 0.05$).

In summary, PDC and PLC mothers were not entrained to the altered lighting conditions on PND 4–6 and it was also unclear whether PDC mothers were entirely entrained on PND 10–12. It is possible that entrainment in PLC mothers appeared to take place at the later time only because light inhibited activity. Only in PLC mothers was a large increase in activity at the beginning of the dark period observed, indicating strong pressure to begin activity, consistent with results of previous studies (Boon *et al.*, 1997; Benstaali *et al.*, 2001).

Influence of early lighting conditions on circadian rhythm of locomotor activity in adolescent and adult offspring

Figure 4A shows the locomotor activity of NLC, PDC and PLC offspring in adolescence. Compared with NLC offspring, locomotor activity in PDC offspring was higher at 10:00 and 13:00 h ($P = 0.016$ and $P = 0.04$). Locomotor activity in PLC offspring was lower at 09:00 h ($P = 0.001$) and higher at 10:00 and 11:00 h ($P = 0.003$ and $P = 0.009$). Figure 4B shows the locomotor activity of NLC, PDC and PLC offspring in adulthood. Compared with NLC offspring, PDC offspring exhibited no significant difference in locomotor activity. Locomotor activity in PLC offspring was lower at 09:00 h ($P = 0.004$) and higher at 19:00 h ($P = 0.001$). There were no significant differences among the three groups during the dark active phase in both the adolescent and adult animals.

ANOVA revealed that daily means (counts/min) of activity did not differ among the three groups at any time point.

Anxiety phenotypes in adolescent and adult offspring from normal lighting conditions, prolonged dark phase conditions and prolonged light phase conditions

Elevated plus-maze test in adolescent and adult offspring

ANOVA revealed that the ratio of time spent in the open arms (vs. total time) in the elevated plus-maze was significantly affected by ELC in both adolescent ($F_{2,45} = 18.38$, $P < 0.001$) and adult ($F_{2,51} = 12.47$, $P < 0.001$) animals (Fig. 5A). Analysis of the number of open-arm entries revealed a significant effect of ELC in adolescent ($F_{2,45} = 13.13$, $P < 0.001$) and adult ($F_{2,51} = 16.09$, $P < 0.001$) animals (Fig. 5B). The percentage of time spent in the open arms and the number of open-arm entries were reduced in PDC compared with NLC and PLC at both ages. With PLC, the number of open-arm entries was increased compared with NLC and PDC at both ages. Groups did not differ in the number of closed-arm entries in both adolescent and adult animals (Fig. 5C).

Social interaction test in adolescent and adult offspring

ANOVA revealed that the frequency of social interaction was significantly affected by ELC in both adolescent ($F_{2,51} = 33.14$, $P < 0.001$) and adult ($F_{2,45} = 13.92$, $P < 0.001$) animals (Fig. 6A). PDC decreased the frequency of social interaction compared with NLC and PLC only in adolescence. PLC increased the frequency of social interaction compared with NLC in both adolescence and adulthood. Analysis of time spent in social interaction revealed a significant effect of ELC in adolescent ($F_{2,51} = 3.27$, $P = 0.046$) and adult ($F_{2,45} = 21.86$, $P < 0.001$) animals (Fig. 6B). PDC decreased the time spent in social interaction in both adolescent and adult animals compared with NLC and PLC.

Object recognition memory and the hippocampal N-methyl-D-aspartate receptor subunit expression in adult offspring from normal lighting conditions, prolonged dark phase conditions and prolonged light phase conditions

Table 3 shows the total time spent in exploring two identical objects in the training trial for the 1- and 24-h retention test. ANOVA for total exploration time revealed no differences among groups in both adolescence and adulthood. One-sample *t*-test used to examine whether the discrimination index was zero (chance level) showed that all groups spent similar time exploring each of the two identical objects in the training trial.

In the 1-h retention test, one-sample *t*-test revealed that the discrimination index of NLC offspring was significantly different from zero in both adolescence ($t_{10} = 6.03$, $P < 0.001$) and adulthood ($t_{11} = 6.90$, $P < 0.001$) (Fig. 7A), indicating that rats at both ages readily discriminated the novel object from the familiar object during the 1-h retention test. Moreover, ELC significantly affected the discrimination index in both adolescence ($F_{2,31} = 7.85$, $P = 0.0017$) and adulthood ($F_{2,32} = 16.13$, $P < 0.001$). As shown in Fig. 7A, PDC decreased recognition memory in both adolescent and adult animals compared with NLC and PLC. In contrast, during the 24-h retention trial, no preference for the novel object in the NLC, PDC and PLC rats

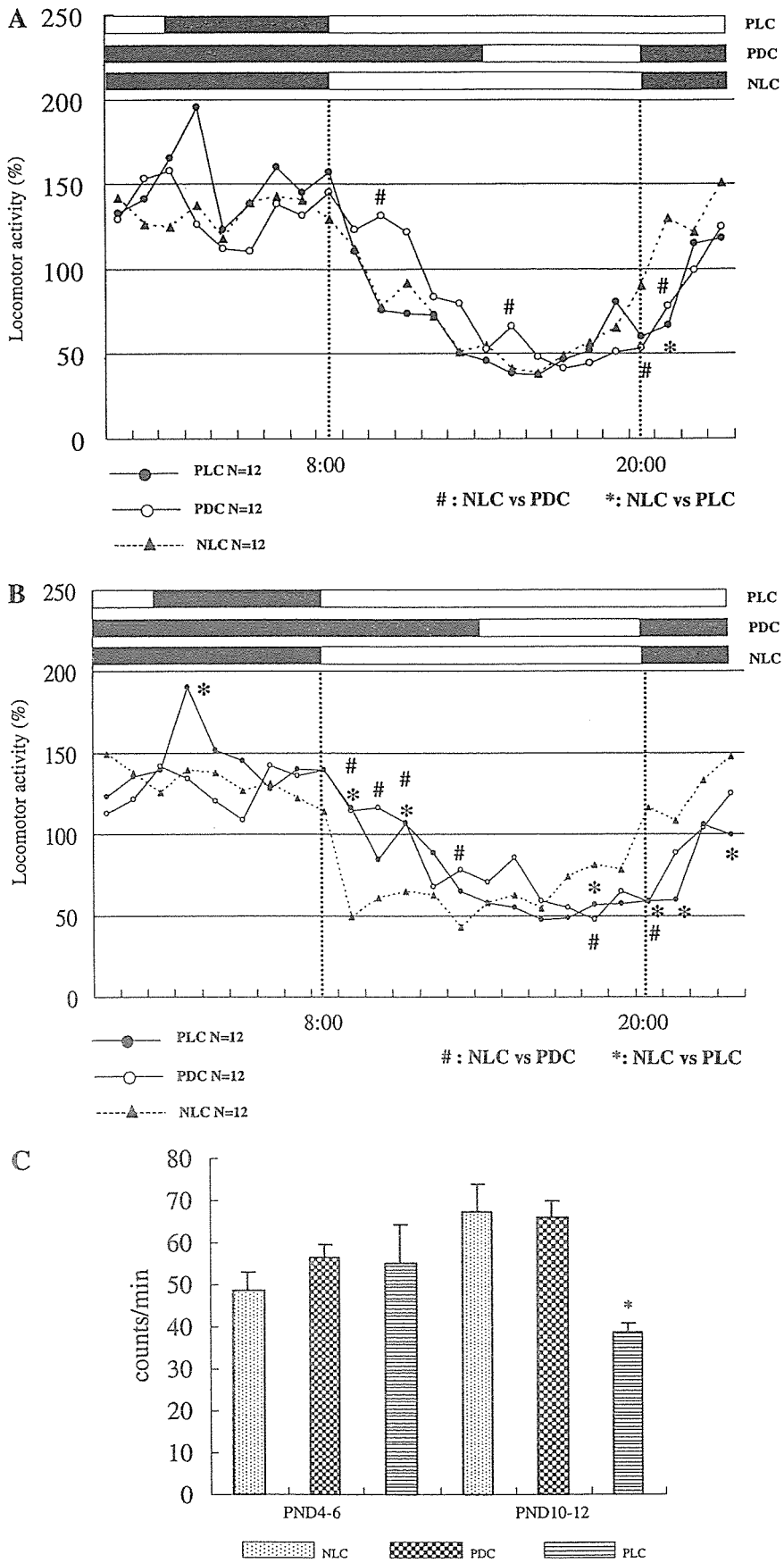

FIG. 3. Comparison of circadian rhythm of locomotor activity among normal lighting conditions (NLC), prolonged dark phase conditions (PDC) and prolonged light phase conditions (PLC) dams. Motor activity as measured by an actimeter of NLC ($n = 12$), PDC ($n = 12$) and PLC ($n = 12$) mothers over 72 h. (A) Seventy-two 1-h percentages of the total mean value of the corresponding group on postnatal day (PND) 4–6 were averaged. Although locomotor activity in PDC mothers was higher at 10:00 h and lower at 21:00 h ($P < 0.05$), that in PLC mothers was lower at 21:00 h ($P < 0.05$). (B) Seventy-two 1-h percentages of the total mean value on PND 10–12 were averaged. Locomotor activity in PDC mothers was higher from 09:00 to 11:00 h ($P < 0.05$) and lower at 18:00 and 20:00 h ($P < 0.05$). (C) Mean values (counts/min) of each group over a 72-h period on PND 4–6 and PND 10–12. On PND 10–12, PLC mothers exhibited significantly less movement than NLC and PDC mothers ($P < 0.05$). Results are means ± SEM. $*^{\#}P < 0.05$ compared with NLC mothers.

