Table 1. Demographic characteristics of the participants.

Variables	Subjects with ASD (n = 15)	TD controls (n = 17)	t value (p)
Age (Range) (SD)	28.2 (20–44) (7.4)	30.3 (21–41) (5.7)	0.906 (.372)
Height, cm (SD)	170.8 (3.9)	174.3 (6.5)	1.8 (.080)
Body weight, kg (SD)	64.1 (9.4)	70.5 (9.8)	1.9 (.068)
SES* (SD)	3.3 (1.0)	1.8 (0.4)	5.5 (<0.001)
Parental SES* (SD)	2.1 (0.5)	2.1 (0.6)	0.2 (.817)
Handedness: Right/Mixed/Left	14/1/0	17/0/0	
IQ**			
FIQ (SD)	104.6 (11.0)	109.2 (7.2)	1.4 (.166)
VIQ (SD)	111.8 (15.3)		
PIQ (SD)	90.0 (14.0)		
Autism spectrum disorder subtype	14 HFA/1 PDD-NOS***		
Autism Diagnostic Interview-Revised			
Social (SD)	13.4 (6.3)		
Communication (SD)	12.1 (3.5)		
Repetitive (SD)	4.6 (2.4)		
Autism Spectrum Quotient (SD)	37.4 (7.1)	13.9 (4.9)	10.3 (<0.001)

*Socioeconomic status assessed using the Hollingshead. Higher scores indicate lower status.
**The intelligence quotients were measured using the Wechsler Adult Intelligence Scale in ASD participants and a Japanese version of the National Adult Reading Test in the TD participants.
***HFA: High-functioning autism; PDD-NOS: Pervasive developmental disorder-not otherwise specified.
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and expressive prosody. To exclude unnatural and unrealistic stimuli, healthy Japanese adults (T.W., N.Y., Y.K., and H.Y.) examined whether the movies were natural and realistic or not. Only the movies that were rated as the most realistic and natural were used as the stimuli in the present experiment.

As verbal information, words with high emotional valence and arousal were selected from the list of Affective Norms for English Words (40 words had a positive valence, whereas the other 40 had a negative valence) [40]. Overly aggressive words were excluded for ethics considerations. As nonverbal information, the professional actors were instructed to display happy or disgusted facial expressions and prosody while speaking each word. The emotional directionality of the facial expressions was always the same as that of the accompanying voice prosody. Thus, there were four types of stimuli (Fig. 1A): a disgusted facial expression and prosody paired with a negative word (i.e., negative (-) nonverbal (NV) information and negative (-) verbal (V) information; NV-V-, e.g., Video S1), a disgusted facial expression and prosody paired with a positive word (NV-V+, e.g., Video S2), a happy facial expression and prosody paired with a negative word (NV+V-, e.g., Video S3), and a happy facial expression and prosody stimuli with a happy word (NV+V+, e.g., Video S4). There were 20 videos of each type.

The emotional directionality of the nonverbal information was validated in an independent behavioral experiment using a separate group of 12 TD male participants (24–34 years old) who did not undergo the subsequent fMRI experiments. To validate the nonverbal information conveyed by the actors' facial expressions, the participants were instructed to evaluate silent movies by indicating a score on an 8-point happy–disgust scale, with one being the happiest and 8 being the most disgusted. In contrast, to validate the prosody, we had the participants perform the same evaluation of the voice stimuli without the visual component of the movie (i.e., only listening to voice). In these evaluations of voice prosody, the participants were instructed to ignore the verbal content of the speech as much as possible. The mean scores and standard deviations for the facial expressions were 1.9 ± 0.3 in the NV+V-, 1.8 ± 0.2 in the NV+V+, 7.1 ± 0.6 in the NV-V-, and 6.9 ± 0.5 in the NV-V+ conditions. The mean scores and standard deviations for the voice prosody 3.0 ± 0.3 in the NV+V-, 2.7 ± 0.4 in the NV+V+, 6.8 ± 0.5 in the NV-V-, and 6.6 ± 0.4 in the NV-V+ conditions. There was no main effect of verbal information in a repeated-measures analysis of variance (ANOVA) comparing the positive versus negative words. All scores were significantly different from neutral facial expressions or prosody ($P = 0.002$, one-sample t-tests). These results indicate that the intended nonverbal information was appropriately conveyed to the participants, for both the facial expressions and voice prosody.

The fMRI scanning consisted of two runs, each of which involved 10 NV-V-, 10 NV-V+, 10 NV+V-, and 10 NV+V+ stimuli, pseudo-randomly ordered. During scanning, the participants were presented with the movies sequentially, and were instructed to judge whether they believed the actor in the movie was a friend or a foe by pressing a button. They were asked to make an intuitive judgment of the actors without deep consideration. Each movie stimulus was presented immediately before a 2,000-ms response period, followed by a 2,500–4,500-ms waiting period (Fig. 1B). The mean length of each trial across all 80 trials was 7,000 ms. For optimization of the event-related design, we also added 10 7,000 ms dummy trials in each run. Therefore, each run took approximately six minutes. Before fMRI scanning, all participants underwent sufficient training to allow them to complete the judgment task.

Behavioral Analysis. Before the following analysis, we needed to exclude the possibility that the friend or foe judgments were strongly biased by any exogenous feature in the stimuli. If this

was the case, the stimuli may have been disproportionately judged as either friends or foes no matter who saw them. To address this issue, we applied a chi-square goodness-of-fit test to the behavioral data obtained from the TD subjects, and we found that there was no significant bias towards judging the stimuli as a friend or a foe across all of the incongruent stimuli (chi-square = 15.7, degree of freedom = 16, $P>0.47$). This suggests that the judgments cannot be explained by the exogenous features of the stimuli rather than internal processes in the participants.

We classified the judgments of the incongruent stimuli into nonverbal-cue-biased and verbal-cue-biased judgments, according to the type of information that primarily affected the judgment [17]. For example, making a judgment that the actor was a foe in response to a NV-V+ stimulus was regarded as a nonverbal-cue-biased judgment, and making a judgment that the actor was a foe in response to a NV+V- stimulus was regarded as a verbal-cue-biased judgment. To clarify the behavioral characteristics of the ASD group, we first conducted repeated-measures mixed-design ANOVAs of response time with one within-participants factor (type of stimuli: congruent/incongruent stimuli) and one between-participants factor (group: ASD/TD). To examine the effects of conflict adaptation [41–43], paired t-tests were conducted between the response time for a congruent stimulus or an incongruent stimulus. We then compared the number of nonverbal-cue-biased judgments of incongruent stimuli between the TD and ASD groups using two-sample t-tests. Note that it is not necessary to compare the number of verbal-cue-biased judgments between the TD and ASD groups, because the number is always the converse of the number of the nonverbal-cue-biased judgments. The influence of type of emotion conveyed by the nonverbal information on the number and mean response time of the nonverbal-cue-biased judgments was also estimated as the interaction between the group and type of emotion conveyed in the stimulus in a repeated-measure mixed-design two-way ANOVA (group: TD/ASD × emotional type: positive/negative). Statistical significance was set at $P<0.05$.

Preprocessing of fMRI Data. The fMRI data were analyzed in SPM8 (http://www.fil.ion.ucl.ac.uk/spm/). Functional images were realigned, slice timing corrected, normalized to the default template with interpolation to a 2×2×2 mm space [44], and smoothed (full width half maximum = 8 mm, Gaussian filter). To remove low-frequency drift from the data, high-pass temporal filtering with a cut-off of 128 s was applied. There was no significant difference in the extent of motion during the scanning between the TD and ASD participants as a repeated-measure mixed-design ANOVA (TD/ASD × absolute values of the six motion parameters: x/y/z/pitch/yaw/row) found neither a significant main effect of the group or interaction ($P>0.4$).

fMRI Analysis

For our event-related fMRI design, in single level analyses, we used a general linear model with eight regressors: four types of stimuli (NV-V-, NV+V-, NV+V-, and NV+V+) × two types of response (friend and foe). Each event-related regressor had an onset at the time of stimulus presentation and a duration that corresponded to the response time. We also added six regressors for the six motion correction parameters. We then defined the brain activity during judgments biased to nonverbal cues as Bnv: ["Foe to NV-V+" and "Friend to NV+V-"] > ["Foe to NV-V-" and "Friend to NV+V+"]. This contrast was based on the assumption that, in judgments of foe to NV-V+, for example, the participants emphasized nonverbal information more explicitly than in judgments of foe to NV-V-. To make this analysis valid, we excluded types of judgments that were rarely selected (e.g., "Friend to NV-V-" and "Foe to NV+V+"). This exclusion was intended to reduce the influence of the stimuli in which the emotional content of the facial expressions and voice prosody were not exactly congruent to the verbal content of the words, though the effect cannot be completely excluded. Using the same logic, we defined the brain activity during judgments biased to verbal cues as Bv: ["Friend to NV-V+" and "Foe to NV+V-"] > ["Friend to NV+V+" and "Foe to NV-V-"].

For the group level analysis, we estimated that activity in different brain regions was specifically related to nonverbal-cues-biased judgments using differences between the Bnv and Bv conditions.

The group level analysis was based on a random-effects model, and we first conducted within-group comparison for each of the groups. Moreover, we employed paired-t-tests and compared brain activity during the nonverbal-cue-biased judgments with that during the verbal-cue-biased judgments (Bnv > Bv). We then conducted between-group comparisons by employing a repeated-measure mixed-design two-way ANOVA of the brain activities (type of judgments: nonverbal- or verbal-cue-biased judgments × group: ASD/TD). Using masked images, significant brain activations were examined in anatomically-defined regions described earlier, which previous studies have suggested are related to empathy, mentalizing, or uncertainty judgments [24,25,45] ($P<0.05$, FDR-corrected, [46,47]). These regions were defined by an automated anatomical labeling (AAL) [48] atlas implemented in WFU PickAtlas (http://www.fmri.wfubmc.edu). To account for individual anatomical differences, we applied Gaussian smoothing (full width half maximum = 8 mm) to these mask images. To investigate the involvement of other brain regions, we also conducted a whole brain analysis ($P<0.05$, FDR-corrected). Furthermore, for the regions found to exhibit activity in these analyses, we examined the effects of the affective valence of the verbal and nonverbal information (i.e., positive/negative) by conducting a repeated measures ANOVA of the activity (positive and negative × verbal and nonverbal information) in each of the subject groups.

To investigate which of the detected regions were more relevant to the nonverbal-cue-biased judgments, we examined Pearson's correlation coefficients between the brain activity in the TD group during the nonverbal-cue-biased judgments and the number of behavioral judgments. This analysis is based on the assumption that activity in brain regions with relevance for certain behaviors significantly correlated with the corresponding behavioral scores across subjects [49–53]. The brain activity was extracted as an averaged percent signal change in 4-mm spheres around the coordinates of interest [54]. The significance level was set at $P<0.05$ after Bonferroni correction among the detected regions (i.e., because five regions were found, the threshold was defined as $P<0.01 = 0.05/5$ regions). To test whether these correlations were less strong in the ASD group, we also calculated the correlation in the ASD group, and compared the correlation coefficients between the TD and ASD groups using Fisher's transformation of correlation coefficients. For further validation of regions detected by the analyses, we examined correlations between brain activity in the ASD group and the ADI-R sub-scores (social and communication). ADI-R-communication scores were included because these scores include items related to social interactions using nonverbal communicative cues (e.g., "failure to compensate verbal communication through gestures (B1)" and "lack of spontaneous imitation of actions and imitative social play (B2)"). Brain activity was extracted in the same way as described above in the analysis of behavior.

Finally, to exclude the possibility that the ASD subjects may show less overall brain activity than the TD subjects, we estimated differences in the amygdala response to congruent emotional stimuli between the groups, because previous studies reported enhanced activation of the amygdala in ASD individuals [55,56]. We employed an FDR-corrected $P<0.05$ for this analysis in the amygdala.

