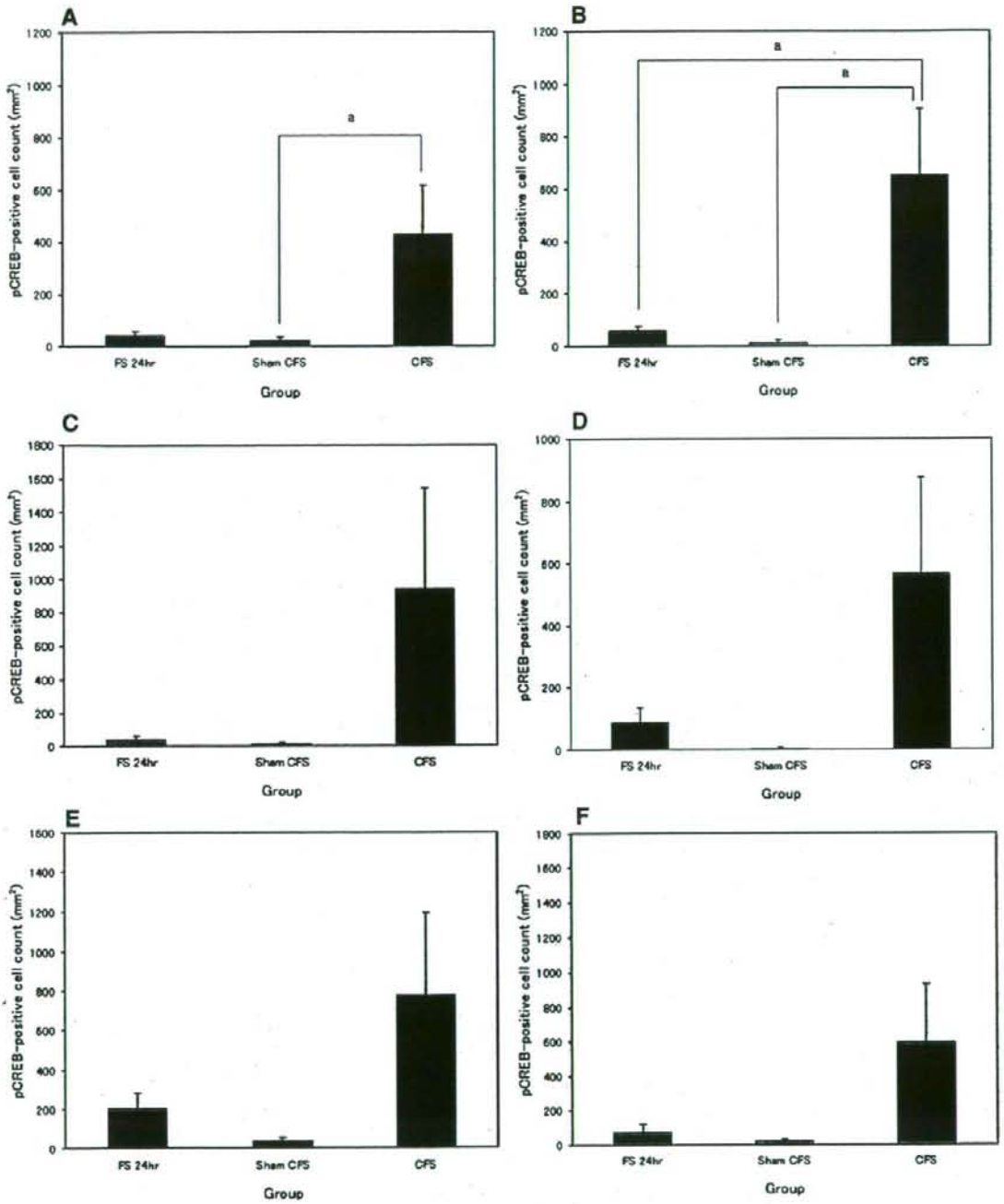

Fig. 3. Effect of sham footshock, footshock, sham conditioned fear stress, and conditioned fear stress on pCREB immunoreactivity in the amygdala of rats. Results are means with S.E.M. and are expressed as pCREB positive cell nuclei per mm². (A) lateral nucleus of the amygdala; (B) basal nucleus of the amygdala; (C) accessory basal nucleus of the amygdala; (D) central nucleus of the amygdala; (E) medial nucleus of the amygdala; (F) cortical nucleus of the amygdala. *$p < 0.05$ The number of rats per group was as follows: FS 24 h group, $n = 5$; sham CFS 2 h group, $n = 6$; CFS 2 h group, $n = 5$. FS, footshock; CFS, conditioned fear stress.

value=50% higher than the background gray value), (2) nuclei diameter of 4–12 μm (to exclude cell debris and artifacts). The background gray value was determined in a part of each unit area containing no nuclei. The measurement of the background gray value and the pCREB positive cell counting were repeated 3 times, and values were averaged. Semiquantitative cell counting was performed by an investigator who was blinded to the treatment.

2.5. Experimental procedures

2.5.1. Experiment 1: CREB phosphorylation in the amygdala under various conditions

Amygdala CREB phosphorylation was assessed under various experimental conditions. The rats were assigned to one of the following 5 groups. (1) Sham footshock 2 h group: The rats were individually placed in the shock chamber without footshocks and 5 min later, they were returned to their homecages (sham footshock). One hour and 45 min later, they were anesthetized in their homecages and then perfused 15 min later: (2) Footshock 2 h group: After the 5-min footshock, the rats were returned to their homecages. One hour and 45 min later, they were anesthetized and perfused similarly: (3) Footshock 24 h group: After the 5-min footshock, the rats were returned to their homecages. Twenty-four hours later, they were anesthetized and perfused similarly: (4) Sham CFS 2 h group: The rats were individually placed in the shock chamber without footshocks and 5 min later, they were returned to their homecages. Twenty-four hours later, they were individually placed in the shock chamber again without footshocks and 5 min later, they were returned to their homecages (sham CFS). One hour and 45 min later, they were anesthetized and perfused similarly: (5) CFS 2 h group: After the 5-min footshock, the rats were returned to their homecages. Twenty-four hours later, they were individually placed in the shock chamber again without footshocks and 5 min later, they were returned to their homecages. One hour and 45 min later, they were anesthetized and perfused similarly. The number of rats per group was as follows: sham footshock 2 h, $n=6$; footshock 2 h, $n=2$; footshock 24 h, $n=5$; sham CFS 2 h, $n=6$; CFS 2 h, $n=5$. Because the number of rats in the footshock 2 h group was relatively small, statistical analysis was performed to compare only the footshock 24 h, sham CFS 2 h and CFS 2 h groups.

2.5.2. Experiment 2: Effect of extinction on CFS-induced freezing behavior and CREB phosphorylation in the amygdala

Changes of CFS-induced freezing behavior and CREB phosphorylation in amygdala were assessed relative to the extinction process of CFS. Rats were assigned to one of the following 6 groups. (1) Sham CFS expression×1 group: One hour and 45 min after above-mentioned sham CFS, the rats were anesthetized and perfused similarly: (2) Sham CFS expression×2 group: Twenty-four hours after sham CFS, the rats were subjected to the second sham CFS. One hour and 45 min later, they were anesthetized and perfused similarly: (3) Sham CFS expression×3 group: Twenty-four hours after the second sham CFS, the rats were subjected to the third sham CFS. One hour and 45 min later, they were anesthetized and perfused similarly: (4) CFS expression×1 group: One hour and 45 min after CFS, the rats were anesthetized and perfused similarly: (5) CFS expression×2 group: Twenty-four hours after CFS, the rats were subjected to the second CFS. One hour and 45 min later, they were anesthetized and perfused similarly: (6) CFS expression×3 group: Twenty-four hours after the second CFS, the rats were subjected to the third CFS. One hour and 45 min later, they were anesthetized and perfused similarly. During the 5-min sham CFS or CFS operation, the behavior of the rats was recorded on videotape. In the behavior experiment, the number of rats per group was 6, and in the histological experiment, the number of rats per group was as follows: sham CFS expression×1–3 groups, $n=6$; CFS expression×1 group, $n=5$; CFS expression×2, 3 groups, $n=4$.

2.6. Data analysis

Multiple group comparisons were performed using one-way and two-way analysis of variance (ANOVA) and the Bonferroni–Dunn's post hoc test. Simple linear regression analysis was used to evaluate the correlations between percent freezing rate and pCREB positive cell count and between times of expression and pCREB positive cell count using the data from sham CFS expression×1–3 and CFS expression×1–3 groups.

3. Results

3.1. Experiment 1: CREB phosphorylation in the amygdala under various conditions

One-way ANOVA indicated a significant effect of CFS footshock compared to the footshock 24 h group and sham CFS group in the lateral nucleus ($F(2, 9)=4.9$, $p=0.026$) and basal nucleus ($F(2, 9)=6.6$, $p=0.011$), but not in the accessory basal nucleus, ($F(2, 9)=6.6$, $p=0.12$), central nucleus ($F(2, 9)=6.6$, $p=0.074$), medial nucleus ($F(2, 9)=6.6$, $p=0.093$) and cortical nucleus ($F(2, 9)=6.6$, $p=0.092$) of the amygdala (Fig. 2, Fig. 3A–3F). In the lateral nucleus, post hoc comparison showed that the number of pCREB positive cells was greater in the CFS 2 h group than in the sham footshock 2 h group ($p=0.014$) (Fig. 2, Fig. 3A). In the basal nucleus, post hoc comparison showed that the number of pCREB positive cells was greater in the CFS 2 h group than in the sham footshock 2 h group ($p=0.011$) and the footshock 24 h group ($p=0.0054$) (Fig. 2, Fig. 3B).