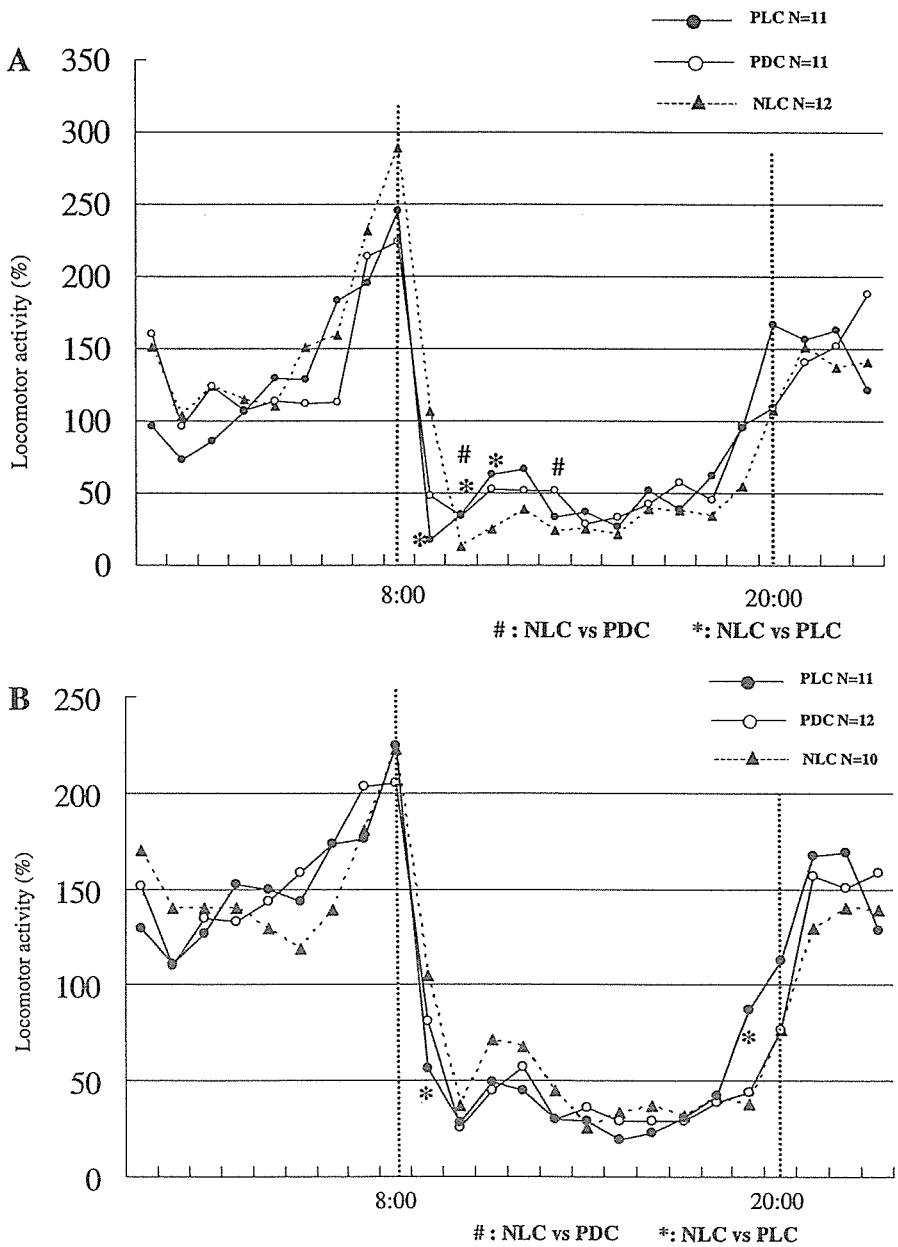

FIG. 4. Circadian rhythms of locomotor activity in normal lighting conditions (NLC), prolonged dark phase conditions (PDC) and prolonged light phase conditions (PLC) offspring. Motor activity as measured by an actimeter of NLC, PDC and PLC offspring over 72 h. Vertical bars represent the change in lighting condition (light/dark, 12/12 h, lights on 08:00–20:00 h). (A) Seventy-two 1-h percentages of the total mean value of the corresponding group on postnatal day (PND) 42–44 were averaged. Compared with NLC offspring, locomotor activity in PDC offspring was higher at 10:00 and 13:00 h ($P < 0.05$). Locomotor activity in PLC offspring was lower at 09:00 h and higher at 10:00 and 11:00 h ($P < 0.05$). (B) Seventy-two 1-h percentages of the total mean value of the corresponding group on PND 84–86 were averaged. Locomotor activity in PLC offspring was lower at 09:00 h and higher at 19:00 h ($P < 0.05$). *$P < 0.05$ compared with NLC offspring.

was exhibited in both adolescence and adulthood (Fig. 7B). As shown in Table 3, ELC did not influence the total amount of time exploring the two objects after the 24-h retention interval.

ANOVA revealed that ELC affected hippocampal mRNA levels of NR2B ($F_{2,21} = 5.06$, $P = 0.016$) (Fig. 8C) but not NR1 (Fig. 8A) and NR2A (Fig. 8B). Levels of NR2B were lower in PDC compared with NLC offspring. Down-regulation of NR2B is consistent with the finding of impaired object recognition memory in PDC offspring.

Corticosterone response and hippocampal glucocorticoid receptor mRNA levels in adult offspring from normal lighting conditions, prolonged dark phase conditions and prolonged light phase conditions

The increased plasma corticosterone response to stress in PDC offspring was significantly sustained compared with NLC and tended to be sustained compared with PLC offspring (Fig. 9). Statistical analysis revealed a significant lighting manipulation × time interaction