Results

Behavioral Results

A repeated-measure mixed-design two-way ANOVA of the response time data (stimulus type: incongruent and congruent stimuli × group: ASD and TD) revealed neither significant main effects of group nor any significant interaction between the group and stimulus type ($P>0.39$). However, there was a main effect of stimulus type ($F(1,30)=6.3$, $P=0.01$). A post-hoc paired t-test revealed that response times in both the ASD and TD individuals were longer for incongruent than congruent stimuli ($P<0.001$, Fig. 1C). This supports the hypothesis that both the TD and ASD participants sensed the incongruity and attempted to resolve this conflicting information, though it is hard to definitively test this hypothesis.

Furthermore, a paired t-test did not detect any significant differences between response times to incongruent stimuli that were presented immediately following congruent stimuli compared to response times to incongruent stimuli that were presented immediately following incongruent stimuli. This finding allowed us to exclude conflict-adaptation effects in the following analysis [41–43].

A two-sample t-test of the number of judgments of the incongruent stimuli indicated that the ASD subjects made significantly fewer nonverbal-cue-biased judgments than the TD subjects (ASD: 23.2 ± 1.8, TD: 26.1 ± 1.2, mean $\pm$ s.e.m.; $P<0.05$, Fig. 1D). Within the nonverbal-cue-biased judgments, a repeated-measure mixed-design ANOVA of the number of the nonverbal-cue-biased judgments did not detect a significant interaction between the group (TD and ASD) and the emotional valence of the nonverbal cues (positive and negative) ($P>0.4$). These results suggest that the decrease in nonverbal-cue-biased judgments in the ASD subjects were commonly observed for both emotional valences of nonverbal information. The response time in the nonverbal-cue-biased judgments was not different between the ASD and TD groups (Fig. 1E). A repeated-measure ANOVA of the response times during the nonverbal-cue-biased judgments did not detect a significant interaction between the group and the emotional valence of the nonverbal information ($P>0.5$).

As a whole, these behavioral results suggest that, compared with the TD subjects, the ASD subjects showed altered and less frequent use of nonverbal content in incongruent verbal-nonverbal social information, which is consistent with previous behavioral findings [17].

fMRI Results

Within-group analysis. Before comparing the neural mechanism for the use of the nonverbal content between the ASD and TD groups, we first conducted a within-group analysis, and searched for the brain regions that showed greater activity in the nonverbal-cue-biased judgments than in the verbal-cue-biased judgments for each of the groups. In the TD group, the bilateral AI, bilateral IFG, dorsal mPFC, bilateral superior parietal lobe (SPL), and left inferior parietal lobe (IPL) showed significantly greater activity in the nonverbal-cue-biased judgments ($t(16)>3.5$, $P<0.05$, FDR-corrected; Fig. 2A; Table 2). In the ASD group, the left amygdala and right superior temporal pole (STP) exhibited significantly stronger activity specific to the nonverbal-cue-biased judgments ($t(16)>3.6$, $P<0.05$, FDR-corrected; Fig. 2A; Table 2). Furthermore, in both the TD and ASD groups, the emotional valences of the nonverbal and verbal cues did not influence the activity in these brain regions during nonverbal-cue-biased judgments (main effect of the emotional valence: $P>0.3$ in one-way ANOVA). These findings provide a qualitative suggestion that the ASD participants used a different neural mechanism to process nonverbal information than the TD participants.

Between-group Analysis

To quantitatively elucidate the differences in brain activity between the ASD and TD subjects, we next conducted a repeated-measure mixed-design two-way ANOVA of the per-voxel brain activity (types of judgments: nonverbal- and verbal-cue-biased judgments × group: ASD and TD). Voxel-level brain activity was extracted from the anatomical brain regions using the same procedures as the within-group analysis. Although there were no significant main effects, we found a significant interaction between the group and the judgment types in the anterior cingulate cortex/ventral mPFC (ACC/vmPFC), dorsal mPFC (dmPFC), bilateral AI, right IFG, left amygdala, and right superior temporal pole (STP) ($F(1,30)>12$, $P<0.05$, FDR-corrected). In contrast, the exploratory whole brain analysis did not find any significant activation ($P<0.05$, FDR-corrected).

A post-hoc analysis of the interaction defined as (Bnv > Bv) × (TD > ASD) found that the ACC/vmPFC, dmPFC, bilateral AIs, and right IFG showed significantly greater activity in the TD group than in the ASD group ($t(30)>3.5$, $P<0.05$, FDR-corrected; Fig. 2B and 2C, Table 2). Another post-hoc analysis using the interaction defined as (Bnv > Bv) × (ASD > TD), the left amygdala and right STP showed significantly greater activity in the ASD group than in the TD group (Fig. 2B and 2C, Table 2). These results suggest that brain activity in these regions underlies the impaired use of nonverbal information in ASD individuals.

Characterization of the Detected Brain Regions

We then investigated which of the 8 brain regions examined exhibited activity that was related to the nonverbal-cue-biased judgments by correlating brain activity with the number of the nonverbal-cue-biased judgments. Consequently, in the TD group, activity in only the ACC/vmPFC and dmPFC exhibited significant positive correlations with the number of nonverbal-cue-biased judgments ($P<0.05$, Bonferroni-corrected among the eight regions in Fig. 2B and 2C; Fig. 3A). In contrast, significant correlations were not found in any of these eight brain regions in the ASD group. In particular, the correlations in the ACC/vmPFC and dmPFC were significantly steeper in the TD than in the ASD group (ACC/vmPFC: $Z=3.3$, $P=0.001$; dmPFC; $Z=2.9$, $P=0.002$; Fig. 3A). These results suggest that these two regions are primarily responsible for nonverbal-cue-biased judgments and imply that that decreased activity in these regions may underlie impaired nonverbal-cue-biased judgments in ASD individuals.

Correlation between fMRI Signals and Symptom Severity

To further examine the relationship between impaired nonverbal-cue-biased judgments and brain activity, we investigated the influence of diminished activity in the ACC/vmPFC and dmPFC on the severity of autistic symptoms related to social interaction

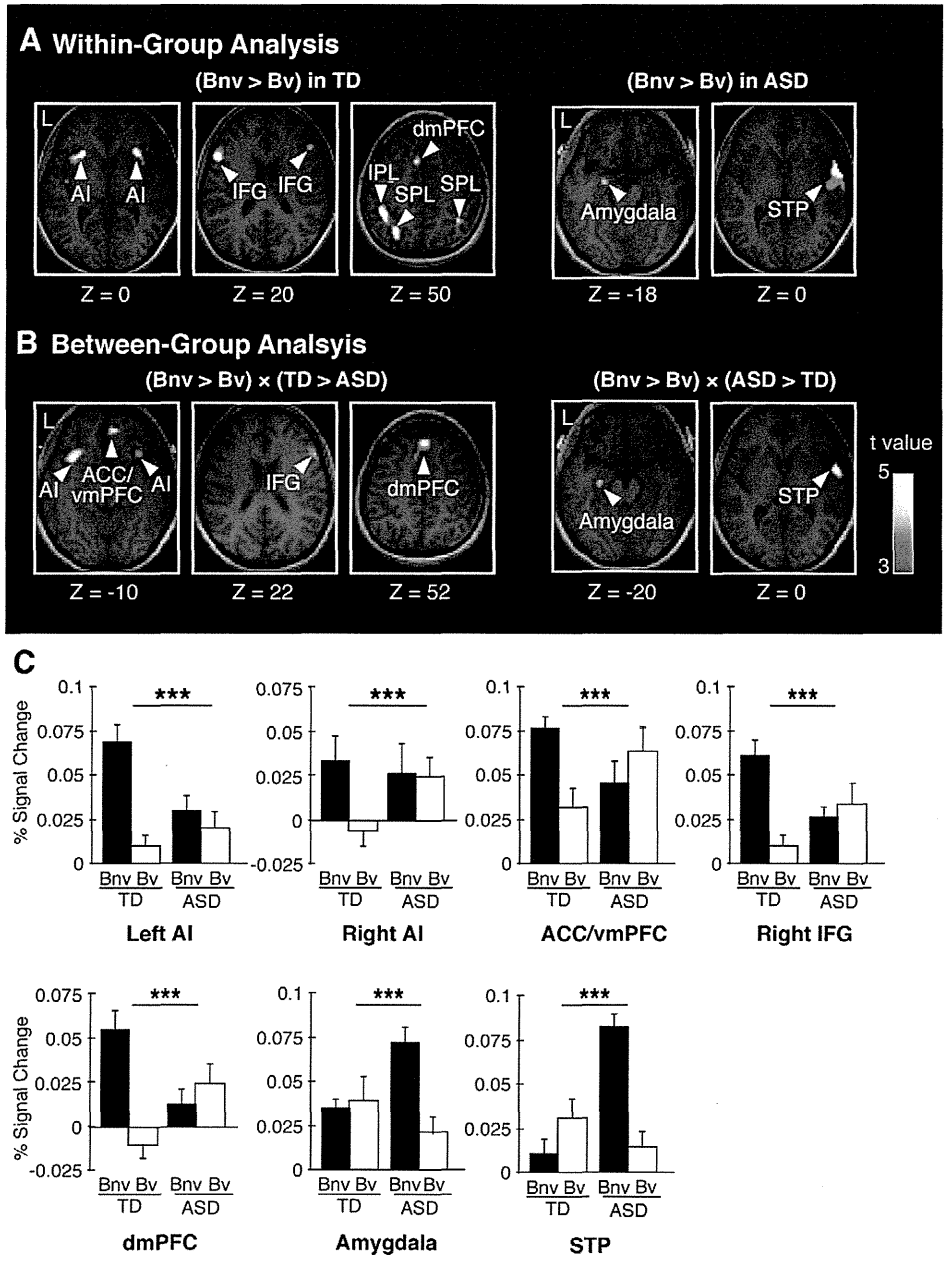

Figure 2. fMRI results (A) Brain regions specific to nonverbal-cue-biased judgments in TD or ASD individuals. The left three panels show the brain regions in which activity was significantly greater during the nonverbal-cue-biased judgments (Bnv) than during the verbal-cue-biased judgments (Bv) in the TD individuals (P<0.05, FDR-corrected). AI: anterior insula, IFG: inferior frontal gyrus, dmPFC: dorsal medial prefrontal cortex, IPL: inferior parietal lobe, SPL: superior parietal lobe. The right two panels show the three brain regions that had significantly greater activity during the nonverbal-cue-biased judgments than during the verbal-cue-biased judgments in the ASD individuals (P<0.05, FDR-corrected). STP: superior temporal pole. **(B) Brain regions whose activity was diminished or increased in ASD individuals.** The left three panels show the five brain regions that had a significant interaction between the type of judgment and the group, as defined as (Bnv > Bv) × (TD > ASD) (P<0.05, FDR-corrected). That is, in these regions, brain activity specific to the nonverbal-cue-biased judgments was larger in the TD group than in the ASD group. ACC/vmPFC: anterior cingulate cortex/ventral medial prefrontal cortex. The right two panels show the three brain regions in which brain activity specific to the nonverbal-cue-biased judgments was larger in the ASD group than in the TD group (P<0.05, FDR-corrected). **(C) Brain activity in the regions showing a significant interaction between judgment and group.** The bar graphs show the percent signal changes in the brain regions that were shown in panel (B). ***: P<0.001. error bar: s.d.
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(i.e., ADI-R social and communication scores). In both regions, the brain activity significantly and negatively correlated with ADI-R communication scores (ACC/vmPFC: $r = -0.67$, $P = 0.008$; dmPFC: $r = -0.62$, $P = 0.01$; both were equivalent to $P<0.05$, Bonferroni-corrected among the two regions and two scores; Fig. 3B), whereas there was no significant correlation between brain activity in these regions and ADI-R social scores (ACC/vmPFC: $r = -0.31$, $P = 0.26$; d mPFC: $r = -0.21$, $P = 0.44$). These results support the hypothesis that autistic deficits in communica-

Table 2. Between-group and within-group fMRI results.