3.2. Experiment 2: Effect of extinction on CFS-induced freezing behavior

Two-way ANOVA (CFS×expression) indicated a significant main effect of CFS ($F(2, 66)=47.1$, $p<0.0001$) and expression ($F(2, 66)=19.0$, $p<0.0001$) in freezing behavior. Two-way ANOVA also indicated a significant interaction between CFS ($F(2, 66)=47.1$, $p<0.0001$) and expression ($F(2, 30)=21.1$, $p<0.0001$). One-way ANOVA across 6 groups indicated a significant effect of treatment ($F(5, 66)=36.3$, $p<0.0001$). Post hoc comparison indicated a significant difference between CFS expression×1 group and CFS expression×2 group ($p<0.0001$) and

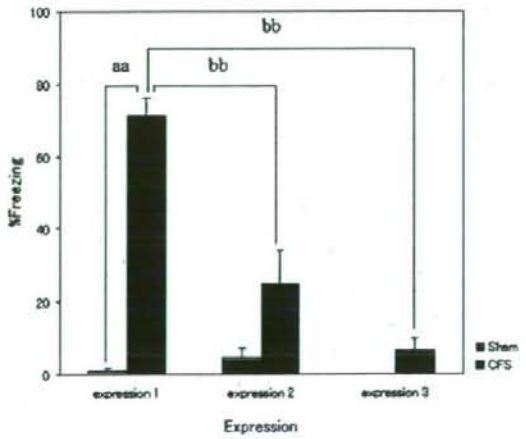

Fig. 4. Effect of repeated exposure to shock box on CFS-Induced freezing behavior in rats. Results are the mean percentage with S.E.M. of freezing scored for a 5-min observation period. $^{aa}p<0.01$, $^{bb}p<0.01$, $n=6$. Expression 1: groups subjected to sham CFS or CFS one time. Expression 2: groups subjected to sham CFS or CFS two times. Expression 3: groups subjected to sham CFS or CFS three times. Sham, sham conditioned fear stress; CFS, conditioned fear stress; N.S., not significant.

between CFS expression×1 group and CFS expression×3 group ($p<0.0001$). Post hoc comparison also indicated significant difference between sham CFS expression×1 group and CFS expression×1 group ($p<0.0001$), but the difference between sham CFS expression×2 group and CFS expression×2 group and the difference between sham CFS expression×3 group and CFS expression×3 group were not statistically significant (Fig. 4).

3.3. Experiment 2: Effect of extinction on CFS-induced CREB phosphorylation in the amygdala

In the basal nucleus of the amygdala, two-way ANOVA (CFS× expression) indicated a significant main effect of CFS ($F(2, 25)=10.2$, $p=0.0037$) and a significant interaction between CFS and expression ($F(2, 25)=21.1$, $p<0.0001$), but the main effect of expression was not statistically significant ($F(2, 25)=10.2$, $p=0.10$). One-way ANOVA across 6 groups indicated a significant effect of treatment ($F(5, 25)=4.7$, $p=0.0037$) in the basal nucleus. Post hoc comparison indicated a significant difference between the sham CFS expression×1 group and CFS expression×1 group ($p=0.0003$), but the difference between the sham CFS expression×2 group and CFS expression×2 group and the difference between the sham CFS expression×3 group and CFS expression×3 group were not statistically significant (Fig. 5B). In the lateral and accessory basal nucleus of the amygdala, the main effects of CFS ($F(2, 25)=1.4$, $p=0.25$; $F(2, 25)=3.8$, $p=0.064$, respectively), the main effects of expression ($F(2, 25)=1.09$, $p=0.35$; $F(2, 25)=1.6$, $p=0.21$, respectively) and the interactions between CFS and expression ($F(2, 25)=2.8$, $p=0.080$; $F(2, 25)=1.8$, $p=0.19$, respectively) were not significant (Fig. 5A, C). In the central, medial and cortical nucleus of amygdala, two-way ANOVA (CFS×session) indicated that the main effects of CFS ($F(2, 25)=5.7$, $p=0.025$; $F(2, 25)=7.1$, $p=0.013$; $F(2, 25)=7.1$, $p=0.014$, respectively) were significant, but the main effects of expression ($F(2, 25)=1.8$, $p=0.19$; $F(2, 25)=1.6$, $p=0.22$; $F(2, 25)=1.4$, $p=0.28$, respectively) and the interactions between CFS and expression ($F(2, 25)=2.4$, $p=0.12$; $F(2, 25)=1.7$, $p=0.20$; $F(2, 25)=1.4$, $p=0.28$, respectively) were not significant. Post hoc comparisons of

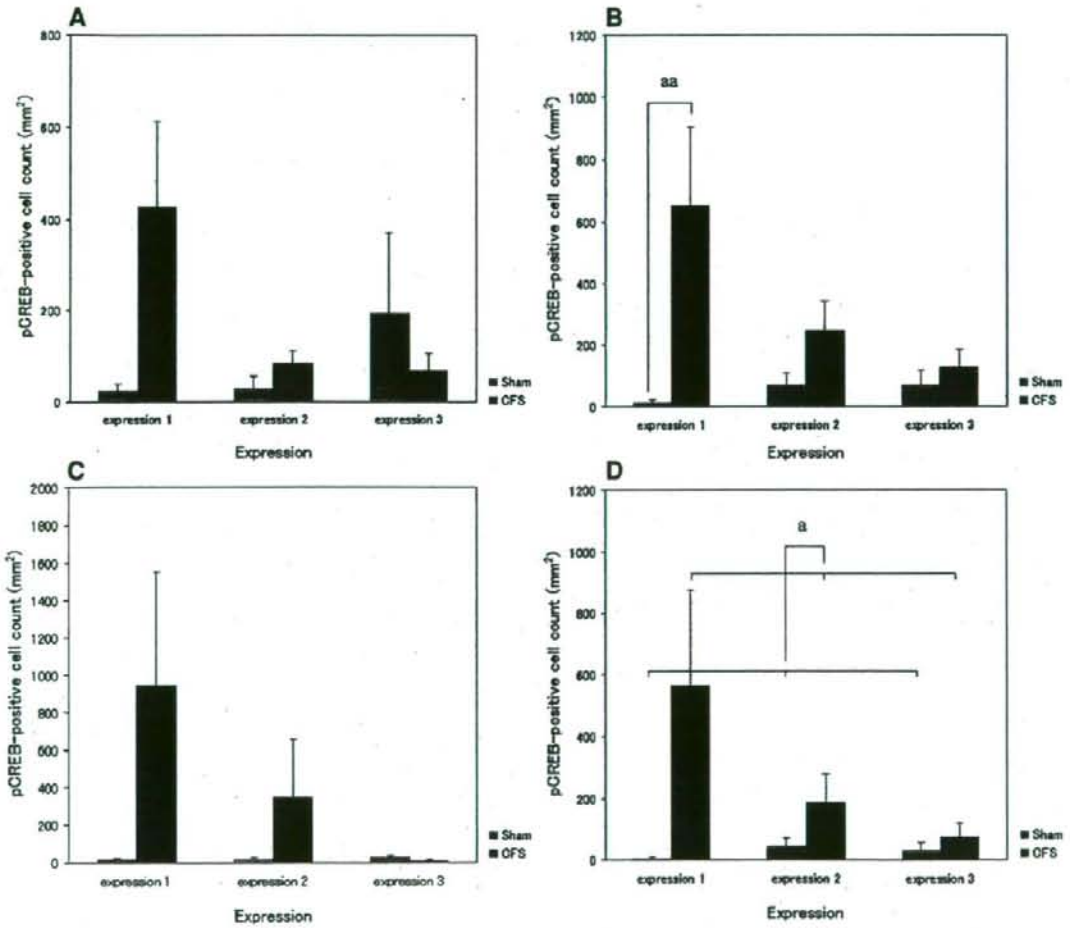

Fig. 5. Effect of repeated exposure to shock box on pCREB immunoreactivity in the amygdala of rats. Results are means with S.E.M. and are expressed as pCREB positive cell nuclei per mm². (A) lateral nucleus of the amygdala; (B) basal nucleus of the amygdala; (C) accessory basal nucleus of the amygdala; (D) central nucleus of the amygdala; (E) medial nucleus of the amygdala; (F) cortical nucleus of the amygdala. $^{a}p<0.05$, $^{aa}p<0.01$ The number of rats per group was as follows: sham CFS expression×1–3 groups, $n=6$; CFS expression×1 group, $n=5$; CFS expression×2, 3 groups, $n=4$. Expression 1: groups subjected to sham CFS or CFS one time. Expression 2: groups subjected to sham CFS or CFS two times. Expression 3: groups subjected to sham CFS or CFS three times. CFS, conditioned fear stress; Sham, sham conditioned fear stress; N.S., not significant.

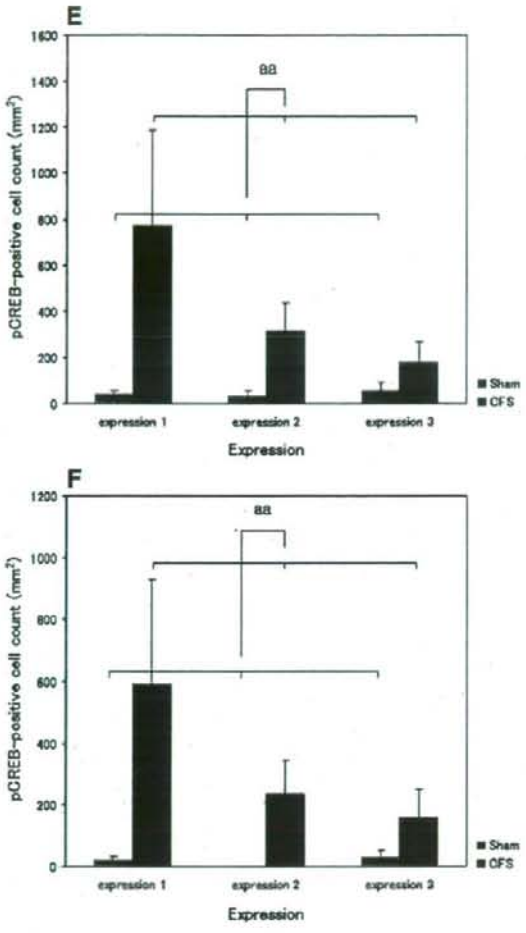

Fig. 5 *(continued).*

two-way ANOVA indicated a significant difference between the sham CFS groups (expression×1 group+expression×2 group+expression×3 group) and CFS groups (expression×1 group+expression×2 group+expression×3 group) in these 3 nuclei ($p=0015$; $p=0.083$; $p=0.0088$, respectively) (Fig. 5D, E, F).