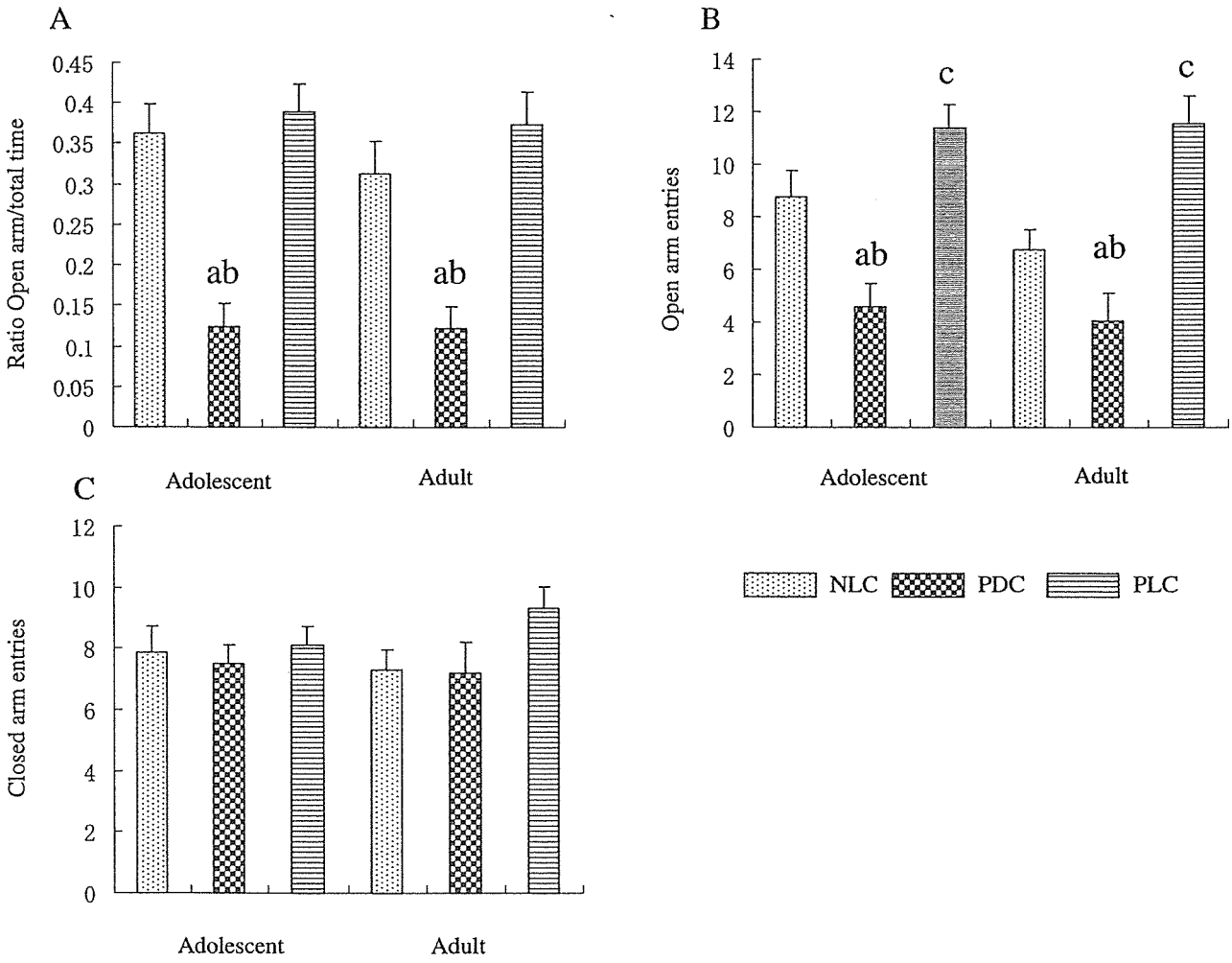

FIG. 5. Prolonged dark phase cycle during neonatal period produces a high-anxiety phenotype in adolescence and adulthood. Results of elevated plus-maze test on postnatal day (PND) 42 and PND 84 in male offspring. (A) Ratio of time spent in open arms over open arms plus closed arms. (B) Number of open-arm entries. (C) Number of closed-arm entries. Percentage of time spent in open arms and the number of open-arm entries were reduced in prolonged dark phase conditions (PDC) compared with normal lighting conditions (NLC) and prolonged light phase conditions (PLC) at both ages. With PLC, the number of open-arm entries was increased compared with NLC and PDC at both ages. Results are means ± SEM. $^{a}P < 0.05$, NLC vs. PDC; $^{b}P < 0.05$, PDC vs. PLC; $^{c}P < 0.05$, NLC vs. PLC.

($F_{2,72} = 2.95$, $P = 0.026$). ELC had no effect on basal ($F_{2,36} = 0.76$, not significant) and peak ($F_{2,36} = 2.05$, not significant) levels of corticosterone but significantly ($F_{2,36} = 4.52$, $P = 0.018$) affected levels at 120 min after starting stress. Posthoc analysis revealed that plasma corticosterone responses at 120 min after the start of stress were significantly higher in PDC than in NLC rats ($P = 0.018$) and tended to be higher compared with PLC rats ($P = 0.085$).

ANOVA revealed that ELC affected hippocampal mRNA levels of GR ($F_{2,19} = 6.19$, $P = 0.0085$) (Fig. 10). Levels of GR were lower in PDC than in NLC offspring. Down-regulation of GR mRNA expression is consistent with the finding of impaired negative feedback of corticosterone in PDC offspring.

Discussion

The study had four major findings. (i) PDC can alter both the quality and quantity of maternal behavior. (ii) Later in life, PDC affects the emotionality of offspring, through underlying alterations of the HPA system. (iii) PDC can also affect memory functioning of offspring by changing hippocampal NR2B receptor expression. (iv) It is assumed that PDC-induced alterations of maternal care can contribute to their offspring's neurobehavioral phenotype. As PLC did not induce a clear alteration in maternal care by dams and an active coping style in offspring in this study, these findings did not support our primary prediction. However, from the results it can be postulated that the altered levels of maternal care in response to different ELC, especially PDC, are involved in the development of the defensive phenotype of offspring. Thus, the effects of ELC are partially consistent with the 'maternal mediation hypothesis' earlier formulated by Levine (1967) and Smotherman & Bell (1980), and enlarged upon by Champagne & Meaney (2001) and Champagne *et al.* (2003a).

Effects of early lighting conditions on maternal behavior

In rodent studies, it was revealed that the dam's genetic background (Ahmadiyeh *et al.*, 2004; Neumann *et al.*, 2005), levels of maternal care received by the dam (Francis *et al.*, 1999a, 2000; Champagne & Meaney, 2006) and various stress exposures during the peripartum period (Pardon *et al.*, 2000; Darnaudery *et al.*, 2004; Macri *et al.*, 2004; Ruedi-Bettschen *et al.*, 2004; Smith *et al.*, 2004; Champagne &

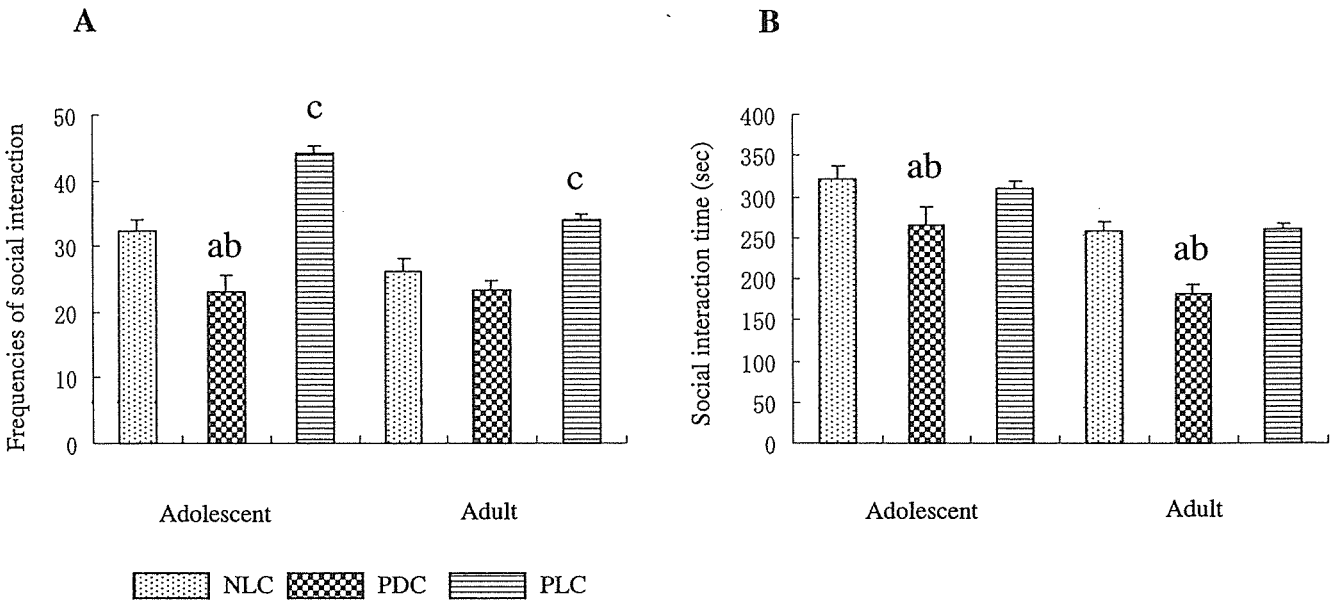

FIG. 6. Prolonged dark phase cycle also reduced social interaction in adolescence and adulthood. Results of social interaction test on postnatal day (PND) 42 and PND 84 in male offspring. (A) Frequency of social interaction. Prolonged dark phase conditions (PDC) decreased the frequency of social interaction in comparison with normal lighting conditions (NLC) and prolonged light phase conditions (PLC) in adolescence. PLC increased the frequency of social interaction compared with NLC in both adolescence and adulthood. (B) Total time spent in social interaction. PDC decreased the time spent in social interaction in both adolescent and adult animals compared with NLC and PLC. Results are means ± SEM. $^{a}P < 0.05$, NLC vs. PDC; $^{b}P < 0.05$, PDC vs. PLC; $^{c}P < 0.05$, NLC vs. PLC.

TABLE 3. Total exploration time in object recognition test

ELC	Training	Retention
1h retention interval, adolescent		
NLC	29.5 ± 1.4	25.1 ± 2.7
PDC	26.4 ± 1.5	17.4 ± 2.0
PLC	26.5 ± 0.9	23.9 ± 0.8
1h retention interval, adult		
NLC	24.7 ± 0.9	20.9 ± 3.2
PDC	25.2 ± 1.8	16.8 ± 1.8
PLC	24.7 ± 1.0	25.9 ± 3.5
24h retention interval, adolescent		
NLC	26.3 ± 1.5	24.3 ± 1.7
PDC	24.0 ± 1.7	22.9 ± 1.8
PLC	26.5 ± 1.0	25.3 ± 1.4
24h retention interval, adult		
NLC	23.8 ± 2.0	22.2 ± 2.9
PDC	23.7 ± 1.2	21.8 ± 2.2
PLC	23.9 ± 1.3	19.0 ± 1.5

Total time spent exploring the two objects (two identical objects for the training trial, and a familiar and a novel object for the test trial) expressed as means ± SEM in seconds. Statistical analysis is described in Results (n = 11–12/group). ELC, early lighting conditions; NLC, normal lighting conditions; PDC, prolonged dark phase conditions; PLC, prolonged light phase conditions.

Meaney, 2006) modulate the characteristics of maternal behavior. In our present study, similar alterations of maternal behavior were observed by merely lengthening the duration of the dark or light phase even during the stress hyporesponsive period of dams (Neumann, 2001), indicating the robust influence of ELC.