Right/Left	Anatomical label	MNI coordinate x	y	z	cluster size	t value
Within-group analysis						
(Bnv > Bv) in TD						
Right	AI	32	24	2	271	4.2
Left	AI	−26	22	−2	362	4.5
Right	IFG	50	28	22	154	3.5
Left	IFG	−54	18	20	377	4.1
Left	dmPFC	−4	20	50	201	3.8
Right	SPL	40	−54	56	144	3.9
Left	SPL	−30	−64	48	421	4.2
Left	IPL	−44	−44	52	387	5.5
(Bnv > Bv) in ASD						
Left	Amygdala	−26	−4	−18	251	4.1
Right	STP	60	4	0	728	4.2
Between-group analysis						
(Bnv > Bv) × (TD > ASD)						
Left	ACC/vmPFC	2	34	8	283	4.2
Left	dmPFC	0	30	52	325	4.3
Right	IFG	54	28	22	201	3.6
Right	AI	38	42	−4	148	4.3
Left	AI	−38	16	−8	710	3.5
(Bnv > Bv) × (ASD > TD)						
Left	Amygdala	−26	−6	−20	114	4.1
Right	STP	56	2	0	253	4.3

ACC: Anterior cingulate cortex; vmPFC: ventral medial prefrontal cortex; dmPFC: dorsal medial prefrontal cortex; IFG: inferior frontal gyrus; AI: anterior insula; STP: superior temporal pole. P<0.05 FDR-corrected.
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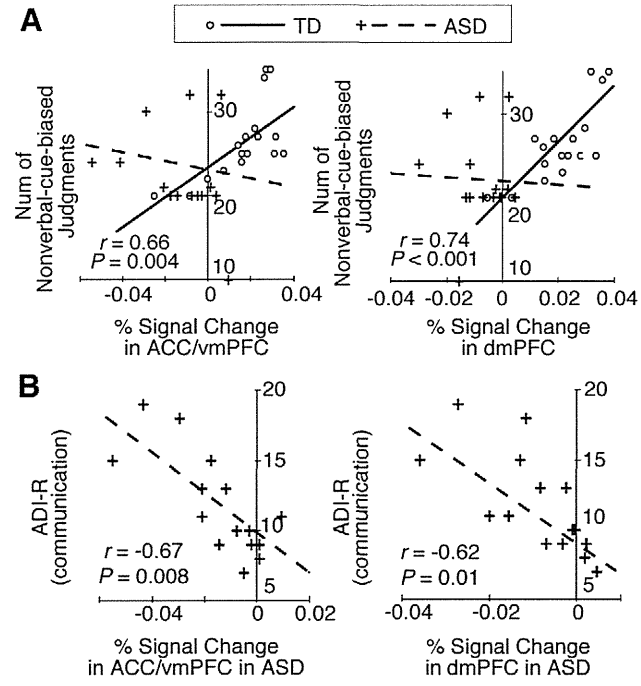

Figure 3. Comparison of fMRI signal and behavioral/clinical indices. (A) Correlation between fMRI signals and the number of nonverbal-cue-biased judgments. In the TD group, among the four regions in shown Fig. 2B, only the activity in the ACC/vmPFC and dmPFC showed a significant positive correlation with the number of nonverbal-cue-biased judgments (P<0.05, Bonferroni-corrected). In the ASD group, there was no significant correlation between this behavioral metric and activity in either region. Circles and lines: data of TD participants; + and dashed lines: data of ASD participants. **(B) Correlation between fMRI signal and clinical metrics.** In ASD group, the fMRI signals in the ACC/vmPFC and dmPFC exhibited significant negative correlations with ADI-R communication sub-scores.
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tion are partially due to diminished brain activity in the ACC/vmPFC and dmPFC.

Differences in Amygdala Activity

The possibility that ASD group exhibited less overall brain activity than the TD group was excluded by the finding that the ASD group exhibited significantly greater activity in the amygdala than the TD group during responses to congruent emotional stimuli ([36, −2, −28], t = 3.9, P<0.05, FDR-corrected in the amygdala) as well as during nonverbal-cue-biased judgments (Fig. 2D, Table 2).

Discussion

To the best of our knowledge, the present study is the first to elucidate the neural mechanisms underlying impaired social judgments of incongruent verbal-nonverbal information in ASD individuals. We found that ASD participants showed diminished activity in the bilateral AI, pIFG, ACC/vmPFC and dmPFC. Among these five brain regions, the ACC/vmPFC and dmPFC were the regions primarily involved in nonverbal-cue-biased judgments in the TD participants. In addition, the reduction in brain activity in these regions was predictive of the severity of the ASD participants' communication deficits. These results indicate that impaired recruitment of the ACC/vmPFC and dmPFC in ASD individuals is associated with their impairments in the social judgment of incongruent verbal-nonverbal information.

The current study employed short movies where a trained professional actor spoke an emotional word while displaying an emotional facial expression and voice prosody. These realistic video stimuli were chosen to maximize the similarity of the psychological task to real-life social situations. In contrast, previous studies have used nonverbal stimuli in the form of a narrator [4,5], static pictures [55,56] or cartoons [20]. The current paradigm allowed us to examine the neural basis of more spontaneous social judgments, which is considered to be distinctively impaired in ASD individuals [19]. It is the case that the current short movies may still evoke some unnatural feelings in the participants due to its monochrome style and possible incongruity between disgustful facial expression and contents of some negative words that are unrelated to disgust, and future study is expected to control such incongruity.

Although the present psychological task was a reaction time task, it had no correct response and allowed the participants to express what they felt in a relatively unrestrained manner. Therefore, the present task allowed the participants to respond in a more spontaneous manner than forced-choice tasks. This spontaneity not only reduced the differences between the task and real-life situations, but also enabled us to detect autistic behavioral and neural impairments, which are often difficult to capture in adult individuals with high functioning ASD [57]. ASD individuals

with higher verbal abilities can often solve social tasks appropriately when they are given enough verbal information and time to respond [58–61]. This superficial, adequate behavior in the adult high functioning ASD individuals is based on ASD-specific forms of the "theory of mind" [58]. In contrast, even individuals with high functioning ASD show difficulties in psychological tasks that require spontaneous mentalizing [3,18,19,62,63]. In addition, ASD individuals are more likely to exhibit impaired social judgments when psychological tasks utilize nonverbal stimuli [19]. Therefore, in the present study, the task paradigm of allowing the participants to respond more spontaneously than forced-choice tasks may have increased the sensitivity of the task to behavioral and neural impairments in high-functioning ASD individuals.

The number of participants in the present study is comparable to that in previous studies [64,65], and was large enough to detect a neural basis for impaired social judgments in ASD individuals. Based on the effect size of the detected significant differences in brain activity between the ASD and TD groups (Cohen's d >1.28), the powers of the present findings were sufficient, even for the weakest activation (>0.93 for the activation in the left AI, $t = 3.5$; Table 2). Furthermore, the differences between the groups in the present study in terms of sex, age, race, intellectual ability, pharmacological status, and psychiatric comorbidity were at least as small as in previous studies employing ASD individuals [5,46,64,65]. As such, the group sizes and homogeneity were sufficient to support our findings.

By demonstrating significant contributions of the ACC/vmPFC and dmPFC in nonverbal-information-biased judgments, the present results suggest that both areas play important roles in the pathophysiology of communication in ASD individuals. Previous studies have suggested that the vmPFC is involved in self-referential mentalizing [46,66,67], and that the dmPFC is associated with non-self-referential mentalizing and deeper objective reasoning [68–70]. That is, both of these subregions of the mPFC are involved in mentalizing [23], and impairments of their activity may disrupt social judgments of hostility. The presently reported results are consistent with those previous findings, and suggest more specific functions of these regions during the processing of incongruent verbal-nonverbal social stimuli. Furthermore, this specific role for these brain regions (i.e., social judgments based on nonverbal information) is in accord with previous findings that explicit attention to nonverbal information during an irony comprehension task increased activity in the anterior portion of the dorsal mPFC in ASD children [20].

The right IFG and bilateral AI also exhibited significantly reduced activity during nonverbal-information-biased judgments in the ASD group compared with the TD group. Previous studies in TD individuals have repeatedly reported significant contributions of these brain regions to empathic processing of the emotions and sensations of other people (reviewed in [25,71]). Functional and structural deficits in these brain regions in ASD individuals have been reported to be associated with clinical deficits in understanding other's emotional states and in communicating with others [72–74]. Notably, consistent with the current results, previous studies have also highlighted a crucial role of the AI in judging and categorizing uncertainty [75].

In contrast to the mPFC, the amygdala showed larger activity in the ASD group than in the TD group. This enhanced activation was observed both during nonverbal-cue-biased judgments and during responding to the congruent stimuli. Anatomical and functional abnormalities in the amygdala of ASD individuals have been reported in a series of previous studies [55,57,76,77]. In particular, previous fMRI studies have suggested that ASD participants show greater activity in the amygdala than TD individuals when they see emotional facial expression [55,56]. This finding is consistent with the present observation of enhanced amygdala activation during responding to congruent stimuli. Moreover, the present study provided further evidence to support this observation because the enhanced activation in the ASD subjects during the nonverbal-cue-biased judgments could be interpreted as a consequence of an enhanced response to nonverbal information, such as facial expressions.

To validate our findings, we excluded the possibility that all brain regions in the ASD subjects exhibited less brain activity than those in the TD subjects. Even after global normalization, we detected enhanced activation in the amygdala in response to congruent emotional cues in the ASD subjects. This enhanced activation in the amygdala furthermore excludes the possibility that training-related effects influenced our findings. In the present study, before the fMRI scanning, the ASD participants required a longer training period to learn the task than the TD participants (ASD: nine times; TD: three times). If the ASD participants became more habituated to the emotional stimuli during training than the TD controls, they may have exhibited less activity in the amygdala [47]. The enhanced activation in the amygdala in the ASD subjects minimizes this possibility and further validates our main findings.

The behavioral pattern in the response times to incongruent stimuli data excluded the possibility that the ASD participants may have been unable to perceive nonverbal information. If this was the case, ASD individuals would not have been able to sense the incongruence between nonverbal and verbal information. However, as in the TD participants, the response times to incongruent stimuli were significantly prolonged compared to the response times to congruent stimuli in the ASD participants (Fig. 1C), which is consistent with a previous study [5]. In addition, the response times did not differ between the ASD and TD groups, for either incongruent or congruent stimuli, as was observed in another previous study [4]. This behavioral pattern suggests that the ASD participants were able to perceive the verbal and nonverbal information and sense the incongruence of the stimuli, which is in line with a previous behavioral study of conflicting verbal-nonverbal information in ASD individuals [17].

Finally, by estimating the effects of the interaction among the types of judgments and the groups, we also excluded the possibility that activation in the brain regions in which the ASD subjects exhibited less activity reflected conflict monitoring rather than the effects of impaired nonverbal-cue-biased judgments. The detected activations were based on differences in brain activity during the nonverbal-cue-biased judgments compared to verbal-cue-biased judgments. Therefore, we can assume that the influence of the conflict monitoring on these activations was minimal.

In conclusion, the present study demonstrated that significantly decreased brain activity in the right IFG, bilateral AI, and ventral and dorsal mPFC was associated with impaired social judgments of incongruent verbal-nonverbal information in high-functioning non-medicated adult males with ASD. In particular, the nonverbal-cue-biased judgments recruited the ACC/vmPFC and dmPFC in the TD group, and diminished activity in these two regions in the ASD subjects was predictive of the extent of their impairments in communicating with other people. Through disrupting important cognitive systems such as mentalizing, empathy, and judging uncertainty, dysfunction in these brain regions may play a role in shaping the characteristic

tendencies of individuals with ASD to make impaired social judgments.