Regression analysis indicated a significant correlation between percent freezing rate and pCREB positive cell count in the lateral ($r=0.43$, $p=0.015$), basal ($r=0.63$, $p=0.001$), accessory basal ($r=0.51$; $p=0.031$), central($r=0.63$, $p=0.002$), medial ($r=0.55$, $p=0.0015$) and the cortical ($r=0.48$, $p=0.0064$) nucleus of amygdala, but there was no significant correlation between times of expression and pCREB positive cell count in any of the subnuclei of amygdala.

4. Discussion

Two different studies have investigated the time course of stress-induced CREB phosphorylation in brain. Stanciu et al. (2001) indicated that contextual-dependent conditioned fear resulted in two peaks of CREB phosphorylation in the parietal cortex, CA1, dentate, basolateral amygdala, and the central amygdala of mice. The first peak occurred 7 min after stress and disappeared at 90 min after stress. The second peak appeared at 180 min after stress and persisted at 360 min after stress. However, they did not investigate CREB phosphorylation at 120 min after stress. Bilang-Bleuel et al. (2002) also showed that forced swimming resulted in two peaks of CREB phosphorylation in

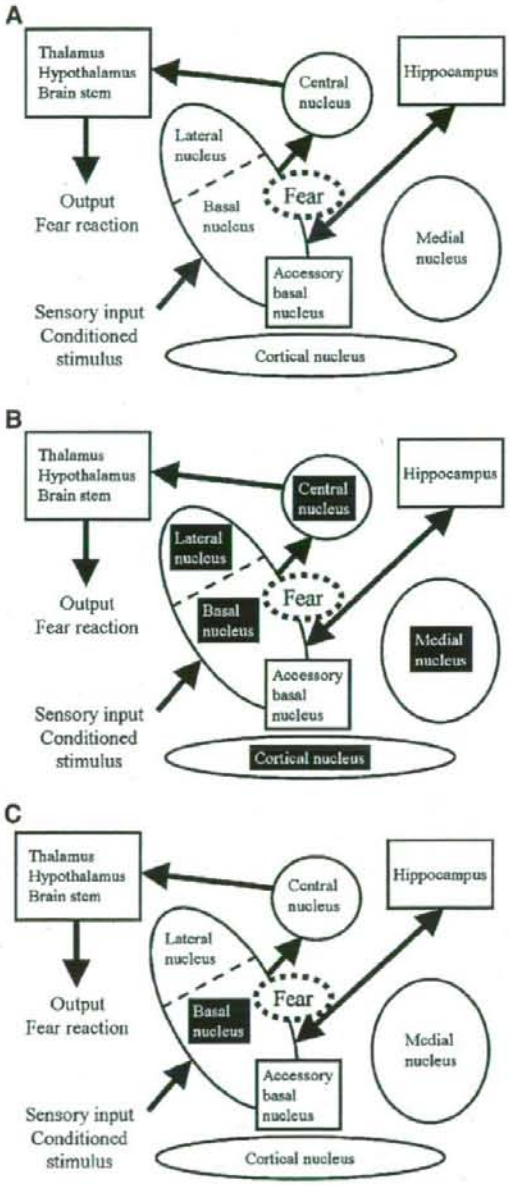

Fig. 6. Scheme representing the changes of CREB phosphorylation in amygdala subnuclei by the expression and extinction of contextual conditioned fear. (A) Neural pathway concerning the expression of contextual conditioned fear. (B) The region where CREB phosphorylation was increased by the expression of conditioned fear (indicated by white letters and black background). (C) The region where CREB phosphorylation was increased in association with the extinction of conditioned fear (indicated by white letters and black background).

the dentate and the neocortex in rats. The first peak occurred at 15 min after stress and disappeared at 60 min after stress. The second peak appeared at 120 min after stress and persisted at 48 h after stress. Based on these studies, the expectation was that stress-induced CREB phosphorylation would result in two peaks, the first within 1 h and the second at more than 2 h. However, the time point of peaks is variable according to the animal species, type of stress, intensity and duration of stress, and the brain location. Data from the present study reflect the second peak of CREB phosphorylation. Further study would be of benefit to investigate the difference in the biological mechanisms between these two peaks.

In Experiment 1, pCREB levels of the lateral and basal nucleus of the amygdala were also increased in the CFS 2 h group. Because amygdala pCREB levels did not increase in the footshock 24 h group, increases in pCREB levels in the amygdala in the CFS 2 h group were not considered to be the result of the footshock 24 h before. Moreover, amygdala pCREB levels in the sham CFS 2 h group did not increase, suggesting that placing rats in the shock chamber without administrating shocks itself did not play a role in this phenomenon. Thus, the increase in pCREB levels in the lateral and basal nucleus in CFS 2 h group likely reflect the neural process of the expression of context-dependent CFS. Indeed, Hall et al. (2001) reported expression of tone-dependent CFS-induced CREB phosphorylation in the lateral and basal nucleus of the amygdala in rats, which is consistent with data from the present study.

In Experiment 2, CFS-induced freezing behavior significantly decreased as the expression of CFS was repeated; this suggests that extinction of CFS occurred. CFS-induced CREB phosphorylation in the basal nucleus also decreased as the expression was repeated. While there was a significant difference between the sham CFS group and the CFS group in expression 1, there was no difference between these groups in expressions 2 and 3, which suggests that CREB phosphorylation in the basal nucleus decreased in parallel with the extinction of CFS-induced freezing behavior. In the central, medial and the cortical nucleus of amygdala, CREB phosphorylation was greater in CFS groups than in the sham CFS groups; however, no time-course effect was observed, as seen in the basal nucleus.

Experiments 1 and 2 demonstrated that CREB phosphorylation was increased by CFS in the lateral, basal, central, medial and the cortical nucleus of amygdala, but CREB phosphorylation was decreased in association with extinction of CFS only in the basal nucleus (Fig. 6B, C). Indeed, Lin et al. (2003) reported that the tone-dependent CFS-induced CREB phosphorylation in the amygdala including the lateral and basal nuclei, decreased as the extinction session was repeated in rats, which is at least partially consistent with our result.

In a previous study, we reported that CFS-induced c-Fos expression in the basal nucleus, but not in the central nucleus of amygdala (Izumi et al., 2006). Although other studies have reported that CFS-induced c-Fos expression in many cortical and subcortical region, the basal nucleus of amygdala was the common location that was associated with CFS (Izumi et al., 2006). Further, pCREB is upstream of c-Fos in the intracellular signal pathway, and pCREB enhances c-Fos production as well as production of other transcription factors. Therefore, functional mapping of pCREB is likely distinct from that of c-Fos. Regardless, the present and previous studies have consistently demonstrated a significant change in the basal nucleus with contextual CFS.

Anatomical and lesion studies have characterized the nuclei of the amygdala that participate in the expression of CFS. Anatomical study indicated that the lateral, basal and central nuclei of the amygdala receive moderate to heavy projections from the sensory-related cortices and the thalamus and that these nuclei are expected to mediate sensory input to the amygdala (Pitkanen, 2000). Further, the central and medial nuclei provide moderate to heavy projections to the subcortical nuclei and the brain stem, and these nuclei are expected to mediate motor, autonomic and hormonal output from the amygdala (Pitkanen, 2000).

Studies regarding tone-dependent CFS indicated that the tone stimulus is received at the auditory organ and is converted to neural information, which subsequently passes through the thalamus and auditory cortex. This neural information is received by the lateral nucleus, which subsequently induces a fear reaction via the central nucleus (LeDoux, 2000). In context-dependent CFS, the contextual stimulus (e.g., exposure to the shock chamber), is received at the sensory organ and is converted to the neural information, which subsequently passes through the thalamus and sensory-related cortices. After being checked against memory in the hippocampus, this information is received by the basal nucleus, which induces a fear reaction via the central nucleus (LeDoux, 2000) (Fig. 6A). In the present study, context-dependent CFS-induced CREB phosphorylation in the basal nucleus and in the lateral nucleus, suggesting that the sensory stimuli except context (e.g., tone or smell) is partly related to fear-conditioning.

Two different neural plasticity-related phenomena occur in brain after the fear memory is retrieved; reconsolidation and extinction. The effects of these two processes on rat behavior are contrary, the former maintains fear related behavior, while the latter reduces it when the fear memory retrieval is repeated (Nader et al., 2000; Debiec et al., 2002). In the present study, the percent freezing rate approximately 70% in the first retrieval, 20% in the second, and 10% in the third. The fact that freezing behavior reduced rapidly in parallel with levels of CREB phosphorylation suggests that the extinction process was dominant over the reconsolidation process in our experimental design. Thus, neural activity of the basal amygdala reflects the extinction process in the case of context-dependent aversive classical conditioning.

We previously reported that CFS-induced c-Fos expression in the basal nucleus (Izumi et al., 2006). The present study demonstrated that CREB phosphorylation in the basal nucleus of the amygdala decreased with the extinction of context-dependent conditioned fear-induced freezing behavior. These data suggest that the basal nucleus of the amygdala plays an essential role in the expression of context-dependent conditioned fear, and this is the first study to demonstrate that CREB phosphorylation in the basal nucleus of the amygdala changes in parallel with the extinction of context-dependent conditioned fear.

Acknowledgements

This study was supported by the Japanese Ministry of Education Grants No. 05454308 (T. K.), No. 06770740 (T. I.), No. 16659299 (T. K.) and by the Itoh Foundation. We thank Dr. Hideki Nakamura for his technical assistance.

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Psychiatry and Clinical Neurosciences 2008; 62: 591–596 doi:10.1111/j.1440-1819.2008.01853.x

Regular Article

Pharmacokinetic interaction between tandospirone and fluvoxamine in the rat contextual conditioned fear stress model and its functional consequence: Involvement of cytochrome P450 3A4

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Aims: In a previous study it was demonstrated that the anxiolytic action of tandospirone, a 5-hydroxytryptamine (5-HT_{1A}) receptor agonist, is facilitated by cytochrome P450 (CYP) 3A4 inhibitors, such as ketoconazole and cimetidine. It is also known that fluvoxamine, a selective serotonin re-uptake inhibitor (SSRI), inhibits CYP3A4. The purpose of the present study was to clarify the pharmacokinetic interaction between tandospirone and fluvoxamine and to evaluate their combined effect in the rat anxiety model.