Both acutely and continuously, PDC decreased active nursing throughout the observational period. However, the PLC-induced elevation of active nursing did not reach significance in the 24 h of intermittent observation. These effects of ELC were so selective that ELC had no effect on the total time spent with pups under PDC and PLC. Maternal behavior is regulated by the neural circuit that involves the medial preoptic area of the hypothalamus (Numan *et al.*, 1977) and is mediated by several hormones, such as prolactin (Bridges *et al.*, 1974, 1985; Neumann, 2003), oxytocin (Pedersen & Prange, 1979; Pedersen *et al.*, 1982; Francis *et al.*, 2000; Bosch *et al.*, 2005; Champagne & Meaney, 2006), estrogen (Siegel & Rosenblatt, 1975a,b; Champagne *et al.*, 2003b, 2006) and corticosterone (Neumann, 2001, 2003). Classically, Schelstraete *et al.* (1992) reported that inverting the light/dark cycle and temperature every 72 h, designated as atypical zeitgeber, disrupted the temporal distribution of maternal care. Particularly in female rats, the duration of light exposure regulates the prolactin surge and subsequently controls reproduction (Pieper & Gala, 1979; Leadem, 1988; Nelson *et al.*, 1994; Sterner & Cohen, 1995). It can be assumed that the manipulation of ELC changed the total amount and temporal distribution of maternal behavior by modulating these neural networks.

In general, changes in the light cycle are associated with stress and are commonly correlated with an increase in glucocorticoid secretion (Stephens, 1980; Munck *et al.*, 1984). As PDC not only lengthen the active period of the dam but also demand a longer duration for entraining to a new lighting condition (Pittendrigh & Daan, 1976; Honma *et al.*, 1985; Boon *et al.*, 1997; Refinetti, 2004; Weinert *et al.*, 2005), PDC dams are postulated to be under stressful situations resembling jet-lag, thus reducing the provision of maternal care. However, in rodents, darkness increases activity whereas light suppresses it, so it could be predicted that the low activity level induced by PLC, which was observed on PND 10–12, might lengthen the time on the nest and maternal interaction with pups in PLC mothers. However, we found no clear increment in either active nursing or contact time with pups among PLC mothers. Although without significance, maternal care in PLC mothers transiently changed, first decreasing and then increasing compared with NLC mothers. Repeated chronic circadian changes have been reported to apparently induce stressful effects on organisms (Stephens, 1980; Munck *et al.*, 1984). However, a single phase-shift did

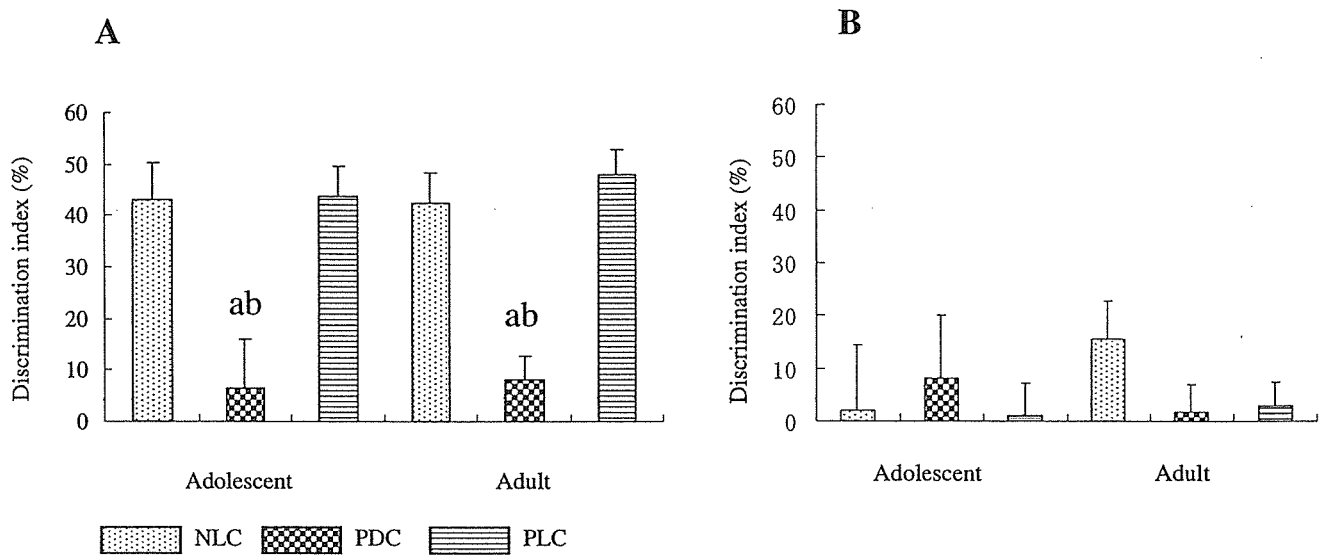

FIG. 7. Prolonged dark phase cycle impaired object recognition memory in adolescence and adulthood. (A) Discrimination index of 1-h retention trial. Prolonged dark phase conditions (PDC) decreased recognition memory in both adolescent and adult animals compared with normal lighting conditions (NLC) and prolonged light phase conditions (PLC). (B) Discrimination index of 24-h retention trial. Results are means ± SEM. $^{a}P < 0.05$, NLC vs. PDC; $^{b}P < 0.05$, PDC vs. PLC.

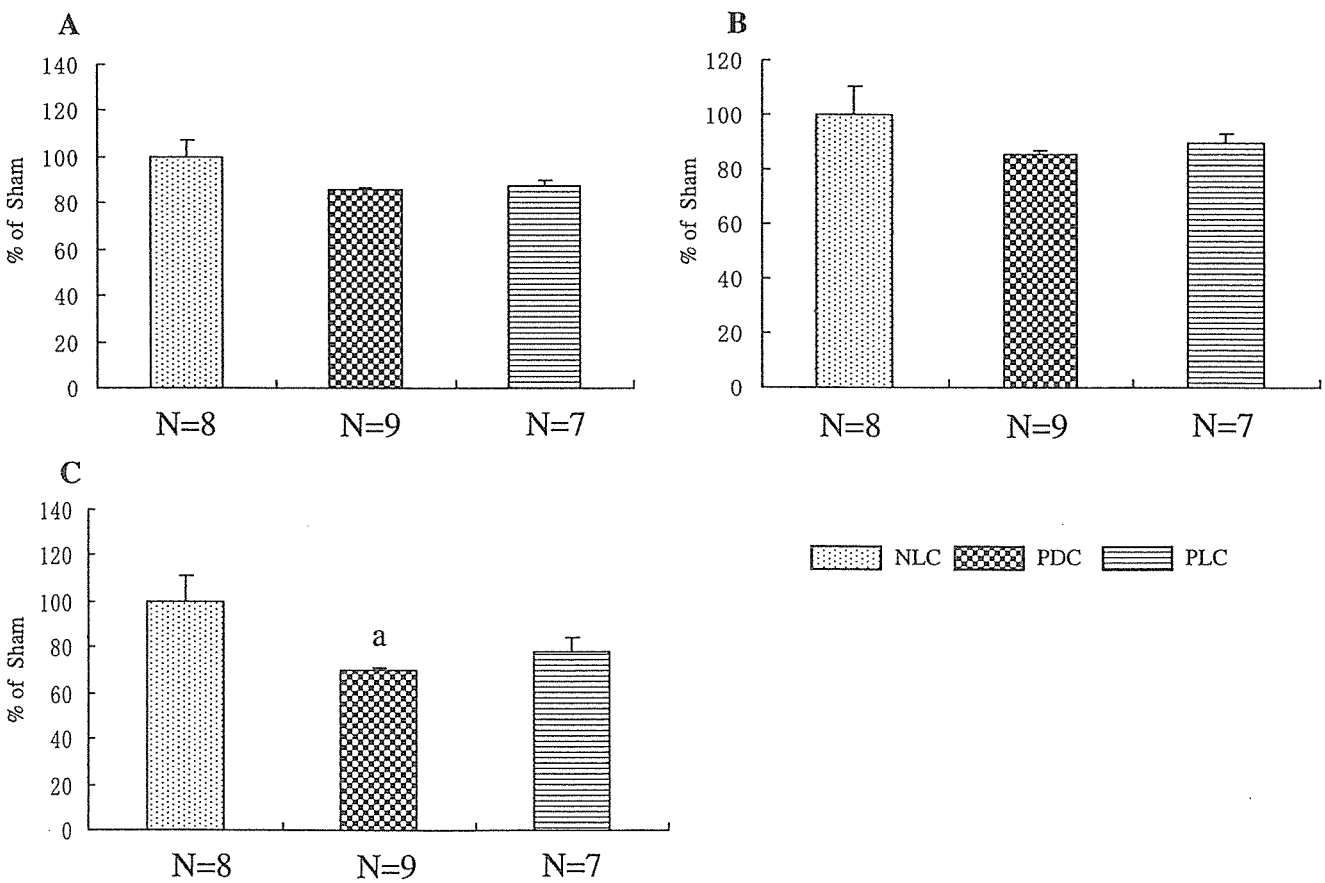

FIG. 8. Influence of early lighting conditions (ELC) on *N*-methyl-D-aspartate receptor (NR)1, NR2A and NR2B mRNA expression in the hippocampus as determined by real-time-quantitative polymerase chain reaction. (A) Influence of ELC on NR1 mRNA levels. (B) Influence of ELC on NR2A levels. (C) Influence of ELC on NR2B levels. Levels of NR2B were lower in prolonged dark phase conditions (PDC) compared with normal lighting conditions (NLC) offspring. $^{a}P < 0.05$, NLC vs. PDC.