Supporting Information

Video S1
(MP4)

Video S2
(MP4)

Video S3
(MP4)

Video S4 Each video is a representative stimulus for each type of stimuli used in the present study. In a video, one of 20 professional actors (10 males and 10 females) appears and speaks an emotional word (positive or negative) with emotional facial expression and prosody (happy: positive or disgust: negative). In VideoS1.mp4 and VideoS3.mp4, the actor speaks "(You look) Distressed" in Japanese with disgust or happy facial expression and prosody, respectively. In VideoS2.mp4 and VideoS4.mp4, he speaks "(You are) Friendly" in Japanese with disgust or happy facial expression and prosody, respectively.
(MP4)

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Author Contributions

Conceived and designed the experiments: HY. Performed the experiments: TW HY OA NY HI YT NI TN YA HT HS WG MM MK AK Y. Kawakobo HK HM KJT Y. Kano NK. Analyzed the data: TW HY. Wrote the paper: TW HY YM KK.

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Decreased Serum Levels of Mature Brain-Derived Neurotrophic Factor (BDNF), but Not Its Precursor proBDNF, in Patients with Major Depressive Disorder

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Abstract

Background: Meta-analyses have identified serum levels of brain-derived neurotrophic factor (BDNF) as a potential biomarker for major depressive disorder (MDD). However, at the time, commercially available human ELISA kits are unable to distinguish between proBDNF (precursor of BDNF) and mature BDNF because of limited BDNF antibody specificity. In this study, we examined whether serum levels of proBDNF, mature BDNF, and matrix metalloproteinase-9 (MMP-9), which converts proBDNF to mature BDNF, are altered in patients with MDD.

Methodology/Principal Findings: Sixty-nine patients with MDD and 78 age- and gender-matched healthy subjects were enrolled. Patients were evaluated using 17 items on the Structured Interview Guide for the Hamilton Depression Rating Scale. Cognitive impairment was evaluated using the CogState battery. Serum levels of proBDNF, mature BDNF, and MMP-9 were measured using ELISA kits. Serum levels of mature BDNF in patients with MDD were significantly lower than those of normal controls. In contrast, there was no difference in the serum levels of proBDNF and MMP-9 between patients and normal controls. While neither proBDNF nor mature BDNF serum levels was associated with clinical variables, there were significant correlations between MMP-9 serum levels and the severity of depression, quality of life scores, and social function scores in patients.

Conclusions/Significance: These findings suggest that mature BDNF may serve as a biomarker for MDD, and that MMP-9 may play a role in the pathophysiology of MDD. Further studies using larger sample sizes will be needed to investigate these results.

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Introduction

Accumulating evidence suggests that brain-derived neurotrophic factor (BDNF) plays a key role in the pathophysiology of major depressive disorder (MDD), as well as the therapeutic mechanisms of antidepressants [1–6]. Previously, we reported that BDNF serum levels in patients with MDD were significantly lower than those of healthy controls, and that there was a negative correlation between BDNF serum levels and the severity of depression in patients [7]. Furthermore, decreased serum levels of BDNF in antidepressant naïve patients with MDD, recovered to levels associated with amelioration of depressive symptoms, after antidepressant treatment [7]. Three meta-analyses [8–10] and a study using a large sample size [11] confirmed our previous findings. Therefore, it is likely that the accurate measurement of blood BDNF levels could serve as a potential biomarker for MDD [6].

Mature BDNF is initially synthesized as a precursor protein, preproBDNF, in the endoplasmic reticulum. Following cleavage of the signal peptide, proBDNF is converted to mature BDNF by extracellular proteases, such as matrix metalloproteinase-9 (MMP -9) and plasmin [6,12–16]. It was initially thought that only secreted mature BDNF was biologically active, and that proBDNF, which localizes intracellularly, served as an inactive precursor. However, new evidence shows that proBDNF and mature BDNF elicit opposing effects via the $p75^{NTR}$ and TrkB receptors, respectively, and that both proBDNF and mature BDNF play important roles in several physiological functions [6,12–14]. Considering the important roles of both proBDNF and mature BDNF in physiological functions, it would be informative

to measure individual levels of proBDNF and mature BDNF in the body fluids of human subjects [6,17]. Although BDNF levels in human blood can be measured using commercially available human BDNF ELISA kits, due to the limited specificity of the BDNF antibody, early versions of these kits were unable to distinguish between proBDNF and mature BDNF [18]. Very recently, we reported that serum levels of proBDNF and mature BDNF in healthy subjects were measurable using newly available human proBDNF and BDNF ELISA kits [18].

This study aimed to determine whether serum levels of proBDNF and mature BDNF were altered in patients with MDD. We also investigated MMP-9 serum levels, as MMP-9 plays a role in the conversion of proBDNF to mature BDNF [15,16]. Since it is also known that patients with MDD suffer cognitive impairment [19–21] even in remission [22], we examined the correlation between serum levels of proBDNF, mature BDNF, and MMP-9, with clinical variables, including depression and cognition, in patients with MDD.

Materials and Methods

Participants

Sixty-nine patients with MDD and 78 age-matched healthy controls were enrolled (**Table 1**). All patients were outpatients and met DSM-IV criteria for MDD [23]. There were no specific medication criteria for inclusion. Sixty-five patients were treated with antidepressants. Two of the four patients who were antidepressant therapy naïve, had been treated with anxiolytics. Control subjects were screened using the Structured Clinical Interview for DSM-IV Axis I Disorders, Non-Patients Edition, and were required to not have an Axis I disorder, according to DSM-IV criteria [23]. Study investigators made a concerted effort to recruit healthy controls who matched patients on age, male/female ratio, education, premorbid intelligence quotient (IQ) (as assessed by the Japanese Adult Reading Scale-25 version, which is the Japanese version of the National Adult Reading Test), body mass index, and smoking status. Smoking status was dichotomized into current smokers versus non-smokers. Exclusion criteria for subjects in both groups included any current or past history of neurological disorders, including head injury, cerebral vascular disorders, epilepsy, alcohol or drug abuse. Subjects who rarely used personal computers were excluded from the study. Prior to commencement of the study, all subjects provided written informed consent, after receiving a full explanation of the study as well as any potential risks and benefits of study participation. Our samples in the current study consist of all such patients with MDD who are not severe depressive state and possess the ability to agree to join the research. The study was approved by the Ethics Committee of Chiba University Graduate School of Medicine (Chiba, Japan) and performed in accordance with the Declaration of Helsinki II.

Assessment of clinical variables

Depression was assessed using 17 items of the Structured Interview Guide for the Hamilton Depression Rating Scale (SIGH-D) [24]. Quality of life (QOL) was assessed using WHOQOL-BREF. Social function was assessed using the Japanese version of the Social Adaptation Self-evaluation Scale (SASS) [25], which is a validated self-evaluation scale for assessment of social functioning [26].

Cognitive impairment was assessed using the Japanese language version of the CogState battery, a rapid, automatically administered computerized battery which assesses verbal learning, visual learning, speed of processing, attention/vigilance, visual working memory, spatial working memory, reasoning and problem solving, and social cognition [27]. The primary measure from each task of the CogState battery was standardized by creating Z-scores. The healthy control mean was set to zero and the standard deviation set to one, following the methodological procedure used by Keefe et al. [28]. A composite score was calculated by averaging all Z-scores from the eight primary measures of the CogState battery. The composite score of the CogState battery correlated well with the composite score of the Brief Assessment of Cognition in Schizophrenia (BACS), Japanese-language version [27] and the composite score of the MATRICS Consensus Cognitive Battery [29]. Therefore, we used these composite scores as representative values for cognition.

Measurement of proBDNF, mature BDNF, and MMP-9 serum levels

Serum samples from control subjects were collected between 9:00 and 15:00, and stored at −80°C until use. Serum levels of proBDNF, mature BDNF, and MMP-9 were measured using the human proBDNF ELISA Kit (Adipo Bioscience, Santa Clara, CA, USA), the human BDNF ELISA Kit (Adipo Bioscience, Santa Clara, CA, USA), and the human MMP-9 ELISA Kit (R&D Systems, Minneapolis, MN, USA), respectively. To minimize assay variance, serum levels of proBDNF, mature BDNF, and MMP-9 from each subject were measured on the same day. All experiments were performed in duplicate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (Emax; Molecular Devices, Sunnyvale, CA, USA).

Statistical analyses

The data show the mean ± standard deviation (SD). Chi-squared test was used for categorical variables. Parametric Student's *t*-test or non-parametric Mann-Whitney test was used to compare two groups. Correlations with clinical variables were performed using Pearson's correlation or Spearman's correlation. A p value of <0.05 was judged as statistically significant. All analyses were carried out using SPSS version 19.0 (SPSS Inc, Chicago, IL, USA).

Results

Demographic data and clinical variables

The demographic information and clinical variables of subjects are presented in **Table 1**. Age, gender, estimated premorbid IQ, education, body mass index, and smoking status did not differ between the two groups. The WHOQOL-BREF and SASS scores in patients with MDD were significantly lower than those of controls, indicating that patients with MDD had a decreased QOL and poor social function. Furthermore, the composite scores of the CogState battery in patients with MDD were significantly lower than those of healthy controls, indicating cognitive impairment in this patients group (**Table 1**).

Serum levels of proBDNF, mature BDNF, and MMP-9

In 20 patients and 29 controls, serum levels of proBDNF were below the minimum detectable concentration (0.5 ng/mL) of the proBDNF ELISA kits. Chi-squared testing showed no difference ($x^2 = 1.106$, $p = 0.293$) between these groups. Serum levels of proBDNF (10.68±11.28 ng/mL) in patients ($n = 49$) were not different ($p = 0.974$) from those (8.90±7.98 ng/mL) in controls ($n = 49$) (**Figure 1A**).

Table 1. Demographic data of subjects.

Characteristics	Patients (n = 69)	Controls (n = 78)	P values
Gender (male/female)	32/37	32/46	0.514
Age (year olds)	40.5±9.7 (20–60)	37.2±9.8 (20–59)	0.098
Smoking status (current/non-smoker)	20/49	19/59	0.526
Premorbid IQ	105.6±9.4 (85–120)	104.0±8.2 (87–118)	0.308
Education (years)	13.8±2.2 (9–18)	14.0±2.0 (12–18)	0.589
Body mass index	22.7±4.3 (15.0–35.9)	22.0±3.3 (17.1–34.3)	0.156
WHOQOL-BREF score	2.63±0.54 (1.27–3.69)	3.78±0.38 (1.96–2.88)	<0.001*
SASS score	26.5±8.3 (8–44)	41.7±5.4 (29–56)	<0.001*
CogState composite score	−0.46±0.80 (−2.85–1.02)	0.00±0.352 (−0.85–0.83)	<0.001^{+}
Age of first depressive episode	33.0±10.0 (11–55)		
Duration of illness (years)	7.2±7.3 (0–29)		
Duration of untreated illness (years)	1.0±1.7 (0–9)		
SIGH-D score	11.8±5.5 (0–24)		

Data show the mean ± SD. Figures in parenthesis represent the range.
WHOQOL-BREF: World Health Organization Quality of Life-Short Version.
SASS: Social Adaptation Self-evaluation Scale, SIGH-D: 17 items of the Structured Interview Guide for the Hamilton Depression Rating Scale.
*Student's t-test, $^{+}$Mann-Whitney's U-test.
doi:10.1371/journal.pone.0042676.t001

In contrast, serum levels of mature BDNF (21.09±5.60 ng/mL) in patients (n = 69) were significantly (p = 0.035) lower than those (23.11±5.90 ng/mL) of controls (n = 78) (**Figure 1B**).

Serum levels of MMP-9 (4.52±1.69 ng/mL) in patients (n = 69) were no different (p = 0.453) from those (4.63±2.69 ng/mL) in controls (n = 78) (**Figure 2**).

Correlations with clinical variables

In subjects with measurable serum levels of proBDNF, there was no correlation between this molecule and clinical variables, such as smoking status, body mass index, duration of illness, duration of untreated illness, SIGH-D scores, WHOQOL-BREF scores, SASS scores, and composite scores of the CogState battery.