Methods: The anxiolytic action of co-administration of tandospirone and fluvoxamine was examined using the rat contextual conditioned fear stress model. After testing the conditioned fear, plasma concentrations of tandospirone and its major metabolite 1-(2-pyrimidyl) piperazine were determined.

Results: One day after fear conditioning, both tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) significantly inhibited conditioned freezing and their combination effect was additive. In addition, plasma concentration of tandospirone was increased by fluvoxamine.

Conclusions: There is a CYP3A4-related drug–drug interaction between tandospirone and fluvoxamine. Therefore, fluvoxamine may facilitate the anxiolytic effect of tandospirone via CYP3A4 inhibition.

Key words: conditioned fear, cytochrome P450 3A4, drug interaction, fluvoxamine, tandospirone.

TANDOSPIRONE, AN AZAPIRONE derivative with serotonin-1A (5-HT_{1A}) receptor agonistic action, is used in Japan and China and is similar to buspirone and ipsapirone, the US and European equivalents. These 5-HT_{1A} receptor agonists are used for the treatment of generalized anxiety disorder.[1–4]

It is known that azapirones are mainly metabolized by cytochrome P450 (CYP) 3A4 isoforms.[5–8] Tandospirone is also a substrate of the CYP3A4 isoforms. Several groups have reported that tandospirone is primarily metabolized by CYP3A4 isoforms, and to a lesser extent by CYP2D6, in human liver microsomes.[7,8] In addition we have previously shown that plasma concentrations of tandospirone and buspirone in rats are increased by co-administration of CYP3A4 inhibitors such as ketoconazole and cimetidine, and that the anxiolytic action of these drugs, as evaluated by contextual conditioned freezing, is facilitated via CYP3A4-related drug–drug interaction.[9]

In addition to known CYP3A4 inhibitors, fluvoxamine, a selective serotonin re-uptake inhibitor (SSRI), also possesses moderate CYP3A4 inhibitory

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Received 24 January 2008; revised 13 June 2008; accepted 18 June 2008.

activity, although it is well known as a potent CYP1A2 and CYP2C19 inhibitor.[10–12] SSRI are widely used as first-line drugs for treatment of anxiety and major depressive disorders. If patients fail to respond to SSRI treatment, however, 5-HT_{1A} receptor agonist (i.e. tandospirone or buspirone) augmentation of SSRI might be performed as one of the augmentation strategies.[13–15] Therefore, co-administration of an azapirone and an SSRI with CYP3A4 inhibitory activity may result in drug–drug interaction. Indeed, interaction between buspirone and fluvoxamine in human healthy volunteers has been reported,[16,17] but drug–drug interaction between tandospirone and fluvoxamine has not been clarified. In the present study we investigated the effects of fluvoxamine on plasma concentration of orally administered tandospirone and evaluated the combinative action of these drugs in the rat contextual conditioned fear stress model as a functional consequence.

METHODS

Animals

Male Sprague–Dawley rats (250–350 g), obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were housed in groups of four in a temperature-controlled environment (22 ± 1°C) with free access to food and water. The animals were maintained on a 12-h light/dark cycle (light phase: 06:30–18:30) and tested during the light phase after 1-week acclimatization period. Thirty-two rats were tested for contextual conditioned fear stress (vehicle, tandospirone, fluvoxamine, tandospirone + fluvoxamine; $n = 8$). The tandospirone ($n = 8$) and tandospirone + fluvoxamine ($n = 8$) groups, after testing for conditioned fear stress, were used for the determination of plasma concentration of tandospirone. In a separate experiment, 32 rats were tested for motor activity (vehicle, tandospirone, fluvoxamine, tandospirone + fluvoxamine; $n = 8$)

All experiments were approved by the Hokkaido University School of Medicine Animal Care and Use Committee, and were in compliance with the Guide for the Care and Use of Laboratory Animals.

Drugs

Tandospirone citrate (a gift from Dainippon Sumitomo Pharma, Osaka, Japan) and fluvoxamine maleate (a gift from Solvay Pharmaceuticals, Weesp, The Netherlands) were dissolved in 0.9% sterile saline.

Contextual conditioned fear stress

The rats were individually subjected to inescapable electric footshocks for a total of 2.5 min (five footshocks [2.5-mA scrambled shock, 30 s duration] delivered at intershock intervals of 35–80 s [mean, 60 s]) in a shock chamber with a grid floor (19 × 22 × 20 cm; Medical Agent, Kyoto, Japan). Electric shocks were produced by a shock generator (Model SGS-02D, Medical Agent). One day after footshocks, the rats were again placed in the shock chamber without footshocks and observed for 5 min. During the observation period (i.e. testing) the duration of freezing behavior was recorded using a time-sampling procedure.[18] Every 10 s the behavior that the animal was currently engaged in was classified as either freezing or activity. Freezing was defined as the absence of all observable movement of the skeleton and the vibrissae, except those related to respiration. All other behavior was scored as activity. The animal was classified as either freezing or active according to its behavior throughout the entire 10-s period. The percentage freezing score (freezing (%)) represented the number of entire 10-s periods for which the animal froze.

Drug administration

One day after footshocks, the rats received a single oral administration of tandospirone citrate (60 mg/kg) 1 h before testing. Fluvoxamine maleate (60 mg/kg) was orally administered 4 h before testing (i.e. 3 h prior to administration of tandospirone citrate). Tandospirone citrate and fluvoxamine maleate were given in a volume of 5 mL/kg.

Determination of tandospirone and its major metabolite (1-[2-pyrimidyl] piperazine) concentrations in plasma

After testing, the rats were immediately decapitated and the blood was collected into heparin-containing tubes. Plasma samples were prepared by centrifuging the blood samples at 1000 *g* for 15 min and stored at −20°C until analysis. The free base concentrations of tandospirone and 1-(2-pyrimidyl) piperazine (1-PP) in the plasma samples were determined using liquid chromatography with a tandem mass spectrometry

(LC/MS/MS) system. An appropriate internal standard solution (50 μL) and distilled water (550 μL) were added to each plasma sample (100 μL) and mixed thoroughly. The mixture was then applied to a solid-phase extraction cartridge (Oasis HLB 60 mg/3 mL, Waters Corporation, Tokyo, Japan) preconditioned with water. The cartridge was washed with water (2 mL) and elution objectives were performed using methanol (3 mL). Following addition of 2% (v/v) propylene glycol (500 μL), the samples were refluxed to dryness under N_2 gas and the residues were dissolved in 10 mmol/L ammonium acetate (500 μL). A total of 20 μL of each sample was injected into a high-performance liquid chromatography (HPLC; 10A, Shimazu, Kyoto, Japan) with a YMC Hydrosphere C18 column (5 μm particle size, 75 × 2.0 mm, YMC, Kyoto, Japan). For analysis of tandospirone and 1-PP, the mobile phase consisted of 10 mmol/L ammonium acetate (solvent A) and methanol (solvent B). Initially, the mobile phase consisted of 80% A and 20% B and then changed to 20% A and 80% B with a linear gradient over 30 s and a flow rate of 0.3 mL/min. Tandospirone and 1-PP were assayed on an MS/MS system using positive-ion electrospray ionization (API4000, Sciex, Toronto, Ontario, Canada). The lower limits for quantification of tandospirone and 1-PP were 0.1 ng/mL and 1 ng/mL, respectively. The standard curves for tandospirone and 1-PP were linear up to 50 ng/mL and 500 ng/mL, respectively. If the measured value of tandospirone or 1-PP plasma concentration was not linear on the standard curve, the sample was diluted with blank plasma and re-assayed.

Measurement of motor activity

Rats motor activity was measured for tandospirone (60 mg/kg, p.o.), fluvoxamine (60 mg/kg, p.o.), and their co-administration. The rats were habituated to the testing room within their housing cages for 1 day. Tandospirone and fluvoxamine were administered 1 h and 4 h prior to testing, respectively. In a separate experiment, rats received co-administration of tandospirone and fluvoxamine. During testing, rats were individually subjected to the testing cage and motor activity was automatically recorded for 5 min by an infrared sensor that detected thermal radiation from the animals.[19] Horizontal movements were digitized and fed into a computer. Locomotion predominantly contributed to the count, but other body movements also contributed when they contained substantial horizontal components.

Data analysis

All data are presented as mean ± SEM of individual values for each rat in all groups. Statistical analysis was performed using Welch test for two groups, or two-way ANOVA for the drug–drug interaction.

RESULTS

Effects of co-administration of tandospirone and fluvoxamine on rat conditioned fear stress

Previous studies have shown that acute administration of tandospirone (30–100 mg/kg, p.o.) and fluvoxamine (30–100 mg/kg, p.o.) dose-dependently reduces conditioned freezing 1 day after fear conditioning in rats.[9,20] In accordance with these studies, the doses of tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) were chosen as the minimal effective doses.

Figure 1 shows the combined effect of tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) on rat conditioned freezing. Two-way

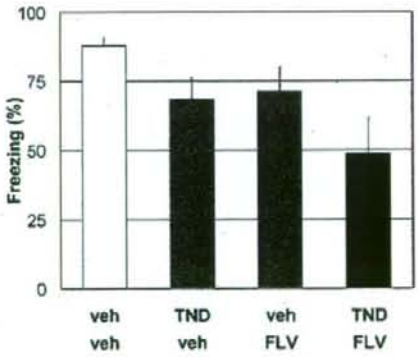

Figure 1. Effect of co-administration of tandospirone and fluvoxamine on contextual conditioned fear stress in rats. One day after footshocks, tandospirone (TND; 60 mg/kg, p.o.) and fluvoxamine (FLV; 60 mg/kg, p.o.) were administered 1 h and 4 h before testing. There was a significant main effect of TND and a nearly significant main effect of FLV, but no interaction. $n = 8$. Veh, vehicle (0.9% sterile saline).