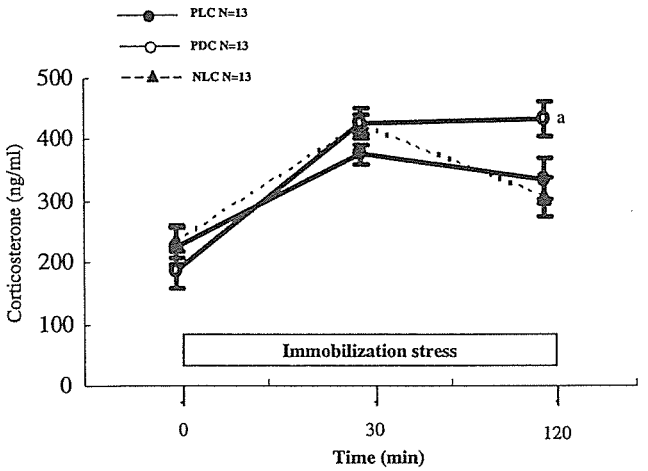

FIG. 9. Prolonged dark phase cycle attenuated negative feedback of corticosterone response to stress. Plasma corticosterone responses at 120 min after the start of stress were significantly higher in prolonged dark phase conditions (PDC) than in normal lighting conditions (NLC) rats ($P < 0.05$) and tended to be higher compared with prolonged light phase conditions (PLC) rats ($P < 0.10$). Results are means ± SEM. $^{a}P < 0.05$, NLC vs. PDC.

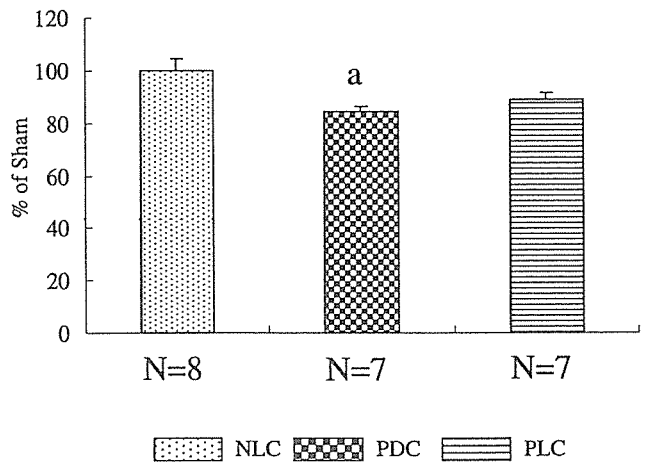

FIG. 10. Influence of early lighting conditions on glucocorticoid receptor mRNA expression in the hippocampus as determined by real-time-quantitative polymerase chain reaction. Levels of glucocorticoid receptor were lower in prolonged dark phase conditions (PDC) than in normal lighting conditions (NLC) offspring. Results are means ± SEM. $^{a}P < 0.05$, NLC vs. PDC. PLC, prolonged light phase conditions.

not cause clear stress responses in rats (Sei *et al.*, 2003). Based on these findings, the severity of stress derived from our paradigm of ELC may be lower than that in chronic circadian changes but higher than in a single phase-shift to some extent. In this context, the reason we could not observe an alteration of maternal care in PLC mothers might be that the stressful influence of light-induced circadian change in the first few days is antagonized by the positive effect of a long photoperiod on maternal behavior.

Effects of early lighting conditions on offspring's activity rhythm

In the neonatal period, maternal care and/or its absence affect diurnal rhythms of activity (Viswanathan & Chandrashekaran, 1985; Shimoda *et al.*, 1986; Viswanathan, 1999; Ohta *et al.*, 2003), corticosterone secretion (Hiroshige *et al.*, 1982a,b,c; Yamazaki & Takahashi, 1983) and Clock gene expressions in the suprachiasmatic nucleus of pups (Ohta *et al.*, 2002, 2003). As early as PND 6, this maternal entrainment gradually decreased in parallel with the development of retinohypothalamic synaptogenesis of pups, becoming directly entrained to the light/dark cycle (Duncan *et al.*, 1986). Our results indicated that the circadian rhythm of adolescent and adult offspring was almost completely synchronized with the normal light/dark cycle. It can be assumed that the altered distribution of the active/inactive cycle in the dam affected the pups' sleep/wake rhythm in the neonatal period but that later these effects could no longer be observed as pups independently entrained to environmental lighting conditions.

Effects of early lighting conditions on offspring's fearfulness and hypothalamic-pituitary-adrenocortical system

In the series of studies using high/low LG dams, the lower nursing activity in dams contributed to the decrement of an active and exploratory coping style and enhanced HPA reactivity to stress among the pups (Liu *et al.*, 1997; Caldji *et al.*, 1998, 2003; Francis *et al.*, 1999b; Menard *et al.*, 2004; Zhang *et al.*, 2005). This was later confirmed by other paradigms using two different strains that vary in the levels of active nursing (Ahmadiyeh *et al.*, 2004; Priebe *et al.*, 2006). As maternal behavior retains the humidity and body temperature of the pup and controls catecholaminergic and HPA activity (Levine, 1967; Stanton *et al.*, 1988; Stanton & Levine, 1990; van Oers *et al.*, 1998; Schmidt *et al.*, 2002), it is conceivable that early adversity, such as low maternal care, produces long-lasting adverse effects on the development of a defensive phenotype, even in adulthood (Liu *et al.*, 1997; Francis *et al.*, 1999b; Gonzalez *et al.*, 2001; Ruedi-Bettschen *et al.*, 2004; Yamazaki *et al.*, 2005; Yoshihara *et al.*, 2005; Priebe *et al.*, 2006).

In examining the corticosterone response to immobilization stress we found that PDC offspring exhibited sustained HPA reactivity to stress but PLC offspring exhibited HPA reactivity similar to that in NLC offspring. Additionally, in two independent behavioral experiments, PDC offspring exhibited a decrement in exploratory behavior in both frequency and duration; however, PLC offspring exhibited an increment in frequency but not in duration of exploratory behavior. The latter inconsistent results of PLC offspring in behavioral parameters might be due to their novelty preference and/or habituation. Although the severity of immobilization stress was quite different from the stress induced by the elevated plus-maze or social interaction test, in accordance with the behavioral phenotype, PDC offspring had a sustained elevated corticosterone response to stress with decreased levels of hippocampal GR mRNA expression compared with NLC offspring. These results are similar to those in other reports of a positive correlation between amounts of active nursing in the dam and the level of active coping style with attenuated HPA response in offspring (Liu *et al.*, 1997; Francis *et al.*, 1999a; Macri *et al.*, 2004; Priebe *et al.*, 2006).

As our early lighting manipulations affected both the dam and litter, it cannot be denied that other factors in addition to active nursing, such as direct effects of the lighting conditions, influenced the development of offspring. However, based on previous studies, it is natural to think, at least in part, that a PDC-induced decrement of maternal care contributed to fearfulness and a sustained HPA response in PDC offspring in our study.

Effects of early lighting conditions on memory and hippocampal N-methyl-D-aspartate receptor 2B mRNA expression

It is often reported that the early rearing experience, such as high/low maternal care (Liu *et al.*, 2000b; Bredy *et al.*, 2003a,b, 2004) and

maternal separation (Francis *et al.*, 2002; Roceri *et al.*, 2002), alters the offspring's memory functioning with hippocampal neuroplasticity. To investigate memory functioning in offspring, we used the object recognition test, which is usually considered to be associated with relatively low stress, to exclude as much as possible a stress response in different ELC-manipulated rats.

Our results revealed that PDC offspring exhibited impairment of short-term memory with decreased hippocampal NR2B mRNA expression. This correspondence between disordered memory and *N*-methyl-D-aspartate functioning was consistent with results in previous reports that suggested that object recognition memory was related to hippocampal NR functioning (Tang *et al.*, 1999; Rampon *et al.*, 2000; Baker & Kim, 2002). PLC offspring showed neither any change in memory functioning nor hippocampal NR expression compared with NLC offspring. Also, in terms of early rearing environment, these results were similar to previous results that reported impaired memory with decreased NR mRNA expression in offspring reared by low LG mothers (Liu *et al.*, 2000b; Bredy *et al.*, 2003b, 2004).

However, although there were no significant differences in hippocampal mRNA levels of the three NR subunits examined and the GR between NLC and PLC offspring, the mRNA levels of NR2B and GR in PLC offspring tended to be down-regulated to some extent (NR2B, $P = 0.15$; GR, $P = 0.07$ compared with NLC offspring) even though there was no decrease in active nursing by their mothers. Based on the trend of those decreases in PLC offspring as well as a trend toward a decrease in NR1 ($P = 0.087$ compared with NLC offspring) and the significant decrease in NR2B and GR in PDC offspring, it can be speculated that changes in ELC may have partially affected the hippocampal mRNA expression of these receptors in these offspring not only through the length of active nursing but also other factors. Actually, Macri *et al.* (2004) suggested that the temporal distribution of maternal care may contribute to the phenotype of offspring with regard to early handling and maternal separation. In addition to the transient changes in daily amounts of maternal care and the altered diurnal distribution of maternal care in PLC mothers, the direct effects of lighting change on offspring might have additionally contributed to the PLC offspring's intermediate phenotypes. In this context, although the precise mechanism remains unknown, it is likely that the combination of low maternal care and light-change-mediated stress may have induced the significant decrease in both NR2B and GR in the PDC group.