There were significant weak correlations between serum levels of mature BDNF and duration of illness (ρ = 0.282, p = 0.019) and body mass index (r = 0.345, p = 0.005) in patients (n = 69). However, there was no correlation between mature BDNF levels, and other clinical variables, such as smoking status, duration of untreated illness, SIGH-D scores, WHOQOL-BREF scores, SASS scores, and composite scores of the CogState battery.

Interestingly, there were significant correlations between MMP-9 levels and WHOQOL-BREF scores (ρ = −0.366, p = 0.002) (**Figure 3A**), SASS scores (ρ = −0.355, p = 0.003) (**Figure 3B**), and the SIGH-D score (ρ = 0.397, p = 0.001) (**Figure 3C**) in patients (n = 69). In contrast, there was no correlation between MMP-9 levels and other clinical variables, including composite scores of the CogState battery in patients (n = 69) and all subjects (n = 147).

Next, we examined the relationships between clinical variables in all subjects and patients. There was a high positive correlation

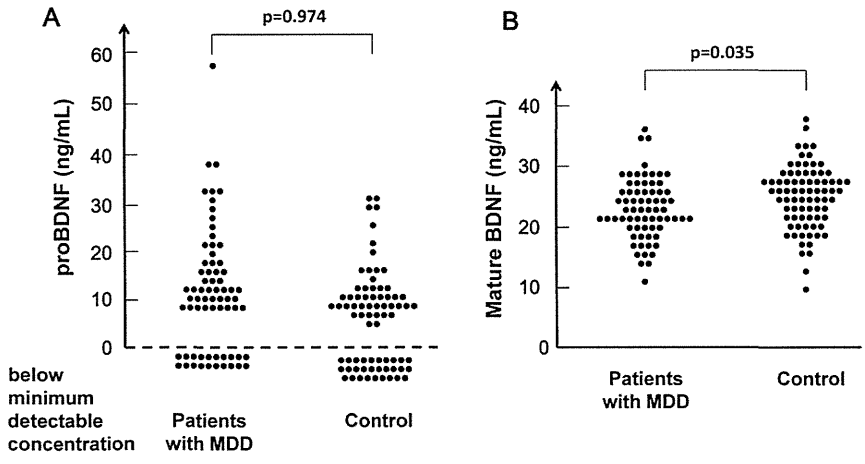

Figure 1. Scatter plot of proBDNF and mature BDNF serum levels in patients with MDD and healthy controls. (A): Serum levels of proBDNF in 20 patients with MDD and 29 healthy subjects were below the minimum detectable concentration (0.5 ng/mL) of the proBDNF ELISA kits. Serum levels of proBDNF in patients with MDD did not differ from those of normal controls. (B): In contrast, serum levels (21.09±5.60 ng/mL) of mature BDNF in patients with MDD, were significantly lower than those (23.11±5.90 ng/mL) of normal controls.
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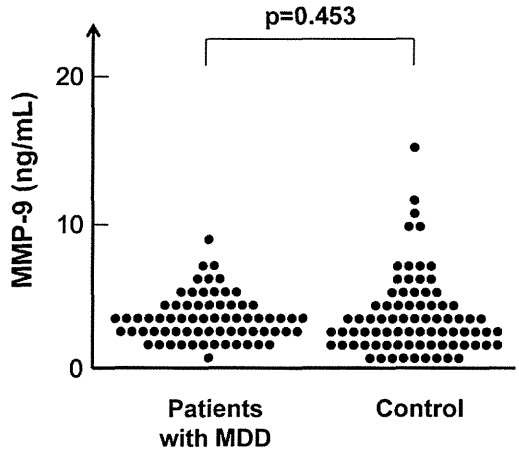

Figure 2. Scatter plot of MMP-9 serum levels in patients with MDD and healthy controls. Serum levels of MMP-9 in patients with MDD did not differ from those of normal controls.
doi:10.1371/journal.pone.0042676.g002

($\rho = 0.828$, $p<0.001$) between SASS and WHOQOL-BREF scores in all subjects ($n = 147$), suggesting a close relationship between QOL and social function. There were negative correlations between SIGH-D and WHOQOL-BREF scores ($r = -0.705$, $p<0.001$) and SIGH-D and SASS scores ($r = -0.579$, $p<0.001$) in patients ($n = 69$) (**Figure 4A and 4B**). Moreover, there were positive correlations between CogState composite scores and WHOQOL-BREF scores ($r = 0.404$, $p = 0.001$) and SASS scores ($r = 0.371$, $p = 0.002$) in patients ($n = 69$) (**Figure 4C and 4D**). These findings suggest that the symptoms of depression and cognition impinge on the QOL and social function of patients with MDD.

Discussion

In this study, we found that for patients with MDD, serum levels of mature BDNF, but not proBDNF were significantly lower than those of age- and gender-matched healthy controls. To the best of our knowledge, this is the first report showing decreased serum levels of mature BDNF in patients with MDD. Three meta-analyses and a large cohort study [8–11] demonstrated that serum levels of BDNF were significantly lower in patients with MDD, although at the time, the commercially available human BDNF ELISA kits were unable to distinguish between proBDNF and mature BDNF because of the limited specificity of the BDNF antibody [18]. It means therefore, that these studies reported on combined levels of proBDNF and mature BDNF, and not the sole levels of mature BDNF [18]. In contrast, the proBDNF and mature BDNF ELISA kits used in the present study are able to distinguish between pro and mature forms of BDNF in human serum [18]. Therefore, it is likely that a reduction in mature BDNF contributes to the decreased levels of BDNF in patients with MDD, observed in earlier reports [8–11].

Previously, we reported that serum levels of BDNF in antidepressant-naïve patients with MDD were significantly lower than those of medicated patients and healthy controls, and that serum levels of BDNF were correlated negatively with the severity of depression [7]. In addition, BDNF serum levels in antidepressant-naïve patients increased after antidepressant treatment [7]. These results were supported by subsequent meta-analyses [8–11]. Taken together these findings imply that serum BDNF levels may function as a state biomarker for MDD. However, in this study, we found no correlation between mature BDNF levels and the severity of depression in patients with MDD. Patients enrolled in this study had moderate, but not severe symptoms of depression (SIGH-D score; 0–24) compared with our previous report [7]. It is likely that the lack of correlation between mature BDNF levels and depression severity seen in our study may be due to the absence of severe disease patients within our cohort. A further study using patients with severe depression will be needed to resolve this issue.

In this study, we were unable to measure serum levels of proBDNF in 49 subjects (20 patients and 29 healthy controls), because their values fell below the minimum detectable threshold of the ELISA kit. The manufacturer's instructions state that the minimum detectable concentrations of the proBDNF and mature BDNF ELISA kits are 0.5 ng/mL and 5–8 pg/mL, respectively, indicating that the sensitivity of the proBDNF kit is markedly lower than that of the mature BDNF kit [18]. As mentioned above, proBDNF and mature BDNF have opposing biological functions in the brain and peripheral organs [6,12–14]. Therefore, accurate measurement of serum proBDNF levels in human samples requires the development of a higher sensitivity ELISA kit than is currently available.

MMP-9 plays a key role in synaptic plasticity of the brain, and acts by converting proBDNF to mature BDNF, which in turn results in TrkB activation [15,16]. A recent study using MMP-9 knock-out mice demonstrated that MMP-9 plays a role in the

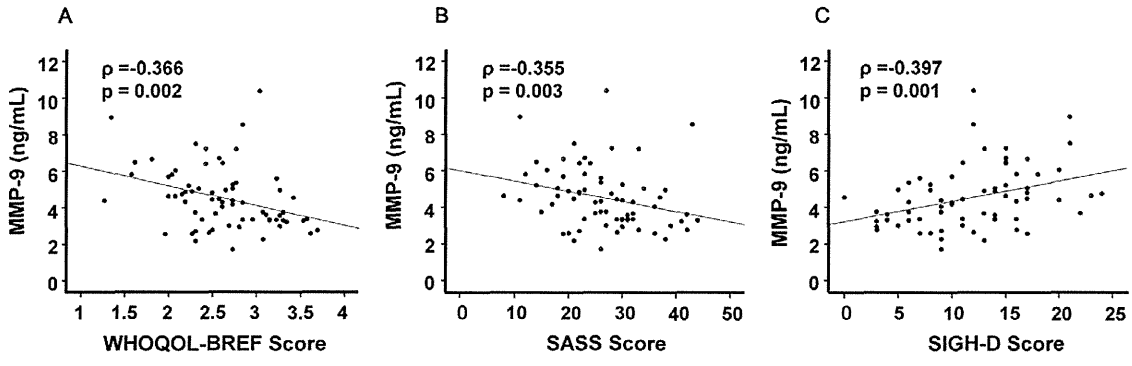

Figure 3. Relationships between MMP-9 serum levels and clinical variables in patients with MDD. (A): There was a significant negative correlation ($\rho = -0.366$, $p = 0.002$) between MMP-9 serum levels and WHOQOL-BREF scores. (B): There was a significant negative correlation ($\rho = -0.355$, $p = 0.003$) between MMP-9 serum levels and SASS scores. (C): There was a significant positive correlation ($\rho = 0.397$, $p = 0.001$) between MMP-9 serum levels and the SIGH-D score.
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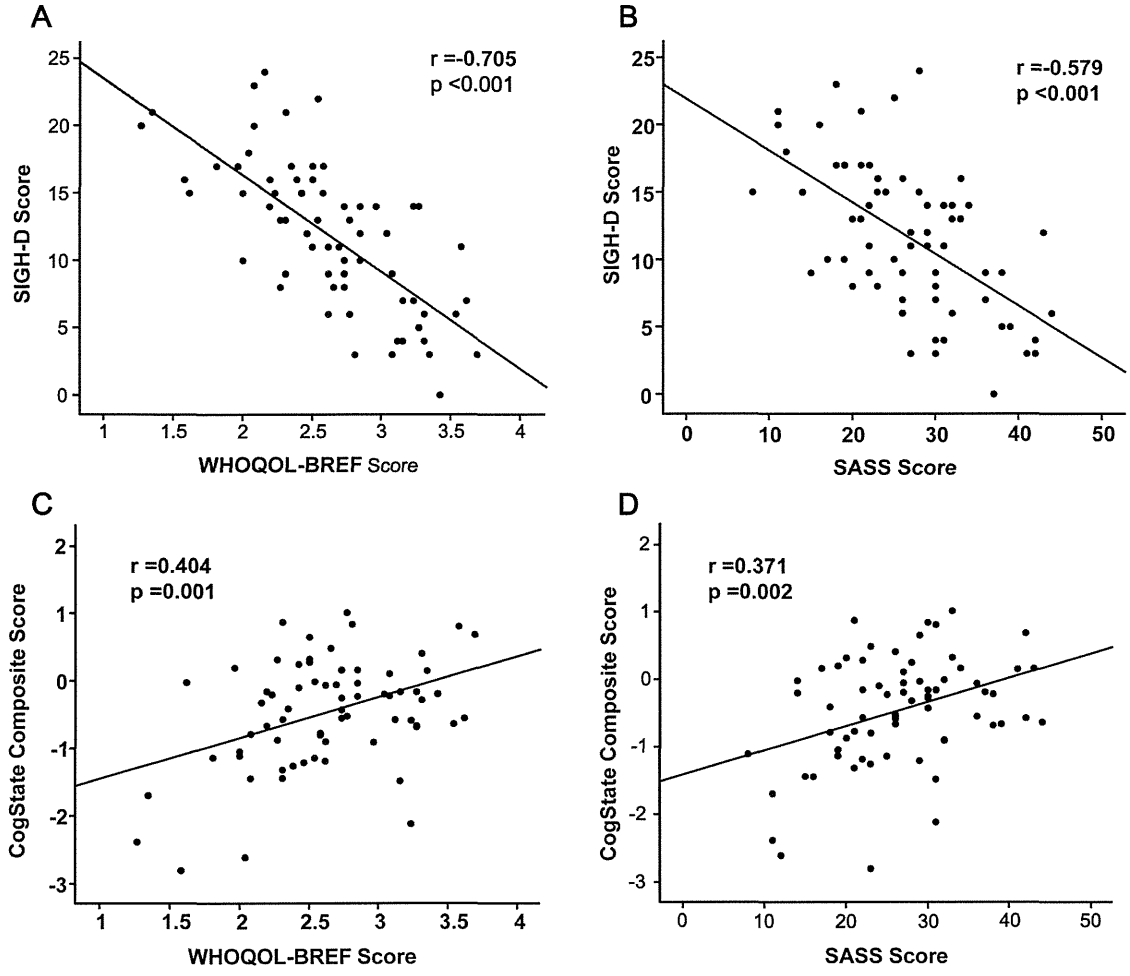

Figure 4. Relationships between clinical variables in patients with MDD. (A): There was a significant negative correlation ($r = -0.705$, $p<0.001$) between the SIGH-D score and WHOQOL-BREF score in patients. (B): There was a significant negative correlation ($r = -0.579$, $p<0.001$) between the SIGH-D and SASS scores in patients. (C): There was a positive correlation ($r = 0.404$, $p = 0.001$) between the CogState composite score and QOL score in patients. (D): There was a positive correlation ($r = 0.371$, $p = 0.002$) between the CogState composite score and SASS score in patients.