Table 1. Effects of fluvoxamine on plasma concentrations of tandospirone and 1-PP in rats (mean ± SEM, $n = 8$)

Treatment (mg/kg)	TND (ng/mL)	1-PP (ng/mL)	1-PP/TND ratio
TND citrate (60) + vehicle	6.4 ± 0.4	345.6 ± 29.3	54.0 ± 3.8
TND citrate (60) + FLV (60)	12.5 ± 2.5*	276.6 ± 17.8**	27.6 ± 4.4***

*$P < 0.05$ vs TND citrate (60 mg/kg) alone; **$P = 0.067$ vs TND citrate (60 mg/kg) alone; ***$P < 0.001$ vs TND citrate (60 mg/kg) alone.
TND was administered as a salt (TND citrate) and detected as the free base.
1-PP, 1-(2-pyrimidyl) piperazine; FLV, fluvoxamine; TND, tandospirone.

ANOVA indicated a significant main effect of tandospirone ($F(1, 28) = 5.65$, $P < 0.05$) and a weak effect of fluvoxamine ($F(1, 28) = 4.19$, $P = 0.0501$). No interaction between tandospirone and fluvoxamine was observed ($F(1, 28) = 0.03$, $P = 0.870$).

Effect of fluvoxamine on the plasma concentrations of tandospirone and its major metabolite, 1-PP

After testing, rats were immediately decapitated and blood was collected to determine plasma concentrations of tandospirone and its major metabolite, 1-PP. As shown in Table 1, plasma concentration of tandospirone was significantly higher in the tandospirone + fluvoxamine-treated group than in the tandospirone alone-treated group ($P < 0.05$, Welch test). Accompanying the increase in plasma concentration of tandospirone, plasma concentrations of 1-PP was slightly, but not significantly, lower in the tandospirone + fluvoxamine-treated group than in the tandospirone alone-treated group ($P = 0.067$, Welch test). Moreover, 1-PP/tandospirone ratio, an index of tandospirone metabolism, was significantly reduced in the tandospirone + fluvoxamine-treated group as compared to the tandospirone alone-treated group ($P < 0.001$, Welch test).

Motor activity

Table 2 shows the combined effect of tandospirone (60 mg/kg, p.o.) and fluvoxamine (60 mg/kg, p.o.) on rat motor activity. Two-way ANOVA indicated significant main effects of tandospirone ($F(1, 28) = 7.69$, $P < 0.01$) and fluvoxamine ($F(1, 28) = 7.49$, $P < 0.05$). No interaction between tandospirone and fluvoxamine was observed ($F(1,28) = 1.17$, $P = 0.288$).

DISCUSSION

In the present study fluvoxamine increased plasma concentration of tandospirone, probably via CYP3A4 inhibition. In accordance with this increase, co-administration of tandospirone and fluvoxamine additively reduced conditioned freezing in rats. These findings suggest that CYP3A4-related drug–drug interaction between tandospirone and fluvoxamine may positively affect their anxiolytic action.

Moreover, we found that co-administration of tandospirone and fluvoxamine additively reduces motor activity in rats. It has been reported that high doses (160–320 mg/kg, p.o.) of tandospirone reduce spontaneous motor activity in mice.[21] In addition, we have shown in a previous study that co-administration of tandospirone and a CYP3A4 inhibitor (i.e. ketoconazole or cimetidine) reduces motor activity in rats.[9] Nevertheless, because two-way ANOVA showed that both tandospirone and fluvoxamine significantly reduced rat motor activity in the present study and no interaction was observed between the two drugs, we could not conclude that the reduced motor activity in the combination group is due to increased plasma

Table 2. Effect of co-administration of tandospirone and fluvoxamine on spontaneous locomotor activities (mean ± SEM, $n = 8$)

Treatment (mg/kg)	Locomotor activity (arbitrary unit)
Vehicle + vehicle	1044.8 ± 101.9
TND (60 mg/kg, p.o.) + vehicle	696.9 ± 88.9
Vehicle + FLV (60 mg/kg, p.o.)	700.0 ± 80.2
TND (60 mg/kg, p.o.) + FLV (60 mg/kg, p.o.)	547.5 ± 88.6

There were significant main effects of tandospirone and fluvoxamine, but no interaction.
FLV, fluvoxamine; TND, tandospirone.

tandospirone concentration. These results, however, strongly suggest that the inhibition of rat conditioned freezing observed with co-administration of tandospirone and fluvoxamine is not a false-positive effect caused by increased motor activity.

In the present study both tandospirone and fluvoxamine were orally administered to clarify their drug–drug interaction. Interestingly, we found in a previous study that co-administration of s.c. tandospirone (0.3 mg/kg) and i.p. fluvoxamine (30 mg/kg), given at subeffective doses, markedly reduced conditioned freezing in rats.[22] In that case, however, plasma concentration of tandospirone was never increased by fluvoxamine, indicating that the enhanced anxiolytic effect observed in the combination group appeared without affecting CYP3A4-related pharmacokinetic drug–drug interaction. Moreover, co-administration of s.c. tandospirone and i.p. paroxetine or citalopram, two SSRI with no CYP3A4 inhibitory activity, significantly reduces conditioned freezing in rats as compared with the vehicle or individual SSRI.[22] These findings elucidate the pharmacodynamic synergistic effect of tandospirone and individual SSRI, which is probably via stimulation of the post-synaptic 5-HT_{1A} receptor.[22] Dissimilar to our previous study (i.e. a combination of s.c. tandospirone and i.p. fluvoxamine), oral co-administration of tandospirone and fluvoxamine significantly increased plasma concentration of tandospirone. The inconsistency between these alterations of plasma tandospirone may be caused by tandospirone's pharmacokinetic properties related to absorption and metabolism. It is known that azapirones, including tandospirone, are rapidly absorbed and undergo extensive first-pass metabolism after oral administration.[23] Therefore, orally administered tandospirone might be more easily affected by CYP3A4 inhibitors than s.c. tandospirone. Indeed, our previous study showed that plasma concentrations of s.c. tandospirone were hardly affected by the CYP3A4 inhibitor ketoconazole in rats.[22] These findings suggest that both pharmacokinetic and pharmacodynamic drug–drug interactions contribute to the combined effect of orally administered tandospirone and fluvoxamine.

In contrast, the enhancement of anxiolytic effects of oral tandospirone by fluvoxamine seemed to be weak, considering both pharmacokinetic and pharmacodynamic drug–drug interactions, because two-way ANOVA on the anxiolytic effect indicated no interaction between tandospirone and fluvoxamine. We could not exclude the possibility that reduced motor activity might have masked the inhibitory effects on rat freezing behavior. In addition, methodological limitations (i.e. we measured both pharmacological behavior and plasma concentrations at a single time point and at a single combination dose) might have influenced the results. Therefore, further studies on the time- and dose-dependent pharmacokinetic and pharmacodynamic interactions between tandospirone and fluvoxamine are needed. The finding that the anxiolytic effect of tandospirone and fluvoxamine combination was additive is useful for estimation of drug–drug interaction in humans. We should pay attention to these drug–drug interactions; in particular, the dose of tandospirone or other azapirones should be sufficiently reduced during concomitant treatment with fluvoxamine. Further studies on drug–drug interaction between tandospirone and fluvoxamine in humans are needed.

CONCLUSIONS

In the present study we investigated the combined effect of tandospirone and fluvoxamine in the rat contextual conditioned fear stress model. Co-administration of oral tandospirone and fluvoxamine additively reduced conditioned freezing in the rat. This effect was accompanied by an increase in plasma concentration of tandospirone. These results indicate pharmacokinetic drug–drug interaction between tandospirone and fluvoxamine. Therefore, the anxiolytic effect of tandospirone may be facilitated by fluvoxamine via CYP3A4-related drug–drug interaction.

ACKNOWLEDGMENTS

This work was supported in part by Japanese Ministry of Education grants No. 16659299 (T. Koyama) and No. 17591191 (T. Inoue). We would like to thank Dainippon Sumitomo Pharma (Japan) for their support.

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Effect of co-administration of a serotonin–noradrenaline reuptake inhibitor and a dopamine agonist on extracellular monoamine concentrations in rats

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Received 3 October 2006; received in revised form 22 January 2008; accepted 6 February 2008
Available online 19 February 2008

Abstract

Recent studies have shown that dopamine agonists are useful for the treatment of not only Parkinson's disease, but also major depressive disorders. However, while these dopamine agonists provide a new treatment strategy for major depressive disorders, such as treatment-resistant cases, the antidepressant effect of dopamine agonists has yet to be investigated. To examine the mechanism of the antidepressive effect of dopamine agonists, we investigated the acute effect of the dopamine receptor agonist, cabergoline, and the serotonin–noradrenaline reuptake inhibitor, milnacipran, on extracellular noradrenaline, dopamine and serotonin concentrations in the rat medial prefrontal cortex. There was a greater increase in extracellular noradrenaline concentrations when acute milnacipran (30 mg/kg intraperitoneally) was administered after acute high-dose cabergoline (1 and 2 mg/kg subcutaneously) than when acute milnacipran was administered following acute vehicle or low-dose cabergoline (0.25 mg/kg subcutaneously). There were no significant differences noted in the dopamine or serotonin concentrations. These results suggest that the addition of cabergoline has the potential to strengthen the antidepressant effects of milnacipran and that the mechanism of action of the antidepressive effect of dopamine agonists might be due to enhancement of induced increases of extracellular noradrenaline.