In natural conditions, energy-conserving, adaptive adjustments occur among individuals, especially in response to decreasing day length, and are believed to promote survival during the harsh conditions of winter (Bronson, 1985). Laboratory rats are sensitive to reproductive inhibition by exposure to a short photoperiod alone and tend to avoid rearing pups under the harsh conditions that would decrease their chance of survival (Leadem, 1988; Heideman & Sylvester, 1997; Lorincz *et al.*, 2001). Although our photoperiodic manipulation was artificial, the PDC-induced decrement of maternal behavior might be considered as one of the corresponding behavioral changes under harsh conditions, not suitable for rearing pups. From another point of view, according to the maternal mediation hypothesis, mothers mediate environmental information into the developing nervous system of pups in the neonatal period via maternal behavior, which in turn determines defensive responses to threatening situations in adult offspring (Cameron *et al.*, 2005). Our observations partially support the hypothesis that a short photoperiod (PDC), which is an environmental cue for harsh conditions, decreases maternal care and communicates the information into the pup's neurodevelopment.

As there are distinct differences in the daily rhythm of maternal behavior and photoperiodic responsiveness between nocturnal and diurnal animals, it is difficult to compare results from rodent studies with those from human studies. However, previous human infant studies indicated the benefits of structured care under a regular 12-h/12-h light/dark cycle on the somatic growth and development of the sleep/wake rhythm (Sander *et al.*, 1972; Mann *et al.*, 1986; Miller *et al.*, 1995). In addition, Ohta *et al.* (2006) recently demonstrated that altered photoperiodic conditions induce acute and lasting effects on the developing biological clocks in mice. Considering findings from rodent studies, including our study, as well as human studies, the possibility that ELC play an important role in the neurodevelopment of offspring through mother–infant interactions is postulated.

In summary, we found that the early lighting environment altered maternal care and directly and/or indirectly affected the development of emotionality and memory functioning in their offspring, which underlies long-standing changes in neurobehavioral systems. These effects are quite similar to those induced by other early adverse paradigms such as maternal separation and low maternal care. Taken together, it is postulated that ELC are, at least in part, important factors controlling early mother–pup interactions and the neurodevelopment of offspring. However, it cannot be ruled out that the effect of early lighting change is involved in our experimental paradigm. Therefore, further studies are required to elucidate whether the effect of photoperiodic duration or circadian change is more critical in early mother–pup interactions and the neurodevelopment of emotionality and memory functioning in offspring.

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Abbreviations

ELC, early lighting conditions; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenocortical; LG, licking/grooming; NLC, normal lighting conditions; NR, *N*-methyl-D-aspartate receptor; PDC, prolonged dark phase conditions; PLC, prolonged light phase conditions; PND, postnatal day; RT-PCR, real-time-quantitative polymerase chain reaction.

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Research report

Long-term outcome of antidepressant-refractory depression: The relevance of unrecognized bipolarity

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Abstract

Background: The long-term outcome of antidepressant-refractory depression is not well known. Therefore, the present study investigated the long-term outcome of 26 antidepressant-refractory patients with depression, whom we had studied and treated in 1995.

Methods: Before being classified as nonresponse, these patients had been treated adequately with at least two tricyclic or heterocyclic antidepressants (a minimum of the equivalent of 150mg of imipramine for 4 weeks). In 1995, 21 of 26 patients were diagnosed with unipolar depression, while 5 were diagnosed with bipolar depression. Mean follow-up was 5.7 years (range: 1–7 years) and changes in diagnosis, remission and treatment efficacy were evaluated.

Results: Following the long-term follow-up, 13 patients achieved full remission and demonstrated high social functioning (mean GAF score, 91). A further four depressed patients experienced full remission; however, subsequent recurrence was observed. In total, 17 of 26 patients experienced remission at least once during the long-term follow-up period despite the chronic depressive episodes observed at study entry. Adjuvant treatment with lithium, dopamine receptor agonists or thyroid hormone was effective for promoting full remission. Among the 21 patients initially diagnosed with unipolar depression in 1995, diagnoses were changed to bipolar disorder in 5 cases.

Limitations: This naturalistic study had a relatively small sample size and treatment was not controlled.

Conclusions: Long-term follow-up revealed that a substantial proportion of antidepressant-refractory depression is comprised of bipolar disorders. In addition, augmentation therapies are effective for promoting full remission among chronically depressed patients without a risk of serious side effects.

Keywords: Antidepressant-refractory depression; Antidepressant-resistant depression; Mood disorder; Augmentation therapy; Lithium; Dopamine receptor agonist

1. Introduction

Antidepressants are clearly beneficial in the treatment of major depression. However, response rates to a variety of antidepressants (classified by more than 50% reduction in depression rating scales) are generally 60–

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70% (Thase and Rush, 1995; Janicak et al., 2002). However, the remaining 30–40% of patients do not sufficiently improve, even if they take the adequate or maximum doses of antidepressants for a sufficient period of time (i.e., a minimum of the equivalent of 150 mg of imipramine for 4 weeks). Half of depressed patients who are non-responders to their first antidepressant may respond following a change to a second antidepressant (typically a drug with a different pharmacological profile) or a pharmacologically reasonable combination therapy consisting of two antidepressants (Thase and Rush, 1995; Lam et al., 2002). Despite multiple pharmacological interventions, 5–10% of patients remain depressed (Inoue et al., 2002). Depressed patients, who are treatment-resistant (or refractory) to multiple, adequate antidepressant treatments, have been widely observed and extensively studied during the last two decades (Thase and Rush, 1995; Stimpson et al., 2002).

As indicated by Roose (1990), the most important issue in treatment-resistant depression is its definition. The term "(antidepressant) treatment-resistant depression" should be applied to patients who do not respond to an antidepressant given in adequate amount for a sufficient duration (Roose, 1990; Halpern and Glassman, 1990), and should be distinguished from intolerant patients, who are unable to tolerate an adequate dose of an antidepressant due to adverse effects, and non-compliant patients; these latter two cases are referred to as pseudorefracotry depression (Möller, 1991). Thase and Rush (1995) defined "(antidepressant) treatment-refractory depression" as treatment nonresponse (i.e., persistence of significant depressive symptoms) despite at least two treatment trials with drugs from different pharmacological classes, each used in an adequate dose for a sufficient period of time (Thase and Rush, 1995). This definition is reasonable because clinical findings suggest that 20–70% of nonresponders will respond to a different type of antidepressant (Thase and Rush, 1995). However, most studies on antidepressant-resistant depression or antidepressant-refractory depression have investigated depressed patients who had not responded to only one adequate antidepressant treatment (for a review, see Thase and Rush, 1995; Stimpson et al., 2002). The primary reason why these studies have not investigated patients who have not responded to two or more antidepressants with different pharmacological properties is the difficulty of obtaining a large sample size as a result of this stricter definition of antidepressant-refractory depression.

The effects of augmentation therapies utilizing lithium or thyroid hormones have been studied in open trials and randomized controlled trials. The results of these studies have shown that these augmentation therapies are effective in the treatment of refractory depression during the relatively short period of the clinical trials (Thase and Rush, 1995; Stimpson et al., 2002). However, there has been no study of the long-term use of augmentation therapies for depression in naturalistic settings.

The long-term outcome and prognosis of antidepressant-refractory depression is not well known, although several clinical studies have investigated the treatment and symptomatology of antidepressant-refractory depression (Roose et al., 1986; Thase and Rush, 1995). We reported the demographic characteristics and symptoms of antidepressant-refractory depression and the efficacy of augmentation therapies (Inoue et al., 1996a). In 1995, 34 depressed patients (9 bipolar, 25 unipolar) were studied and a follow-up study of these patients with antidepressant-refractory depression was conducted. In the present study, outcome for antidepressant-refractory depression is reported by a prospective long-term follow-up study. To assess full remission and improvement of depression, we used the Global Assessment of Functioning (GAF) Scale (DSM-III-R) rather than the Hamilton Depression Rating Scale (HDRS).

2. Methods

2.1. Study design

The present research was a naturalistic follow-up study of antidepressant-refractory depression, including both bipolar and unipolar depressed patients. In 1995, we investigated the demographic characteristics, symptoms and treatment responses to augmentation therapies of 34 antidepressant-refractory depressed patients (9 bipolar, 25 unipolar) (Inoue et al., 1996a). Each patient had satisfied the DSM-III-R criteria for major depression with melancholia or bipolar disorder, depressed in the current depression episode. Inclusion criteria required moderate depressed symptoms after adequate treatment with two or more antidepressants (i.e., a minimum of the equivalent of 150 mg of imipramine for 4 weeks). In 1995, tricyclic and tetracyclic antidepressants were available in Japan; however, monoamine oxidase inhibitors, serotonin-noradrenaline reuptake inhibitors and selective serotonin reuptake inhibitors had not yet been approved. According to the Clinical Global Impressions (CGI) scale (National Institute of Mental Health, 1985), treatment efficacies were evaluated as worse, no

change, minimally improved, much improved or very much improved. Patients rated very much improved or much improved were regarded as the responders. Following the completion of this study, these patients continued to attend our department and receive treatment. Treatment, symptoms and social functioning were prospectively recorded for 7years, from 1995 until 2002.