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development of pentylenetetrazole-induced kindling, by converting proBDNF to mature BDNF in the hippocampus [30]. This study, found no difference in serum MMP-9 levels between patients with MDD and healthy controls, consistent with a previous report that detected no changes from the norm in gingival crevicular fluid MMP-9 levels from female patients with depression [31]. Interestingly, we found a positive correlation between serum MMP-9 levels and the severity of depression in patients with MDD, although the role of MMP-9 in the pathophysiology of MDD is currently unknown. One possibility is that MMP-9 expression is increased as a compensatory response to decreases in mature BDNF, in patients with MDD. A recent proteomic approach using plasma samples from a large case-control cohort, demonstrated that plasma levels of MMP-9 in patients with MDD ($n = 245$) were significantly higher than those of controls ($n = 254$) [32], a finding that is inconsistent with our data. The reasons underlying this discrepancy are currently unknown. It has also been reported that the *MMP-9* gene polymorphisms are associated with cardiovascular disease and neuropsychiatric disorders, including schizophrenia, and bipolar disorder [33], suggesting that this enzyme may be a pathological mediator in these diseases. Given the opposing functions of proBDNF and mature BDNF, it would be of great interest to study the precise mechanisms controlling the cleavage of proBDNF to mature BDNF [6]. Therefore, further detailed studies will be also necessary to examine the role of MMP-9 in the pathophysiology of MDD.

Finally, there are some limitations to this study that need to be mentioned. The main limitation was the very small cohort size of the medication-naïve patients with severe symptoms of depression. Antidepressant medication is known to increase serum levels of BDNF in patients with MDD [6,8–11]. Therefore, further studies using a larger sample size of medication-naïve patients will be needed. Another limitation was the sensitivity of the human proBDNF ELISA kits, a sensitivity which is significantly lower than that of the human mature BDNF kit [18]. Given the key role of proBDNF/mature BDNF/TrkB signaling in the pathophysiology of MDD [7,12–14], it is imperative to develop proBDNF ELISA kits of greater sensitivity. It would also be of great interest to study the relationships between serum levels of proBDNF and mature BDNF along with levels of extracellular peptidases (e.g., MMP-9, plasmin) that convert proBDNF to mature BDNF.

In conclusion, we found that in patients with MDD, serum levels of mature BDNF, but not proBDNF, were significantly lower than those of healthy controls. Furthermore, we found correlations between serum MMP-9 levels and depression, QOL, and social function in patients, although there were no differences in serum MMP-9 levels between patients and healthy controls. Further studies measuring the serum levels of proBDNF, mature BDNF, and MMP-9 using larger cohorts, particularly cohorts of antidepressant-naïve patients, will be needed.

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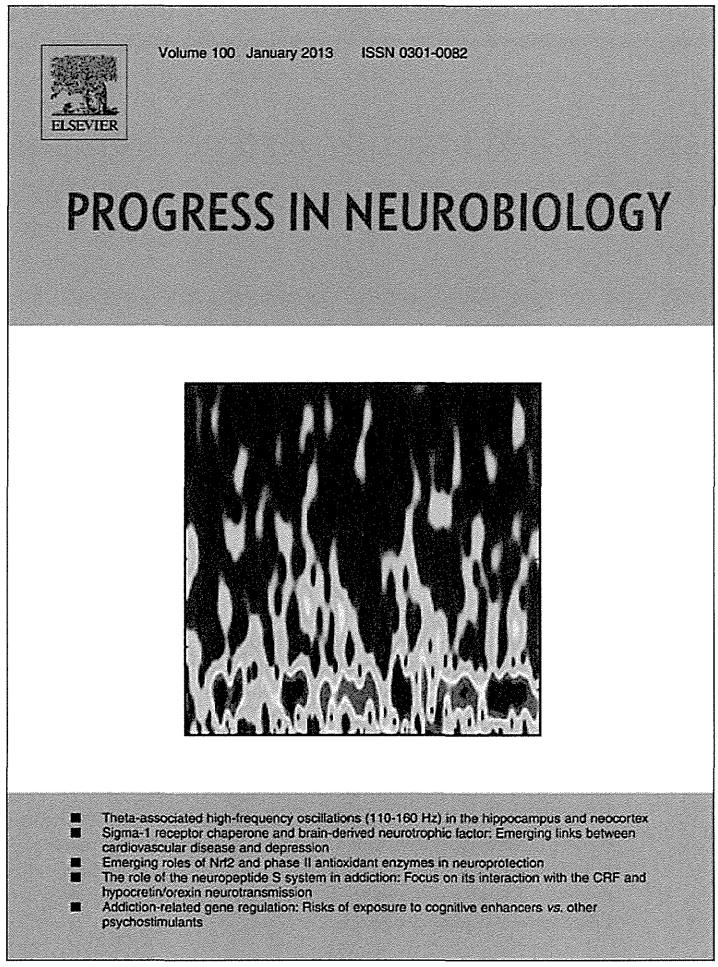
Author Contributions

Performed the experiments: TY KH. Analyzed the data: TY KH. Contributed reagents/materials/analysis tools: TY MI TN MN HW TS AS TH NK TH ME AK MI. Wrote the paper: TY KH.



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Sigma-1 receptor chaperone and brain-derived neurotrophic factor: Emerging links between cardiovascular disease and depression

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ABSTRACT

Epidemiological studies have demonstrated a close relationship between depression and cardiovascular disease (CVD). Although it is known that the central nervous system (CNS) contributes to this relationship, the detailed mechanisms involved in this process remain unclear. Recent studies suggest that the endoplasmic reticulum (ER) molecular chaperone sigma-1 receptor and brain-derived neurotrophic factor (BDNF) play a role in the pathophysiology of CVD and depression. Several meta-analysis studies have showed that levels of BDNF in the blood of patients with major depressive disorder (MDD) are lower than normal controls, indicating that blood BDNF might be a biomarker for depression. Furthermore, blood levels of BDNF in patients with CVD are also lower than normal controls. A recent study using conditional BDNF knock-out mice in animal models of myocardial infarction highlighted the role of CNS-mediated mechanisms in the cardioprotective effects of BDNF. In addition, a recent study shows that decreased levels of sigma-1 receptor in the mouse brain contribute to the association between heart failure and depression. Moreover, sigma-1 receptor agonists, including the endogenous neurosteroid dehydroepiandosterone (DHEA) and the selective serotonin reuptake inhibitor (SSRI) fluvoxamine, show potent cardioprotective and antidepressive effects in rodents, via sigma-1 receptor stimulation. Interestingly, agonist activation of sigma-1 receptors increased the secretion of mature BDNF from its precursor proBDNF via chaperone activity in the ER. Given the role of ER stress in the pathophysiology of CVD and MDD, the author will discuss the potential link between sigma-1 receptors and BDNF-TrkB pathway in the pathophysiology of these two diseases. Finally, the author will make a case for potent sigma-1 receptor agonists and TrkB agonists as new potential therapeutic drugs for depressive patients with CVD.

Contents

Abbreviations: ACTH, adrenocorticotropic hormone; ALLO, allopregnanolone; ANA-12, *N*2-(2-{[(2-oxoazepan-3-yl)amino]carbonyl}phenyl)benzo[b]thiophene-2-carboxamide; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; BiP, binding immunoglobulin protein; CNS, central nervous system; CSF, cerebrospinal fluid; CVD, cardiovascular disease; DG, dentate gyrus; DHEA, dehydroepiandosterone; DHEA-S, dehydroepiandosterone sulfate; 5,7-DHF, 5,7-dihydroxyflavone; 7,8-DHF, 7,8-dihydroxyflavone; 4′-DMA-7,8-DHF, 4′-dimethylamino-7,8-dihydroxyflavone; ELISA, Enzyme-Linked ImmunoSorbent Assay; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GABA, γ-amino butyric acid; GRP78, 78 kDa glucose-regulated protein; HPA, hypothalamus-pituitary-adrenal; HRSD, Hamilton rating scale for depression; HSD, hydroxysteroid dehydrogenase; ICV, intracerebroventricular; IP_3, inositol 1,4,5-triphosphate; LPS, lipopolysaccharide; LV, left ventricle; MAP, mitogen-activated protein; MDD, major depressive disorder; MMP-9, matrix metalloproteinase-9; 1NMPP1, 1-(1,1-dimethylethyl)-3-(1-naphthalenylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; NAc, nucleusaccumbens; NGF, nerve growth factor; NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; PO, pressure overload; PREG, pregnenolone; PREG-S, pregnenolone sulfate; PROG, progesterone; QOL, quality of life; RV, right ventricle; SSRI, selective serotonin reuptake inhibitor; SWA, slow wave activity; Trk, tyrosine kinase; TSPO, translocator protein; UPR, unfolded protein response; VTA, ventral tegmental area.

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1. Introduction

Epidemiological studies have demonstrated a strong association between cardiovascular disease (CVD) and depression (Musselman et al., 1998; Hemingway and Marmot, 1999; Rugulies, 2002; Joynt et al., 2003; van Melle et al., 2004; Taylor et al., 2005; Baune et al., 2012; Nemeroff and Goldschmidt-Clermont, 2012). Depression is highly prevalent in patients with CVD, with between 20 and 40% of patients meeting the criteria for major depressive disorder (MDD) or experiencing an increase in depressive symptoms. These symptoms are often chronic, and are associated with the development and progression of coronary artery disease, a worsening of health-related quality of life (QOL), poor physical functioning, recurrent cardiac events, and a 2- to 2.5-fold increased risk of mortality (Celano and Huffman, 2011). Accumulating evidence suggests that the central nervous system (CNS) may contribute to the worsening of heart failure, although the precise mechanisms underlying this process remain unclear (Joynt et al., 2003; de Jonge et al., 2010).

Considering the comorbidity of depression with CVD, it seems likely that there may be a common pathophysiological pathway for these two diseases. In this review article, the author discusses the roles of the novel endoplasmic reticulum (ER) molecular chaperone sigma-1 receptor and brain-derived neurotrophic factor (BDNF) in the association of depression with CVD. In addition, the author proposes that sigma-1 receptor agonists may act as potential therapeutic drugs for depressive patients with CVD.