Keywords: Cabergoline; Milnacipran; In vivo microdialysis; Noradrenaline; Dopamine; Serotonin

1. Introduction

The introduction of various antidepressants, such as tricyclic antidepressants, has led to a greater efficacy in the treatment of depression. Recently, newer antidepressants that are selective serotonin reuptake inhibitors (SSRIs) and serotonin–noradrenaline reuptake inhibitors (SNRIs) have been developed and these agents exhibit fewer side effects than was previously seen for tricyclic antidepressants. Currently one of the recommended first-line treatments for major depression (mild to moderate, single or recurrent nonpsychotic episode) is the use of SSRIs or venlafaxine, an SNRI (Janicak et al., 2001). However, in spite of the rapid progress in the development of new antidepressants, a number of depression patients continue to be treatment-resistant, having failed to respond to several of the new antidepressant therapeutic trials. In an attempt to increase the antidepressant effect, augmentation therapies that administer lithium carbonate and thyroid hormone in addition to the antidepressants have been recommended (Aronson et al., 1996; Bauer and Döpfmer, 1999). However, these augmentation therapies had no efficacious effect in the large group of patients with treatment-resistant depression. Therefore, it is crucial that the mechanism responsible for treatment-resistant depression be elucidated so that therapeutic regimens can be developed to successfully treat this group of patients.

The biological basis of depression is hypothesized to be due to the lack of neurotransmitters such as serotonin and noradrenaline within the brain. Recent evidence suggests that dopamine may also be related to depression (Kapur and Mann, 1992). Clinically, it has been reported that Parkinson's disease has a large impact on the rate of depression in these patients (Cummings, 1992). Dopamine agonists are clinically efficacious in the treatment of Parkinson's disease and the associated depression (Lemke et al.,

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0014-2999/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2008.02.019

2004). Bromocriptine, a dopamine receptor agonist, has been reported to have an antidepressant effect equal to imipramine and amitriptyline in the treatment of depression (Waehrens and Gerlach, 1981; Bouras and Bridges, 1982; Theohar et al., 1982). Previously, we reported that the addition of the dopamine agonists, bromocriptine and pergolide, in conjunction with antidepressants increased the antidepressant effect on treatment-resistant depression (Inoue et al., 1996; Izumi et al., 2000). It has also been reported that the administration of the dopamine receptor agonist, cabergoline, in conjunction with the SNRI, milnacipran, enhanced the antidepressant effect (Takahashi et al., 2003). More recently, a double-blind study showed that pramipexole, a dopamine receptor agonist, had a significant antidepressant effect as compared with placebo and that this effect was equal to that of fluoxetine (Corrigan et al., 2000). In a naturalistic study on treatment-resistant depression, pramipexole also exhibited an antidepressant effect (Lattanzi et al., 2002). In a small-scale double-blind study that examined bipolar depression, a significant antidepressant effect was additionally observed when pramipexole was added to mood stabilizers (Goldberg et al., 2004).

While there have been several clinical reports that have examined the efficacy of dopamine agonists in affective disorders, the antidepressant mechanism of dopamine agonists has yet to be determined. In this study, we investigated the mechanism of the antidepressant effect of the co-administration of milnacipran, an SNRI, and cabergoline, a dopamine agonist, by measuring the extracellular noradrenaline, dopamine and serotonin concentrations in the medial prefrontal cortex of rats using an in vivo microdialysis method. Cabergoline is highly selective for dopamine D_2 and dopamine D_3 receptors, and the affinities of cabergoline for other receptors, which include the α_2-adrenoreceptor of rats, are very low as compared with that seen for the dopamine D_2 receptors (Mantegani et al., 1999). A few studies have reported that the antidepressant effects of tricyclic antidepressants and nontricyclic antidepressants, which inhibit noradrenaline reuptake and/or serotonin reuptake, were augmented by co-administration of dopamine agonists (Inoue et al., 1996; Izumi et al., 2000), although the antidepressant effect of dopamine agonists added to selective serotonin reuptake inhibitors was somewhat inconclusive (Mattes, 1997; Lattanzi et al., 2002). However in rats, tricyclic antidepressants are rapidly metabolized by demethylation, and thus, it is expected that their inhibitory effects on serotonin reuptake will be smaller in vivo than in vitro (Waldmeier and Stocklin, 1989). Therefore, we examined the combination effect of cabergoline and milnacipran (SNRI), as milnacipran is not metabolized by cytochrome P450 and since it also has a low first-pass metabolism in addition to a low potential for drug interactions (Spencer and Wilde, 1998). We did not use tricyclic antidepressants in this study.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan), weighing 250–340 g, were housed in groups of four and maintained on a 12-h light–dark cycle, (light phase: 06:30–18:30), in a temperature-controlled environment (22±1 °C) with free access to food and water. Experiments began after a 10-day period of acclimatization.

2.2. Drugs

Cabergoline (Pharmacia, Milan, Italy) was dissolved in 0.1 M H_3PO_4 (0.2 ml for each 4.5 mg of cabergoline), with saline used to dilute the solution to the required concentrations (0.25, 1 or 2 mg/ml). Milnacipran (Asahi Kasei, Shizuoka, Japan) was dissolved in distilled water to achieve a final concentration of 30 mg/ml.

2.3. Experimental procedures

All experiments were performed in nonrestrained, freely moving rats. The 32 rats were divided into four groups according to cabergoline dose (A: cabergoline 0 mg/kg; B, 0.25 mg/kg; C, 1 mg/kg; D, 2 mg/kg). Cabergoline was administered subcutaneously 120 min after the first dialysate samples were collected, and milnacipran (30 mg/kg) was administered intraperitoneally to all rats 180 min after the cabergoline injections. In every experiment, 8 rats (2 rats for each group) were examined, with all of the experiments then repeated.

2.4. Microdialysis procedures

Stereotactic implants were placed in the rats under pentobarbital anesthesia (30 mg/kg i.p.) with an AG-4 guide cannulae (Eicom Co., Kyoto, Japan) and the implants abutted the surface of the medial prefrontal cortex at the following coordinates relative to the bregma: A+3.2, ML+0.8, and DV+1.0 mm. Dialysis probes with an outer diameter of 0.22 mm (A-I-4-03; Eicom, Kyoto, Japan) were then inserted into the guide cannulae so that 3.0 mm of each probe was exposed to the medial prefrontal cortex tissue. Rats were housed in individual cages.

One day after the surgery, rats were perfused with artificial cerebrospinal fluid (145 mM NaCl, 3.0 mM KCl, 1.3 mM $CaCl_2$, and 1.0 mM $MgCl_2$) at a flow rate of 2 μl/min. Following an initial perfusion period of 2 h, dialysate samples were collected every 20 min for 480 min and then placed in sample vials containing 50 μl of 0.05 M acetic acid. Extracellular noradrenaline, serotonin and dopamine levels were determined using high-performance liquid chromatography (Eicom, Kyoto, Japan). Determinations of noradrenaline, dopamine and serotonin levels were performed as previously described (Kitaichi et al., 2004).

All procedures were approved by the Hokkaido University Graduate School of Medicine Animal Care and Use Committee, and complied with the Guide for the Care and Use of Laboratory Animals, Hokkaido University Graduate School of Medicine.

2.5. Statistical analysis

All the data are given as the mean values±S.E.M. for the individual rats from each group. The noradrenaline, dopamine and serotonin concentrations of the dialysate samples were expressed as absolute values (pg/fraction). To investigate the effect of cabergoline alone on extracellular noradrenaline,

dopamine and serotonin concentrations, a repeated measures analysis of variance (ANOVA) for absolute values was used during the 0–180 min interval after the cabergoline administration. In addition, a repeated measures ANOVA for absolute values was used to examine the effects of cabergoline on the milnacipran-induced increases of the monoamines (180–360 min). Areas under the curve for the 180 to 360 min periods were compared between the four cabergoline groups. Differences in the area under the curve between the four groups were analyzed using a one-way ANOVA, followed by a Duncan's test. Differences in absolute values measured at each of the collection time points between the four groups were analyzed using a one-way ANOVA, followed by a Duncan's test. $P<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Effect of acute cabergoline (0, 0.25, 1 and 2 mg/kg) treatment on extracellular noradrenaline, dopamine and serotonin during 0–180 min in the medial prefrontal cortex (Fig. 1)

The repeated measures ANOVA (0–180 min) found no significant effect by cabergoline on the extracellular noradrenaline concentrations. There was a significant effect for time [$F(9,243)=3.338$, $P=0.0007$] and a significant interaction between the cabergoline treatment and time [$F(27,243)=1.874$, $P<0.0072$]. However, the Duncan's test indicated that extracellular noradrenaline concentrations following the cabergoline 0 mg/kg treatment were not different from those seen for the other three groups at any of the time points (0–180 min).

The repeated measures ANOVA (0–180 min) found no significant effect for cabergoline or time and there was also no significant interaction between the cabergoline treatment and time on extracellular dopamine concentrations.

The repeated measures ANOVA (0–180 min) found no significant effect of cabergoline or no significant interaction between cabergoline treatment and time on the extracellular serotonin concentrations. However, there was a significant effect observed for time [$F(9,234)=5.566$, $P<0.001$].

These results indicate that acute doses of cabergoline at concentrations of 0.25–2 mg/kg did not affect the extracellular monoamine concentrations in the rat medial prefrontal cortex.

3.2. Effect of acute milnacipran (30 mg/kg) on extracellular noradrenaline, dopamine and serotonin concentrations during 180–360 min in the medial prefrontal cortex following cabergoline treatment (Fig. 1)

Acute administration of milnacipran (30 mg/kg) increased the extracellular noradrenaline concentrations (Fig. 1A). Two-way ANOVA with repeated measures (180–360 min) indicated that there was a significant effect for the cabergoline treatment [$F(3,27)=3.424$, $P=0.0312$] and for time [$F(9,243)=44.036$, $P<0.001$] on extracellular noradrenaline concentrations. In addition, the interaction between the cabergoline treatment and time was significant [$F(27,243)=1.755$, $P=0.0145$]. The high-dose cabergoline (1 and 2 mg/kg) treatment groups showed significantly higher concentrations of extracellular noradrenaline as compared with the vehicle and low-dose cabergoline (0.25 mg/kg) groups (Duncan's test, cabergoline 1 mg/kg vs. 0 mg/kg, 260, 340 min, $P<0.05$; cabergoline 1 mg/kg vs. 0.25 mg/kg, 260 min, $P<0.01$, 340 min, $P<0.05$; cabergoline 2 mg/kg vs. 0 mg/kg, 280, 300, 340 min, $P<0.05$; cabergoline 2 mg/kg vs. 0.25 mg/kg, 260, 280, 300, 340 min, $P<0.01$, 240, 320, 360 min, $P<0.05$).