2.2. Patients

Of the subjects in our 1995 study, patients who were followed up for one or more years were enrolled in the present study. Depressed patients with brain MRI or EEG evidence of organic brain disease were excluded from the present study. Patients with concurrent significant medical problems were also excluded from the research. From the 34 patients in the 1995 study, a total of 26 patients (5 bipolar and 21 unipolar, according to the 1995 diagnoses) were investigated.

2.3. Assessment

The authors investigated the current diagnosis, severity of symptoms, medication, social functioning (employment, etc.), GAF scores, whether the patients had experienced full remission for 7years and whether the patients had discontinued medication due to full remission. Treatment efficacies of various augmentation therapies have been evaluated according to the CGI scale (National Institute of Mental Health, 1985).

Clinical pharmacological studies often use a score of 7 or less on the 17-item HDRS (Thase and Rush, 1995) as a definition of remission; however, symptomatic improvement does not fully account for the functional recovery observed in fully remitted patients

Table 1

(A) Summary of diagnoses and final outcomes for bipolar patients with refractory depression in the present study

No.	Age	Sex	1995 Dx	Final Dx	Follow-up (years)	Final GAF	Severity in 1995	Final severity	Social functioning
1	25	M	BPD(I)	BPD(I)	5	30	Mild	Severe	Inpatient
2	66	M	BPD(I)	BPD(I)	7	25	Remission	Severe	Inpatient
3	51	M	BPD(II)	BPD(II)	7	70	Remission	Mild	Laid off
4	37	M	BPD(II)	BPD(II)	7	100	Mild	Remission	Work
5	53	M	BPD(II)	BPD(II)	7	90	Mild	Remission	Living at home
6	43	F	MD(S)	BPD(I)	7	100	Mild	Remission	Work
7	51	F	MD(S)	BPD(II)	6	90	Mild	Remission	Living at home
8	33	F	MD(S)	BPD(II)	7	65	Moderate	Mild	Housewife
9	51	M	MD(S)	BPD(II)	7	60	Moderate	Moderate	Living at home
10	56	M	MD(R)	BPD(II)	7	90	Mild	Remission	Work

(B) Summary of diagnoses and final outcomes among unipolar patients with refractory depression in the present study

1	55	M	MD(S)	MD(S)	7	70	Mild	Mild	Living at home
2	36	M	MD(S)	MD(S)	1	70	Mild	Mild	Work
3	73	F	MD(S)	MD(S)	7	70	Moderate	Mild	Housewife
4	38	F	MD(S)	MD(S)	7	70	Moderate	Mild	Work
5	69	F	MD(S)	MD(S)	7	60	Mild	Moderate	Living at home
6	59	F	MD(S)	MD(S)	2	80	Mild	Remission	Housewife
7	76	F	MD(S)	MD(S)	7	90	Moderate	Remission	Living at home
8	66	M	MD(S)	MD(S)	2	90	Remission	Remission	Living at home
9	29	F	MD(S)	MD(S)	3	90	Mild	Remission	Housewife
10	43	M	MD(S)	MD(S)	4	90	Mild	Remission	Work
11	62	F	MD(R)	MD(R)	7	70	Mild	Mild	Housewife
12	43	F	MD(R)	MD(R)	7	70	Mild	Mild	Housewife
13	66	F	MD(R)	MD(R)	7	50	Remission	Moderate	Living at home
14	54	F	MD(R)	MD(R)	3	90	Remission	Remission	Housewife
15	69	F	MD(R)	MD(R)	2	90	Remission	Remission	Living at home
16	38	M	MD(S)	MD(R)	7	100	Remission	Remission	Work

M, male; F, female; Dx, diagnosis; BPD(I), bipolar disorder I; BPD(II), bipolar disorder II; MD(S), major depression, single episode; MD(R), major depression, recurrent. Age, final Dx, final severity and social function were evaluated at the final follow-up visit. Remission denotes full remission.

(Lenderking et al., 1999; Lecrubier, 2002). Although DSM-III-R defines full remission as "no significant signs or symptoms of the disturbance during the past 2 months", the achievement of an asymptomatic state with a full, functional recovery (i.e., a complete recovery) has traditionally been viewed as full remission in depression and is a fundamental goal for the treatment of depression (Kraepelin, 1913; Weitbrecht, 1973; Lecrubier, 2002). Therefore, a score of 80 or higher on the GAF scale is a good and straightforward indicator of full remission.

3. Results

3.1. Diagnosis

After the mean follow-up period of 5.7 years, among the 15 patients with major depression, single episode, 1 patient was diagnosed with major depression, recurrent, 4 patients were diagnosed with bipolar disorder, while the diagnosis of the remaining 10 patients remained major depression, single episode (Table 1A and B). Of six patients diagnosed in 1995 with major depression, recurrent, one patient was diagnosed with bipolar disorder and five patients were diagnosed with major depression, recurrent. The 1995 diagnoses of five bipolar patients were not changed (Table 1A). Of the 21 unipolar depressed patients, 5 diagnoses were changed to bipolar disorder.

In 2002, the 26 subjects consisted of 10 bipolar patients, 6 patients with major depression, recurrent, and 10 patients with major depression, single episode.

3.2. Outcome

During the long-term follow-up period, we confirmed that 8 of 10 patients diagnosed with bipolar depression and 9 of 16 patients diagnosed with unipolar depression achieved full remission (Table 2A). Recurrence occurred in only two of nine patients diagnosed with remitted unipolar depression and one patient with recurrence remitted again; however, one patient had moderate depression at the final observation after two recurrences and one remission (Table 2B). Among patients diagnosed with bipolar depression, 5 of 10 experienced a recurrence of depression and 7 of 10 experienced hypomanic/manic episodes.

After the follow-up period, 13 of 26 patients (5 of 10 bipolar patients and 8 of 16 unipolar patients) finally achieved full remission and demonstrated high social functioning (mean GAF score, 91) (Table 1A and B). In reality, 8 of 13 remitted patients had returned to work (3 patients as housewives) by the final observation. Among these fully remitted patients, five discontinued antidepressant treatment (one bipolar patient continued to take valproic acid), and eight remained on antidepressants, dopamine receptor agonists or mood stabilizers (data not shown). The four remaining depressed patients experienced full remission but recurrence was observed thereafter. Including these patients with full remission and recurrence, 17 of 26 patients experienced full remission at least once during the follow-up period.

After the follow-up period, 13 patients (5 bipolar, 8 unipolar) still had depression: 2 severe, 2 moderate and 9 mild (Table 1A and B). In 7 of 13 patients,

Table 2

(A) Summary of recurrence, remission and effective augmentation treatments among bipolar patients with refractory depression in the present study

No.	Recurrence after 1995	Remission after 1995	Effective augmentation
1	1M, 1D	Yes	Pergolide
2	1M, 1D	Yes	Li, T4
3	3D	Yes	Bromocriptine, T4
4		Yes	Bromocriptine
5		Yes	Bromocriptine
6	1M, 1D	Yes	
7	1hM	Yes	Li, Pergolide
8	1hM	No	
9	1hM	No	
10	1hM, 1D	Yes	Li, T4

(B) Summary of recurrence, remission and effective augmentation treatments among unipolar patients with refractory depression in the present study

1		No	
2		No	
3		No	
4		No	
5		No	
6		Yes	Li, T4
7		Yes	Bromocriptine
8		Yes	Bromocriptine
9		Yes	Bromocriptine
10		Yes	Bromocriptine
11		No	
12		No	
13	2D	Yes	Bromocriptine
14		Yes	Bromocriptine
15		Yes	Pergolide
16	1D	Yes	Bromocriptine

M, manic episode; hM, hypomanic episode; D, depressive episode (number indicates the number of episodes). Remission denotes full remission. Effective augmentation therapies are indicated when full remission was achieved. T4, L-thyroxine; Li, lithium carbonate.

depression has continued since diagnosis in 1995 despite adequate antidepressant treatment and various augmentation therapies: one moved to a different hospital because of home relocation in year 1; however, the other 6 patients now receive treatment as outpatients at our department.

3.3. Treatment

The 26 patients in this study received two or more antidepressants and various augmentation therapies. Four patients were treated with ECT, but the efficacy was transient and all patients relapsed (data not shown). Augmentation therapies contributory to full remission were noted and shown in Table 2A and B. In 9 of 13 patients with full remission at the final evaluation, the addition of dopamine receptor agonists (bromocriptine or pergolide) to adequate doses of conventional antidepressants was effective. The combination of lithium and dopamine receptor agonists with antidepressants was effective in one patient. The combination of lithium and L-thyroxine with antidepressants was effective in two patients.

All patients received lithium augmentation trials and 4 (1 bipolar, 3 unipolar according to 1995 diagnosis) of 26 patients (15%) were lithium responders. As the diagnoses were changed after the long-term follow-up, these four patients were three bipolar and one unipolar patients at the final evaluation.

Long-term use of various augmentation therapies, such as lithium, thyroid hormone and dopamine receptor agonists, did not cause any serious side effects or any sequelae. No cases of rapid cycling were observed during the follow-up period. Among the patients in this study, the long-term use of antidepressants clearly did not cause mixed or (hypo)manic episodes.