2. Sigma-1 receptor

Sigma receptors were initially proposed as a subclass of opioid receptors to mediate the unique psychotomimetic effects of SKF10,047 (*N*-allylnormetazocine). They were later found to be a distinct pharmacological entity, as the effects of SKF10,047 were resistant to classical opiate receptor antagonists. Receptor binding studies indicated the existence of at least two subtypes of receptors: sigma-1 and sigma-2. The sigma-1 receptor which was cloned in 1996, is an integral membrane protein with a 24-kDa molecular mass, and is expressed predominantly at the ER. The mammalian sigma-1 receptor shares 90% identity and 95% similarity of amino acid sequences across species. These receptors also share 30% identity and 67% similarity with the yeast sterol C8–C7 isomerase, which is involved in post-squalene sterol synthesis (Moebius et al., 1997; Su et al., 2010). TMBase analysis predicts that the first two hydrophobic domains (amino acids 11–29 and 91–109) are transmembrane-spanning helices, separated by a 50 amino acid loop, with a 125 amino acid carboxyl terminus (Aydar et al., 2002; Su et al., 2010). Two hydrophobic domains positioned at the N-terminus and the center of the receptor contains membrane-spanning α-helices (Fig. 1) (Duncan and Wang, 2005; Hashimoto and Ishiwata, 2006; Ishikawa and Hashimoto, 2010; Su et al., 2010; Hayashi et al., 2011). The sigma-2 receptor has not yet been cloned.

Recent findings reveal that the sigma-1 receptor is a novel ER molecular chaperone, which regulates a variety of cellular functions, such as inositol 1,4,5-triphosphate (IP_3) receptor-mediated Ca^{2+} signaling, ion channel firing, protein kinase location/activation, cellular redox, neurotransmitter release, inflammation, cellular differentiation, neuronal survival and synaptogenesis (Su et al., 2010; Hayashi et al., 2011; Hayashi and Su, 2007). In its dormant state, the sigma-1 receptor forms a complex with the chaperone binding immunoglobulin protein (BiP), also

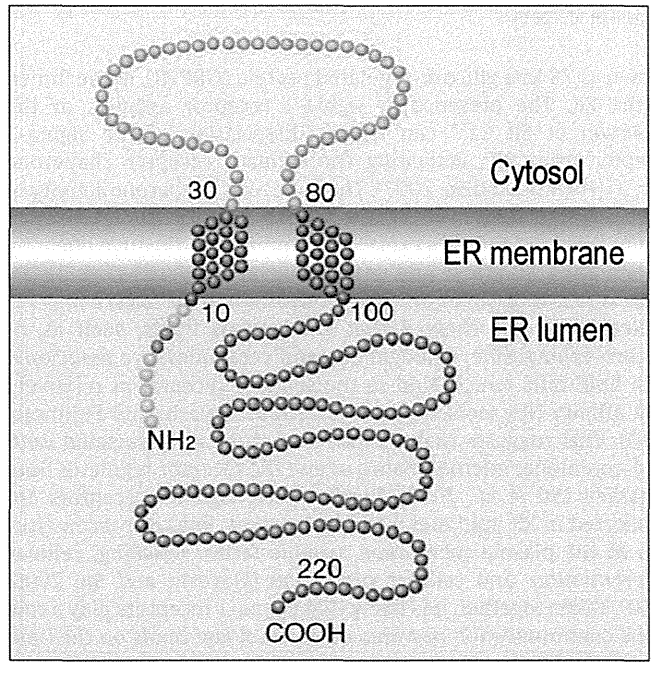

Fig. 1. Putative structure of the sigma-1 receptor.
The sigma-1 receptor is a single polypeptide comprising 223 amino acids and a hydrophobicity plot predicts two membrane-spanning regions in the endoplasmic reticulum (ER). The longer C-terminus (blue) shows complete homology with sterol-binding fungal isomerase. There is also a typical arginine–arginine ER locating signal (red) near the N-terminus.
From Duncan and Wang (2005).

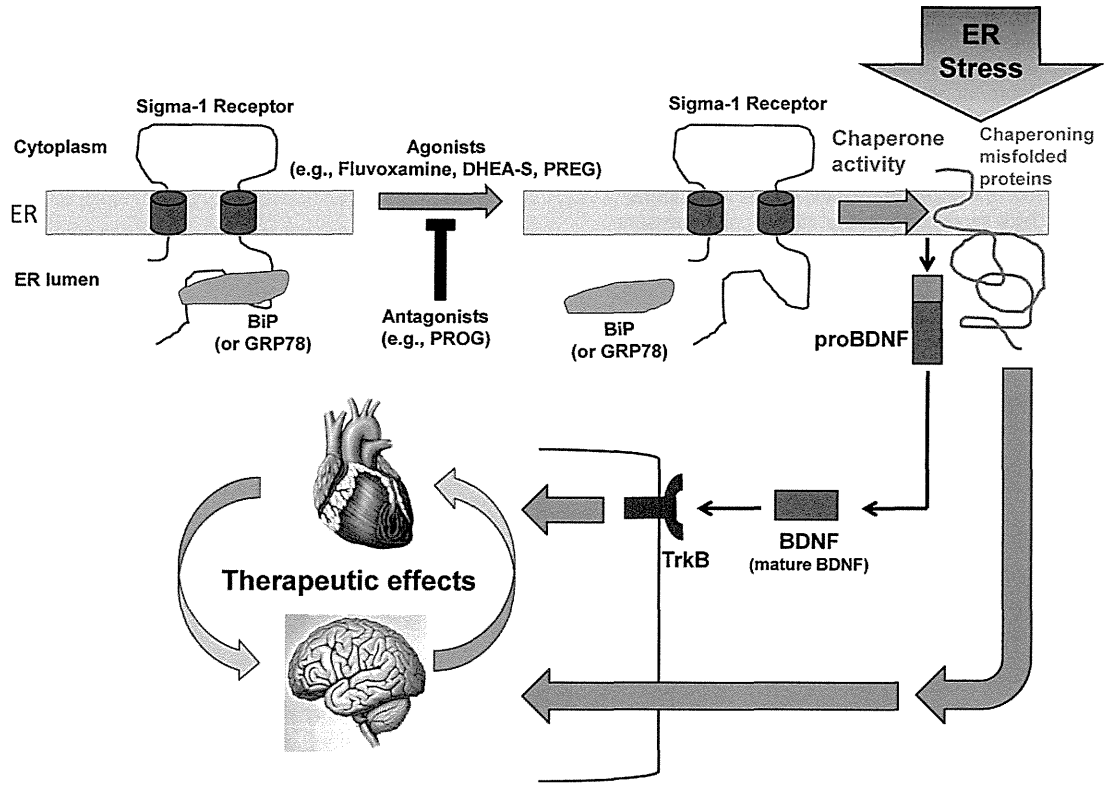

Fig. 2. A possible therapeutic mechanism for sigma-1 receptor agonists in cardiovascular disease.
Stimuli such as oxidative stress, ischemic insult, disturbances in Ca^{2+} homeostasis, and enhanced expression of normal and/or folding-defective proteins lead to the accumulation of unfolded proteins, a condition referred to as ER stress, and, ultimately, to the development of CVD (Minamino and Kitakaze, 2010; Minamino et al., 2010). In control states, the sigma-1 receptor forms a complex with another ER chaperone, BiP (GRP78), which plays a role in CVD (Minamino and Kitakaze, 2010; Minamino et al., 2010). Sigma-1 receptor agonists, such as fluvoxamine, DHEA (or DHEA-S), and PREG, bind to sigma-1 receptors on the ER, and promote dissociation of the sigma-1 receptor from its complex with BiP (Hayashi et al., 2011). This dissociated sigma-1 receptor is free to stimulate chaperone activity, resulting in cardioprotection and the regulation of BDNF secretion. Thus, agonist activation of sigma-1 receptors results in secretion of BDNF from proBDNF via chaperone activity. Mature BDNF can activate BDNF-TrkB signaling pathways in the brain and heart, which in turn, produce cardioprotective and antidepressant effects in depressed patients with CVD. In contrast, endogenous sigma-1 receptor antagonist PROG reinforces the association, blocking the action of sigma-1 receptor agonists (Hayashi et al., 2011). This represents a slight modification from Hayashi et al. (2011).

known as 78 kDa glucose-regulated protein (GRP78), in the lumen of the ER. The presence of sigma-1 receptor agonists or the depletion of ER Ca^{2+} can trigger dissociation of the sigma-1 receptor from BiP, activating the sigma-1 receptor chaperone (Fig. 2) (Hayashi and Su, 2007). The molecular chaperone activity of this receptor is located within the C-terminus, and up-regulation of this receptor by agonists exerts a potent neuroprotective effect by ameliorating ER stress and reactive oxygen radicals (Fig. 2) (Su et al., 2010; Hayashi et al., 2011; Hayashi and Su, 2007).

Recent studies showed that endogenous lipids, such as, D-erythro-sphingosine, sphinganine, and ceramides, are associated with lipid rafts which bind to the sigma-1 receptor at relatively high affinity (Ramachandran et al., 2009; Hayashi and Fujimoto, 2010). This suggests that sigma-1 receptors are associated with lipid-containing microdomains, where the receptor regulates lipid dynamics (Su et al., 2010). Furthermore, sigma-1 receptors are implicated in ER lipid metabolism/transport, lipid raft reconstitution at the plasma membrane, trophic factor signaling, cellular differentiation, and cellular protection (Hayashi and Su, 2005, 2010). Taken together, it is likely that sigma-1 receptors play a role in the communication between cholesterol and lipids on the lipid raft, at the ER membrane (Hayashi and Su, 2005, 2010; Su et al., 2010).

Accumulating evidence suggests that sigma-1 receptor plays an important role in neuronal plasticity, a process implicated in the pathophysiology of neuropsychiatric diseases, such as MDD, and schizophrenia. Therefore, sigma-1 receptor agonists may serve as novel therapeutic drugs to alleviate the accumulation of misfolded proteins in the brain (Duncan and Wang, 2005; Hashimoto and Ishiwata, 2006; Ishikawa and Hashimoto, 2010; Su et al., 2010; Hayashi et al., 2011; Hayashi and Su, 2007; Stahl, 2008; Hashimoto, 2009a,b; Hayashi and Stahl, 2009; Hindmarch and Hashimoto, 2010; Hashimoto and Furuse, 2012; Niitsu et al., 2012; Yoshida et al., 2012c).

Very recently, Shioda et al. (2012) discovered a novel splice variant of the sigma-1 receptor lacking the 47 bp sequence of exon 2. This missing sequence has a detrimental effect on mitochondrial energy production and cell survival. Overexpression of this splice variant decreased mitochondrial Ca^{2+} uptake in response to IP_3 receptor-mediated stimulation, indicating that it antagonizes sigma-1 receptor activity. Thus, this sigma-1 receptor splice variant may enhance ER stress-induced cell death, via down-regulation of adenosine triphosphate (ATP) production and activation of autophagy. Conversely, sigma-1 receptors could protect against ER stress-induced cell death, via up-regulation of ATP production, mitochondrial biogenesis and respiration (Shioda et al., 2012). Further detailed studies are needed to fully determine the underlying association between this splice variant receptor and the sigma-1 receptor ligand.

2.1. Sigma-1 receptors in the heart

Sigma-1 receptors are also widely expressed in peripheral organs, such as lung, liver, adrenal glands, testis, kidney, heart, as well as the brain (Hashimoto and Ishiwata, 2006; Collier et al., 2007; Waterhouse et al., 2007). In the heart, sigma-1 receptors

have been detected in cardiomyocytosis from neonatal rats (Ela et al., 1994) and on the membranes of cardiac myocytes from adult rats (Novakova et al., 1995). Furthermore, the sigma-1 receptor is more abundantly expressed in whole-cell extracts from the left (LV) and right ventricles (RV) compared with brain samples (Bhuiyan et al., 2010; Bhuiyan and Fukunaga, 2011).

Cardiovascular function could be influenced through a series of complex mechanisms, initiated by sigma-1 receptor in the heart binding to agonistic ligands (Bhuiyan and Fukunaga, 2011). Recently, Fukunaga and coworkers reported reduced expression of sigma-1 receptor in LV tissue with the progression of LV hypertrophy, and a significant negative correlation between sigma-1 receptor expression in the LV and heart failure (Bhuiyan et al., 2010; Bhuiyan and Fukunaga, 2009). These findings suggest a role for sigma-1 receptor in the pathophysiology of cardiac hypertrophy and heart failure (Bhuiyan and Fukunaga, 2011).