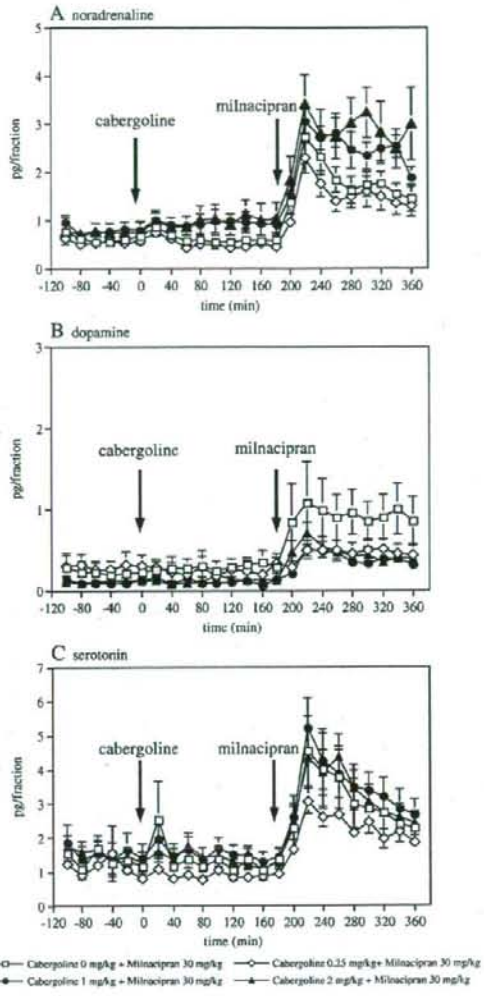

Fig. 1. Effect of acute administration of milnacipran (30 mg/kg intraperitoneally) on extracellular concentrations of noradrenaline, dopamine and serotonin in the medial prefrontal cortex following cabergoline treatment (0, 0.25, 1 or 2 mg/kg subcutaneously). Values represent the mean±S.E.M. (pg/20 min fraction). (A) $N=7$ (cabergoline 0 mg/kg group), $N=8$ (cabergoline 0.25, 1 and 2 mg/kg groups); (B) $N=7$ (cabergoline 0, 1 and 2 mg/kg group), $N=8$ (cabergoline 0.25 group); (C) $N=7$ (cabergoline 0 and 1 mg/kg groups), $N=8$ (cabergoline 0.25 and 2 mg/kg).

Table 1
Effect of acute milnacipran treatment following cabergoline on the area under the curve for extracellular noradrenaline, dopamine and serotonin concentrations in the medial prefrontal cortex

	Cabergoline 0 mg/kg+ milnacipran 30 mg/kg	Cabergoline 0.25 mg/kg+ milnacipran 30 mg/kg	Cabergoline 1 mg/kg+ milnacipran 30 mg/kg	Cabergoline 2 mg/kg+ milnacipran 30 mg/kg
Noradrenaline	318.5±48.6	263.3±38.9	425.6±35.6	488.1±83.0[ab]
Dopamine	161.4±57.1	81.0±18.8	66.4±7.6	80.5±16.8
Serotonin	543.6±133.7	398.5±36.6	614.0±103.8	571.1±87.1

Values represent the mean±S.E.M. (pg min). Data were calculated based on Fig. 1. [a]$P<0.05$ vs. cabergoline 0 mg/kg+milnacipran 30 mg/kg, [b]$P<0.05$ vs. cabergoline 0.25 mg/kg+milnacipran 30 mg/kg. Noradrenaline, $N=7$ (cabergoline 0 mg/kg group), $N=8$ (cabergoline 0.25, 1 and 2 mg/kg groups); dopamine, $N=7$ (cabergoline 0, 1 and 2 mg/kg group), $N=8$ (cabergoline 0.25 group); serotonin, $N=7$ (cabergoline 0 and 1 mg/kg groups), $N=8$ (cabergoline 0.25 and 2 mg/kg).

Acute administration of milnacipran (30 mg/kg) increased the extracellular dopamine and serotonin concentrations (Fig. 1B and C). Two-way ANOVA with repeated measures (180–360 min) indicated neither a significant effect of cabergoline treatment nor an interaction between cabergoline treatment and time on the extracellular dopamine and serotonin concentrations (Fig. 1B and C). Only the effects of time on the extracellular dopamine and serotonin were found to be significant [dopamine, $F(9,207)=7.838$, $P<0.001$; serotonin, $F(9,234)=32.787$, $P<0.001$].

3.3. Effect of acute milnacipran treatment following cabergoline on the area under the curve for extracellular noradrenaline, dopamine and serotonin concentrations in the medial prefrontal cortex

The area under the curve (180–360 min) for the extracellular noradrenaline of the cabergoline 2 mg/kg group was significantly higher than that seen for either the cabergoline 0 mg/kg or the low-dose cabergoline 0.25 mg/kg groups (Duncan's test; $P<0.05$). The areas under the curve (180–360 min) for the extracellular dopamine and serotonin were not significantly different between the four groups (Table 1).

4. Discussion

In this study, co-administration of cabergoline and milnacipran significantly increased extracellular noradrenaline concentrations as compared with that seen for milnacipran alone. However, cabergoline did not increase the basal levels of extracellular noradrenaline, suggesting that cabergoline enhances extracellular noradrenaline concentrations only when increased by SNRIs.

There are several possible mechanisms that could explain the increases in extracellular noradrenaline concentrations. Cabergoline is a dopamine receptor agonist and among the five dopamine receptor subtypes, only high affinities for the dopamine D_2 and dopamine D_3 receptors have been observed (Millan et al., 2002; Newman-Tancredi et al., 2002). Therefore, cabergoline's agonist effect on the dopamine D_2 and dopamine D_3 receptors may lead to the enhancement of the increased extracellular noradrenaline concentrations that are a direct result of noradrenaline reuptake inhibition caused by milnacipran administration. There have been no previous reports prior to this study that used in vivo microdialysis methods to examine the combined effect of noradrenaline reuptake inhibitors and dopamine agonists on the extracellular monoamine concentrations. Future studies will be necessary to elucidate the roles of dopamine D_2 and dopamine D_3 stimulations in the dopamine agonist- and SNRI-caused enhancements, e.g., by local application of dopamine agonists to the brain tissue.

Another possible mechanism involves an increase in extracellular noradrenaline concentrations that are mediated by the stimulation of the dopamine D_1 receptors. This is supported by a previous report that found that local infusion of dopamine to the medial prefrontal cortex increased extracellular noradrenaline concentrations, and that this effect was inhibited by the dopamine D_1 receptor but not by dopamine D_2 receptor antagonists (Pan et al., 2004). However, we found that cabergoline administration did not increase the basal levels of extracellular noradrenaline. In addition, a previous report found that cabergoline had a very low affinity for dopamine D_1 receptors (Millan et al., 2002). Therefore, it is unlikely that dopamine D_1 stimulation by cabergoline mediates the increased extracellular noradrenaline concentrations seen after milnacipran administration. Consistent with this result, it has also been shown that quinpirole, a dopamine D_2 receptor, dopamine D_3 receptor and dopamine D_4 receptor agonist, does not influence extracellular noradrenaline concentrations in the medial prefrontal cortex (Devoto et al., 2001).

The affinity of cabergoline for α_2-adrenoreceptors of rats ($IC_{50}=290$ nM) is very weak as compared to that seen for the dopamine D_2 receptors ($IC_{50}=3$ nM) (Mantegani et al., 1999). Since it has been found that the α_2-antagonist idazoxan increases basal concentrations of extracellular noradrenaline in the prefrontal cortex (Devoto et al., 2001) and that it enhances the effect of venlafaxine, an SNRI, on noradrenaline increases in the medial prefrontal cortex (Weikop et al., 2004), the α_2-antagonism by cabergoline (Newman-Tancredi et al., 2002) might account for the enhanced noradrenaline increases that are observed after the combined administration of cabergoline and milnacipran. Miyagi et al. (1996) reported that there was increased locomotion in mice after administration of cabergoline at a dose of 0.5 mg/kg, but not at lower doses. In our preliminary experiments, we found that administration of cabergoline at 1 mg/kg or more increased locomotion in rats, although this hyperlocomotive effect was not observed at lower doses (0.5 and 0.25 mg/kg) (our unpublished data). Thus, 1 mg/kg of cabergoline is the minimum effective dose required to stimulate dopamine D_2 receptors in rats. Taken together, it seems unlikely that administration of cabergoline at 1 and 2 mg/kg blocks α_2-adrenoreceptors, although this possibility cannot be excluded completely. It is possible that a comparison of dopamine agonists that lack α_2-adrenoreceptor affinity, such as quinpirole, might be able to help clarify whether there is any significant role that the weak α_2-antagonistic property of cabergoline may have with regard to the increased noradrenaline concentrations.

Similar to the noradrenaline reuptake inhibitor reboxetine (Kitaichi et al., 2004), milnacipran increased not only

extracellular noradrenaline concentrations but also extracellular dopamine concentrations in the medial prefrontal cortex. It has been shown that the increases of extracellular dopamine concentrations in the medial prefrontal cortex that are caused by noradrenaline reuptake inhibitors are due to the dopamine reuptake inhibition from noradrenaline transporters (Carboni et al., 1990). A more recent study has reported that dopamine is co-released with noradrenaline from noradrenaline nerve terminals in the cerebral cortex and that the majority of the extracellular dopamine found in the cerebral cortex is derived from noradrenergic nerves (Devoto et al., 2001). Additionally, systemic administration of quinpirole causes only a transient decrease in the extracellular dopamine concentrations in the prefrontal cortex, and when infused locally in the prefrontal cortex, it has no influence on the extracellular dopamine concentrations (Devoto et al., 2001). This could perhaps explain the reason why levels of cabergoline that were not statistically significant in our study still tended to inhibit the extracellular dopamine increases caused by milnacipran in the medial prefrontal cortex. It seems unlikely that the inhibitory effects of cabergoline on the dopaminergic nerves cancel the stimulatory effects of antidepressants on the extracellular dopamine. In line with this, it has been speculated that when SNRIs and cabergoline are administered together, all five dopamine receptors are stimulated by cabergoline and SNRI-induced dopamine.