4. Discussion

In the present study, subsequent to long-term follow-up (mean: 5.7 years, range: 1–7 years), the diagnoses of 5 (24%) of 21 patients with unipolar antidepressant-refractory depression were changed to bipolar disorder. There has been no research conducted on the conversion from unipolar depression to bipolar disorder in antidepressant-refractory depression. However, in non-refractory depression, a similar finding that 70 (12.5%) of 599 unipolar depressed patients were converted to bipolar disorder in a prospective observation period of up to 11 years was reported by Akiskal et al. (1995). In 1995, our patients had been depressed for an average of 5 years, indicating that these unipolar depressed patients were observed for an average of 11 years, a period similar to the study by Akiskal et al. (1995). In comparison to non-refractory depression, there may be more converters from unipolar to bipolar in antidepressant-refractory depression. Recommendation of more intensive use of mood stabilizers might be considered after the completion of future research. Furthermore, the increased tendency for patients diagnosed with unipolar antidepressant-refractory depression to become manic in comparison to non-refractory unipolar depression should be noted. In the end, 10 (38%) of 26 treatment-refractory depressed patients were diagnosed as bipolar at the final evaluation. This relatively high prevalence of bipolar disorder is comparable to the 46% prevalence of bipolar I and II disorders in patients with antidepressant-resistant depression recently observed by Sharma et al. (2005). As antidepressant-refractory depression includes more bipolarity, this suggests that bipolarity plays an important role in the pathophysiology of a subgroup of patients with antidepressant-refractory depression.

Patients diagnosed with depression associated with both unipolar and bipolar mood disorders are believed to be able to completely recover and achieve full remission (Kraepelin, 1913; Weitbrecht, 1973). However, in previous studies, long-term observation until full remission has not been attained. By the 1995 evaluation in the present study, patients with antidepressant-refractory depression had suffered from chronic depression for an average of 5 years, for unipolar depression, and 3.4 years, for bipolar depression. Nevertheless, during the long-term follow-up of the present study, we confirmed that 8 of 10 bipolar depressed patients and 9 of 16 unipolar depressed patients achieved full remission. While full remission of these depressed patients is likely in principle, as observed by Emil Kraepelin (1913), it is clinically important to confirm in naturalistic settings that chronic antidepressant-refractory depression is not a subgroup that is unable to achieve full remission.

During the follow-up period, recurrence occurred in only 2 of 10 remitted unipolar depressed patients and these patients were remitted thereafter again. In bipolar depressed patients, 5 of 10 patients experienced recurrence of depression and 7 of 10 patients experienced hypomanic/manic episodes. Accordingly, as also shown in the 1995 study, bipolar antidepressant-refractory patients had more episodes than unipolar antidepressant-refractory depressed patients. The prevention of such mood episodes by mood stabilizers might be important for these bipolar

antidepressant-refractory depressed patients, although they continue to have chronic episodes of depression for years. However, as half of the bipolar patients had been diagnosed with unipolar depression in 1995 because of a lack of previous manic or hypomanic episodes, they could not receive mood stabilizers until the final diagnosis was made. Therefore, the diagnosis of bipolar depression before the first manic/hypomanic episode is an important issue.

For effective treatment of refractory depression, several limitations of our study must be considered: the choice of augmentation therapies used for our patients was not controlled, and their efficacies were evaluated based on the CGI scale. Nevertheless, we can suggest that lithium, L-thyroxine and dopamine receptor agonists, in combination with antidepressants, are effective treatments for patients with either unipolar or bipolar antidepressant-refractory depression. Meta-analyses based on placebo-controlled double-blind studies have confirmed the efficacy of lithium, but not triiodotyronine, for refractory depression (Aronson et al., 1996; Bauer and Döpfmer, 1999). However, a systematic review indicated that even the evidence for lithium augmentation is very weak (Stimpson et al., 2002). Despite limited evidence, clinicians must pursue effective pharmacological therapies for antidepressant-refractory depression for the benefit of these patients. L-Thyroxine and dopamine receptor agonists have not been investigated by randomized controlled trials for antidepressant-refractory depression; however, their efficacies were reported in open-labeled trials (Inoue et al., 1996b; Bauer et al., 1998). The present study showed that the addition of dopamine receptor agonists was effective in 9 of 13 patients with full remission as the final evaluation, the addition of lithium and a dopamine receptor agonist was effective in 1 patient, and the addition of lithium and L-thyroxine was effective in 2 patients. Accordingly, these augmentation therapies were considered helpful for these remitted patients at the final evaluations in long-term naturalistic observations without any serious side effects.

Interestingly, one of four lithium responders began the follow-up with a bipolar disorder; however, these responders consisted of three bipolar and one unipolar patients by the conclusion of the study. Previous studies have primarily examined the effect of lithium augmentation among unipolar depressed patients. However, with only a small number of bipolar depressed patients, a meta-analysis could not draw conclusion as to whether bipolar patients are more or less likely responders compared with unipolar depressed patients (Bauer and Döpfmer, 1999). Future studies should compare the response rate to lithium augmentation among unipolar and bipolar depressed patients who have been diagnosed based on long-term observation.

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Prefrontal Cortex and Amygdala Volume in First Minor or Major Depressive Episode After Cancer Diagnosis

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Background: *Major and minor depressive episodes in cancer patients are frequent and are frequently seen as the first depressive episode in a patient's life. However, the neurological basis of these depressive episodes remains largely unknown.*

Methods: *Subjects were 51 breast cancer survivors (BCS) who had no history of any depressive episode before the cancer diagnosis (11 BCS with a history of a first minor depressive episode after cancer diagnosis, 11 BCS with a history of a first major depressive episode after cancer diagnosis, and 29 BCS with no history of any depressive episode after cancer diagnosis). We analyzed the prefrontal cortex (PFC) and amygdala volumes in a 1.5-Tesla Magnetic Resonance Imaging scanner. We characterized the structural correlates of depression using two complementary approaches. The first was voxel-based morphometry (VBM) that allowed us to scan the entire brain for reactive gray matter deficit. The second was classical volumetry focusing on the amygdala.*

Results: *Voxel-based morphometry revealed no brain region, including PFC, for which volume was significantly different among the three groups. There were trend-level differences in the left amygdala volume in the manual tracing method among the three groups. The left amygdala volumes in the subjects with a first minor and/or major depressive episode were significantly smaller than in those with no history of any depressive episode.*

Conclusions: *It might be suggested that amygdala volume was associated with a first minor and/or major depressive episode after cancer diagnosis.*

Key Words: Depressive disorder, magnetic resonance imaging, voxel-based morphometry (VBM), prefrontal cortex (PFC), amygdala

Becoming aware of the diagnosis of cancer in oneself is a stressful life event. Although not all cancer patients develop depressive episodes, some patients experience major depressive episodes and others experience minor depressive episodes which are symptomatologically less severe than major depressive episodes. Previous studies have reported that 1% to 54% and 4% to 35% of cancer patients, respectively, experience major and minor depressive episodes (Chochinov 2001; Hotopf et al 2002; Uchitomi et al 2003). These depressive episodes in cancer patients have generally been assumed to be reactive and of short duration and are frequently seen in the clinical oncology setting as the first depressive episode in their patient's life (Chochinov 2001). However, the neurobiological differences and similarities between patients showing first major and/or minor depressive episode after cancer diagnosis remain largely unknown.

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In a previous study, we reported the lack of association between hippocampal volume and the first major depressive episode after cancer diagnosis (Inagaki et al 2004). In this previous report, we did not take into consideration minor depressive episodes or investigate other brain regions potentially associated with these depressive episodes. Minor depressive episodes can also, like major depressive episodes, have a negative impact (Evans et al 1999), such as giving rise to suicidal tendencies (Henriksson et al 1995). Most research in depressive symptomatology indicates that there are no qualitative differences between minor and major depressive episodes (Cuijpers et al 2004; Kendler and Gardner 1998). In a volumetric study using magnetic resonance imaging (MRI), it was demonstrated that subjects with late-life–onset minor depression had also, like patients with major depression, a smaller prefrontal cortex (PFC) as compared with healthy subjects (Kumar et al 1998).

Previous studies have demonstrated disruption of function and decrease in the volume of brain regions associated with emotions/stress response in cases of major depression. It has been demonstrated that the PFC cerebral blood flow and glucose metabolism are pathologically inactivated and that the PFC volume is reduced (Botteron et al 2002; Bremner et al 1997, 2002; Drevets et al 1997) in major depression. On the other hand, cerebral blood flow and glucose metabolism of the amygdala have been reported to be pathologically activated in major depression (Abercrombie et al 1998; Drevets et al 2002), and the volume of the amygdala has been reported to differ from that in healthy control subjects (Frodl et al 2002; Mervaala et al 2000; Sheline et al 1998). Furthermore, these two regions have also been suggested to be associated with emotional processing in major depression (Siegle et al 2002).

Because the PFC has a complex structure and exhibits heterogeneity among subjects, the boundaries of the different areas of the PFC are sometimes difficult to define. Voxel-based morphometry (VBM) is extremely useful in that it allows facile assessment of the anatomical differences among brain regions, including the PFC, without any operational bias toward those brain structures that have identifiable boundaries (Ashburner and

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