2.2. Neurosteroids

Neurosteroids are synthesized from cholesterol in the CNS and peripheral nervous systems (Baulieu et al., 2001; Plassart-Schiess and Baulieu, 2001; Charalampopoulos et al., 2008; Strous et al., 2006; Schüle et al., 2011) (Fig. 3). Pregnenolone (PREG) is synthesized from cholesterol by cytochrome $P450_{SCC}$, which is located in the mitochondria, and can be oxidized to progesterone (PROG) by 3β-hydroxysteroid dehydrogenase (3β-HSD) on the ER. The formation of PREG from cholesterol is also regulated by the translocator protein, TSPO (18 kDa), formerly called peripheral-type benzodiazepine receptor. This protein is located predominantly in the outer mitochondrial membrane (Schüle et al., 2011). Furthermore, PREG is converted to 17α-hydroxypregnenolone, and then to dehydroepiandosterone (DHEA) by 17,20-desmolase. Both PREG and DHEA are found, in part, as sulfate esters (PREG-S and DHEA-S), at concentrations that are frequently equal or superior to those of the corresponding free neurosteroids. PROG is converted to 5α-dihydroprogesterone by 5α-reductase, then, 3α-hydroxysteroid dehydrogenase (3α-HSD) converts it to allopregnanolone (ALLO). 11-deoxycorticosterone is produced from PROG by 21β-hydroxylase, and in turn, is converted to corticosterone by 11β-hydroxylase (Fig. 3).

Both PROG and DHEA are further metabolized to androstenedione, a precursor of testosterone (Fig. 3). Testosterone is then converted to estradiol, by the enzyme aromatase. In an alternative pathway, androstenedione is aromatized to estrone, which is converted to estradiol (Fig. 3). These neurosteroids are well known, potent allosteric modulators at γ-amino butyric acid $(GABA)_A$ receptors (Lambert et al., 1995; Reddy, 2010; Schüle et al., 2011; Wang, 2011).

2.2.1. Neurosteroids and sigma-1 receptors

Su et al. (1988) reported that certain gonadal and adrenal steroids, in particular PROG, bind to sigma receptors in the brain and spleen, suggesting a link between the endocrine, nervous, and immune systems (Su, 1991). In a subsequent study, Maurice et al. (1996) reported on in vivo modulation of sigma-1 receptors in the mouse brain by neurosteroids, including PROG, PREG-S and DHEA-S. DHEA, the most abundant endogenous neurosteroid, is a sigma-1 receptor agonist with moderate affinity, whereas PROG and testosterone are sigma-1 receptor antagonists (Table 1) (Hayashi and Stahl, 2009; Waterhouse et al., 2007; Maurice et al., 2001; Bhuiyan and Fukunaga, 2011). PREG is also a sigma-1 receptor agonist (Hayashi and Stahl, 2009). These findings suggest that, as endogenous sigma-1 receptor ligands, these neurosteroids play a physiological role in both the CNS and peripheral systems (Maurice et al., 1999, 2001, 2006; Monnet and Maurice, 2006; Niitsu et al., 2012; Yoshida et al., 2012c).

2.2.2. Neurosteroids in animal models of depression

Neurosteroids are known to regulate the inhibitory and excitatory balance within the CNS. Multiple lines of evidence suggest that neurosteroids play a role in the pathophysiology of psychiatric diseases such as MDD and schizophrenia (van Broekhoven and Verkes, 2003; Eser et al., 2006; Schüle et al., 2011; Niitsu et al., 2012; Yoshida et al., 2012c). Administration of PREG, DHEA, and their sulfate esters (PREG-S and DHEA-S) induces antidepressant-like effects in animal models, and these effects were antagonized by pretreatment with sigma-1 receptor antagonists, such as BD1047 and PROG (Urani et al., 2001). This study implicates sigma-1 receptors in the antidepressant activity of these neurosteroids. Furthermore, DHEA-S attenuates phencyclidine (PCP)-induced cognitive impairment in mice (Hashimoto et al., 2007a). This effect is reversed by treatment with the sigma-1 receptor antagonist NE-100, suggesting a role for this receptor in its mode of action (Hashimoto et al., 2007a). Interestingly, the antidepressant effects of PREG or DHEA and their respective sulfates may be linked to hypothalamus-pituitary-adrenal (HPA) axis activity and BDNF expression (Naert et al., 2007), suggesting that in part, neurosteroids exert their effect by mediating BDNF expression.

In animal studies, administering the antidepressant fluoxetine to rats induced a rapid increase of ALLO within the brain, without altering the levels of PREG, PROG, or DHEA (Uzunov et al., 1996). Recently, Shirayama et al. (2011) reported that infusion of ALLO into the hippocampus and amygdala produced an antidepressant effect in learned helplessness rats, an animal model of depression. Taken together, it is likely that ALLO plays a role in the pathophysiology of MDD and in the therapeutic mechanisms of antidepressants. It is currently unclear whether sigma-1 receptors play a role in the antidepressant effects of ALLO, although ALLO is able to bind this receptor (Rybezynska et al., 2009).

2.2.3. Neurosteroids in patients with major depressive disorder (MDD)

Decreased cerebrospinal fluid (CSF) levels of PREG, but not PROG, have been linked to depressive symptoms in medication-free patients with MDD (George et al., 1994). A randomized pilot study found that the PREG treated group showed a trend for recovery of depressive symptoms however, cognition remained unchanged (Osuji et al., 2010). In contrast, there are conflicting results with regard to levels of DHEA or DHEA-S in the plasma and salivary fluids of patients with MDD (Schüle et al., 2011). In the majority of clinical studies in MDD, successful pharmacotherapy with antidepressants is associated with a decline in DHEA-S levels (Schüle et al., 2011). Interestingly, double-blind, placebo-controlled studies show that DHEA has an antidepressant effect in patients with MDD (Morales et al., 1994; Wolkowitz et al., 1999).

The plasma and CSF levels of ALLO in patients with MDD were approximately 60% lower than those of healthy controls (Uzunova et al., 1998). In this group of patients, treatment with antidepressants (fluoxetine or fluvoxamine) normalized the CSF levels of ALLO. Interestingly, there was a statistically significant correlation between symptom improvement and the increase in CSF ALLO levels, after fluoxetine or fluvoxamine treatment. CSF levels of PREG and PROG remained unaltered after drug treatment. It is noteworthy that the association between amelioration of depressive symptoms and increases of ALLO was detectable only in CSF, but not plasma (Uzunova et al., 1998; Schüle et al., 2011).

Recently, Marx et al. (2009) reported on a placebo-controlled, double-blinded, randomized trial of adjunctive PREG, for the treatment of cognitive impairment and negative symptoms in patients with schizophrenia. The PREG-treated group showed significantly greater improvement in negative symptoms scores, compared with the placebo-treated group, although the score

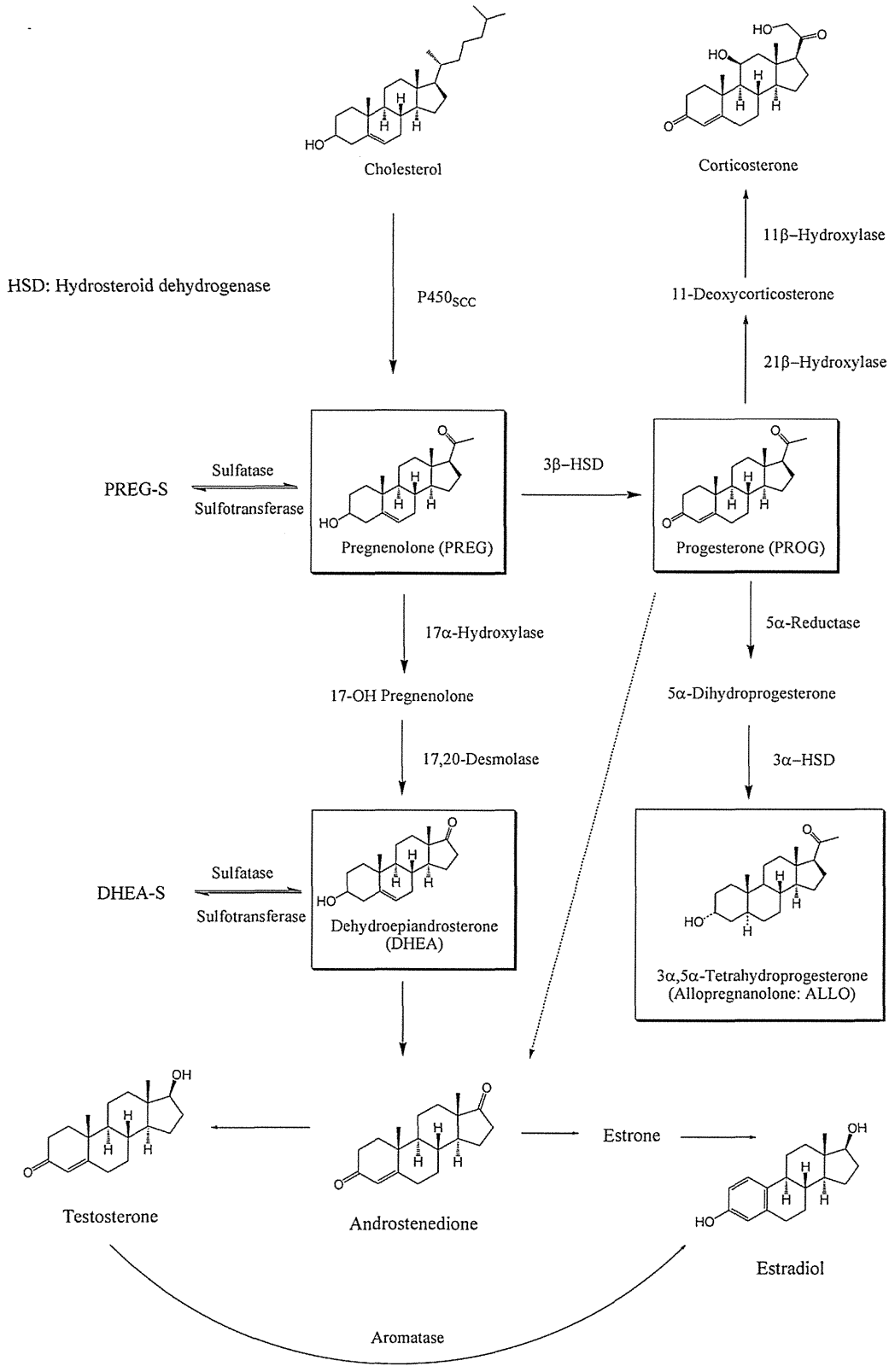

Fig. 3. Biosynthesis of neurosteroids from cholesterol.
Pregnenolone (PREG) is synthesized from cholesterol by cytochrome $P450_{SCC}$, and can be oxidized to progesterone (PROG) by 3β-hydroxysteroid dehydrogenase (3β-HSD). Furthermore, PREG converts to 17α-hydroxypregnenolone and then to dehydroepiandosterone (DHEA) by 17,20-desmolase. Both PREG and DHEA are found, in part, as sulfate esters (PREG-S and DHEA-S). PROG is converted to 5α-dihydroprogesterone by 5α-reductase, then 3α-hydroxysteroid dehydrogenase (3α-HSD) converts into allopregnanolone (ALLO). 11-deoxycorticosterone is produced from PROG by 21β-hydroxylase, and is converted to corticosterone by 11β-hydroxylase. Both PROG and DHEA are further metabolized to androstenedione, which is a precursor of testosterone. Then testosterone is converted to estradiol by the enzyme aromatase. In an alternative pathway, androstenedione is aromatized to estrone, which is subsequently converted to estradiol.