In this study, since the medial prefrontal cortex receives dense dopamine innervation ($A+3.2$ mm, ML 0.8 mm, DV 1.0 mm from bregma), microdialysis probes were inserted into this area. Previous results have indicated that both dopaminergic and noradrenergic neural transmissions contribute to the extracellular dopamine concentrations in the medial prefrontal cortex (Devoto et al., 2003), while most of extracellular dopamine reflects the activity of the noradrenergic neurons in cortical areas other than the medial prefrontal cortex (Devoto et al., 2001, 2003). Thus, this regional specificity of the medial prefrontal cortex may account for the dissociation that is noted between the extracellular noradrenaline and dopamine when there is a combined treatment. In other words, for dopaminergic neurons, cabergoline has a tendency to decrease the extracellular dopamine, while for noradrenergic neurons, it increases the extracellular noradrenaline.

Antidepressant effects of dopamine agonists for treatment-resistant depression have been reported for dopamine agonists when added to tricyclic antidepressants. However, Mattes (1997) observed that when the dopamine receptor agonist, pergolide, was added to SSRIs, it was not effective in treatment-resistant depression. In the current study we found that the co-administration of cabergoline and milnacipran did not enhance extracellular serotonin concentrations as compared with that seen for milnacipran alone. Therefore, dopamine agonists may be more effective for depressive disorders when combined with antidepressants, which have noradrenaline reuptake inhibition abilities.

Recent clinical studies indicate that the co-administration of atypical antipsychotics, especially olanzapine, augments the antidepressant effects of serotonin reuptake inhibitors in treatment-resistant depression (Thase et al., 2007). Koch et al. (2004) reported that olanzapine alone increases both dopamine and noradrenaline release in the medial prefrontal cortex of rats, and that the co-administration of olanzapine with fluoxetine results in further increases in both catecholamines. These neurochemical effects of the combination therapy most likely are responsible for the substantial clinical improvement in the symptoms seen in patients with treatment-resistant depression. Quintin and Thomas (2004) pointed out that the antidepressant effects seen with atypical antipsychotics are due to increases of dopamine in the medial prefrontal cortex, with the effects directly related to the antipsychotics blocking the dopamine D_2 receptors. In our present study, when cabergoline was added to milnacipran, there was an increase in extracellular noradrenaline but not in dopamine concentrations in the medial prefrontal cortex of rats. Since cabergoline acts as a dopamine D_2 agonist, it stimulates dopamine D_2 receptors, thereby increasing net dopaminergic neurotransmissions. Thus, when there is co-administration of cabergoline with antidepressants, the effects on the catecholamines are similar to that seen when there is co-administration of atypical antipsychotics with antidepressants.

In conclusion, this study examined the potential mechanism of action for the combined administration of a dopamine agonist with an antidepressant. The single administration of cabergoline did not alter extracellular noradrenaline, dopamine or serotonin concentrations. Larger increases in the extracellular noradrenaline concentrations in the medial prefrontal cortex were obtained when a combination of high-dose cabergoline and milnacipran administration was used, suggesting that enhancement is the mechanism of the augmentation of the antidepressant effect that is observed in such cases. To clarify this mechanism of action for dopamine agonist-induced augmentations of the antidepressant effects, further studies of chronic administration of more selective dopamine agonists will be necessary.

Acknowledgement

This work was supported in part by grants-in-aid for Scientific Research No. 09470205 (T. K.), No. 17591191 (T. I.) and No. 17790800 (Y. K.) from the Japanese Ministry of Education, Science and Culture.

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Journal of Affective Disorders 110 (2008) 62–69

Journal of Affective Disorders

www.elsevier.com/locate/jad

Research report

Increased expression of splicing factor SRp20 mRNA in bipolar disorder patients

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Received 5 July 2007; received in revised form 20 December 2007; accepted 3 January 2008
Available online 20 February 2008

Abstract

Background: Variations and defects in alternative splicing are well known to be associated with a variety of human diseases and the stress response. We previously reported a decrease in glucocorticoid receptor (GR) α, but not GRβ in mood disorder patients, suggesting an aberrant alternative splicing mechanism. To examine whether altered RNA splicing may underlie the pathophysiology of mood disorder, we evaluated the expression of a variety of SR protein splicing factors, a family of proteins indispensable for proper alternative splicing, in mood disorder patients.

Methods: We used quantitative real-time PCR to measure expressions of SRp20, SRp30c, SC35, SRp40, SRp46, SRp54, SRp55, SRp75, ASF/SF2, and 9G8 mRNA in peripheral white blood cells of 33 mood disorder patients during a depressive episode. In addition, the expressions of SRp20 and SC35 mRNA were quantified for 78 mood disorder patients in a remissive state, and 32 the first-degree relatives of these mood disorder patients.

Result: A significant correlation was observed between SRp30c and the GRβ/GRα ratio in control subjects, but not in mood disorder patients. Increased expression of SRp20 but not SRp30c mRNA was observed in bipolar disorder patients in both the depressive and remissive states. Major depressive disorder patients did not show any significant change in mRNA levels of SR proteins.

Limitation: Subjects were Japanese adults. Patient treatment was not standardized.

Conclusions: These results suggest that aberrant alternative splicing machinery caused by increased SRp20 mRNA expression would be associated with the pathophysiology of bipolar disorder.

Keywords: Alternative splicing; SR protein; SRp20; Mood disorder; Bipolar disorder

1. Introduction

Mood disorder patients often exhibit hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis (Pariante and Miller, 2001; Pariante, 2004; de Kloet et al., 2005). One of the plausible mechanisms to lead the HPA

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0165-0327/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jad.2008.01.003

axis to hyperactivity is the dysfunction of negative feedback regulation in which the glucocorticoid receptor (GR) plays crucial roles (Holsboer, 2000; Pariante and Miller, 2001; de Kloet et al., 2005). Several lines of evidences have indicated the decrease in GR expressions of mood disorder patients in distinct regions of the cortex and hippocampus (Webster et al., 2002; Knable et al., 2004; Perlman et al., 2004), as well as in peripheral blood cells (Pariante, 2004; Matsubara et al., 2006).

GR has several isoforms produced by alternative splicing, two of which (GRα and β) have been the focus of most studies because of their relative abundance (Lu and Cidlowski, 2006). Our previous study indicated that the levels of GRα mRNA were decreased in the peripheral blood cells of mood disorder patients, whereas GRβ mRNA levels were unaltered (Matsubara et al., 2006). Additionally, although healthy control subjects showed a significant inverse correlation between levels of GRα and GRβ mRNAs, neither major depressive disorder (MDD) nor bipolar disorder (BPD) patients showed such correlations. These findings suggest the possibility of a defective alternative splicing mechanism in mood disorder patients.

Alternative splicing is widely regarded as a major source of diversity in the human proteome. Recently, defective alternative splicings were reported to be associated with many neuropsychiatric diseases (Grabowski and Black, 2001; Lee and Irizarry, 2003; Garcia-Blanco et al., 2004). These findings suggest that possible deficits in the alternative splicing mechanism lead to change in gene expression in patients with various psychiatric diseases including mood disorders.

One of the best characterized molecules regulating splicing of RNA transcript is the family of SR protein splicing factors (Graveley, 2000; Black, 2003; Lareau et al., 2007). SR proteins are critical components of constitutive splice site recognition and often bind specifically near alternative splice sites to favor their selection (Zheng, 2004). Among SR proteins, SRp30c is involved in the alternative splicing producing GRβ mRNA from GR pre-mRNA (Xu et al., 2003). Also, SC35 is thought to be increased by stress, and facilitate the shift to alternative splicing of acetyl cholinesterase pre-mRNA in mouse brain (Meshorer et al., 2005). These findings suggest that the aberrant expression and/or function of SR protein might be involved in the pathophysiology of mood and stress-related disorders. However, there are no reports examining the expression of SR proteins in mood disorder patients.

The aim of this study is to examine whether the expressions of SR protein splicing factor mRNAs are altered in the peripheral white blood cells of patients with mood disorders in both the depressive and remissive states, as well as in the first-degree relatives of mood disorder patients. In addition, we examined whether there are any correlations between SR protein mRNA levels, GRα and β mRNA levels, the ratio of GRβ mRNA to GRα mRNA (GRβ/GRα) and serum cortisol concentrations.

2. Methods and materials

2.1. Subjects

A demographic summary of healthy control subjects (n=28), mood disorder subjects (n=33 in a depressive state, n=78 in a remissive state), and the first-degree relatives of the mood disorder subjects (n=32) are shown in Table 1. MDD and BPD patients were diagnosed according to the criteria in the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV). These included both outpatients and inpatients seen by the Division of Neuropsychiatry of the Yamaguchi University Hospital. The severity of the depressive state was assessed by a 21-item "Hamilton Depression Rating Scale" (HDRS). Subjects with an HDRS score of more than 18 were regarded as being in a depressive state, and those with a score of less than 6 and who showed no symptoms of a major depressive episode according to the DSM-IV criteria for more than 2 months were regarded as being in a remissive state. Individuals were excluded from the present study if they had abnormal physical examinations or abnormal results for routine medical laboratory tests such as a complete blood count, renal, liver or thyroid functions. Female subjects who were pregnant or took oral contraceptives were also excluded. All healthy controls subjects and the first degree relatives were genetically unrelated residents living in Japan without either mental past histories, and controls and patients were all of Japanese ethnicity and there is no significant population stratification in Japanese reported in several groups (Kakiuchi et al., 2003; Yamada et al., 2004). This protocol was approved by the Institutional Review Board of Yamaguchi University Hospital. Informed written consent was obtained for all subjects.

2.2. Blood sample preparation

Blood was obtained by venipuncture between 10:00 A.M. and 11:00 A.M., and processed to determine serum cortisol concentration and for total RNA purification.

2.3. RNA isolation and cDNA synthesis

Total RNA was prepared from peripheral white blood cells using QIAamp RNA Blood Mini kit (Qiagen,