Importantly, DHEA(S) is known to possess anti-glucocorticoid properties [17] because it antagonizes the effect of cortisol [13] and inhibits glucocorticoid-induced enzyme activity [18]. In healthy adults, acute administration of DHEA rapidly reduces cortisol levels [19]. Hence, DHEA(S) is assumed to be involved in stress responses [20] as well as in a broad range of behavioral functions [21]. Due to these characteristics, DHEA(S) has been implicated in the pathophysiology of various psychiatric disorders including major depression [22], bipolar disorder [23], dysthymic disorder [24], panic disorder [25], borderline personality disorder [26], eating disorder [27], posttraumatic stress disorder (PTSD) [28], and schizophrenia [29,30]. Several studies have investigated baseline DHEA(S) levels and cortisol/DHEA(S) ratios in schizophrenia patients, although their findings are not necessarily consistent. Nevertheless, based on the fact that the DHEA(S) level is influenced by the negative feedback of DEX administration, some studies have shown that DHEA(S) and/or cortisol/DHEA(S) ratio in response to the DST could be a sensitive marker for HPA axis function [27,31]. However, no studies to date have investigated DHEA(S) as assessed by the DST in schizophrenia.

In this context, the present study aimed to compare the post-DEX cortisol and DHEAS levels and the cortisol/DHEAS ratio between schizophrenia patients and age- and sex-matched healthy controls. We hypothesized that, among these measures, the cortisol/DHEAS ratio would be the most sensitive marker that distinguishes patients from healthy controls. We also expected that schizophrenia patients would be more likely to be associated with either or both of hyper- and hypo-cortisolism as compared to controls.

METHODS

Participants

Forty-three patients with schizophrenia (age range: 16-70 years), who were under treatment at the National Center of Neurology and Psychiatry Hospital or at a nearby hospital or psychiatric clinic, were enrolled. Of the 43 patients, 23 were hospitalized in the emergency ward of the National Center of Neurology and Psychiatry Hospital for the acute treatment of their psychotic symptoms at the time of the neuroendocrine testing. Consensus diagnoses for DSM-IV schizophrenia [32] were made by psychiatrists based on clinical interviews, observations and case notes. Thirty-seven age- and sex-matched healthy volunteers (age range: 23-70 years) were recruited from the community, through advertisements in free local magazines and our website announcement. At the first visit, the healthy participants were interviewed using the Japanese version of the Mini-International Neuropsychiatric Interview [33] by a research psychiatrist, and only those who demonstrated no current Axis I psychiatric disorders were enrolled in this study. In addition, those who demonstrated one or more of the following conditions during a non-structured interview performed by an experienced psychiatrist were excluded from the healthy control group: past or current contact to psychiatric services, and other obvious self-reported signs of past primary psychotic and mood disorders as well as PTSD. Additional exclusion criteria from both the patient and control groups were: having a prior medical history of central nervous system disease or severe head injury, having a history of substance dependence or substance abuse within the past six months, having major systemic medical illnesses, or taking corticosteroids, antihypertensive medications, oral contraceptives or estrogen replacement therapies. The present experiments on our subjects were conducted in accordance with the Declaration of Helsinki. After the nature of the study procedures had been explained, written informed consent was obtained from all subjects. The study was approved by the ethics committee of the National Center of Neurology and Psychiatry.

Dexamethasone Suppression Test

First, participants took 0.5 mg tablet of DEX orally at 23:00 h. For inpatients, a ward nurse gave this tablet to each patient. For the remaining subjects, compliance was monitored at the time of the blood collection by asking them whether they took the tablet as directed on the previous night. On the next day, plasma and serum samples were collected at 10:00 h. Plasma concentrations of cortisol were measured by radioimmunoassay and serum concentrations of DHEAS were measured by chemiluminescent enzyme immunoassay at SRL Corporation (Tokyo, Japan). The detection limit for cortisol was 27.59 nmol/l (= 1.0 μg/dl). Cortisol values under the detection limit were treated as 0 nmol/l. As our hypothesis was that the two extreme ends of cortisol values (i.e., both exaggerated and blunted cortisol reactivity) would be related to schizophrenia, in the main analysis we also adopted the categorical division of participants based on *a priori* defined cut-off values of cortisol, i.e., 27.59 nmol/l (= 1.0 μg/dl) and 137.95 nmol/l (= 5.0 μg/dl), which were derived from several previous studies [12,34-36]. 'Non-suppressors' were defined to be individuals whose cortisol level was equal to or more than 137.95 nmol/l. 'Enhanced-suppressors' were defined as those individuals whose cortisol level was less than 27.59 nmol/l which corresponded to the cortisol level under the detection limit. The remaining individuals were considered to be 'moderate-suppressors'.

Clinical Assessment, Antipsychotic Medication, and Psychological Distress

For schizophrenia patients, symptoms were assessed by an experienced research psychiatrist in 41 of the total 43 patients using the Positive and Negative Syndrome Scale (PANSS) [37]; this yields a total score in addition to scores on positive, negative, and general psychopathology subscales. All patients with schizophrenia were receiving antipsychotics at the time of the neuroendocrine testing. Daily doses of antipsychotics, including depot antipsychotics, were converted to chlorpromazine equivalents using guidelines [38,39].

For healthy controls, subjectively perceived psychological distress during one week preceding the neuroendocrine test was assessed *via* the Hopkins Symptom Checklist (HSCL) [40], a self-report questionnaire consisting of 58 (or 54) items which are scored on five underlying symptom dimensions, i.e., somatization, obsessive-compulsive, interpersonal sensitivity, anxiety, and depression symptoms. In the present study a validated Japanese version of the HSCL [41] comprising 54 items was

used, as described in our previous study [35]. In this questionnaire, subjects were instructed to rate each item based on the distress perceived during the previous week, using a four-point likert scale, with "not-at-all" being scored 1, "occasionally", 2, "sometimes", 3, and "frequently", 4.

Statistical Analysis

Averages are reported as means ± standard deviation (SD). To compare categorical variables, χ^2 test was used. The t-test or Mann-Whitney U-test was used to examine differences between two groups. Plasma cortisol levels, serum DHEAS levels, and cortisol/DHEAS ratio were compared between two groups using the Mann-Whitney U test because these hormonal data did not satisfy the assumptions for parametrical testing, which was revealed by the Kolmogorov-Smirnov test. Correlation between hormonal measures and clinical variables were calculated using the Spearman's rank correlation test. Statistical significance was set at two-tailed $p < 0.05$. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 (SPSS Japan, Tokyo).

RESULTS

Relationships of Hormonal Measures with Demographic Characteristics

Table **1** shows the demographic and clinical characteristics of patients with schizophrenia and healthy controls. Patients and controls were well matched for age and sex. In the patient group, age significantly correlated with the DHEAS level ($\rho = -0.39$, $p = 0.009$), but not with cortisol level ($\rho = 0.05$, $p = 0.76$) or cortisol/DHEAS ratio ($\rho = 0.26$, $p = 0.09$). In the control group, age significantly correlated with the DHEAS level ($\rho = -0.55$, $p < 0.001$) and cortisol/DHEAS ratio ($\rho = 0.41$, $p = 0.013$), but not with cortisol level ($\rho = 0.12$, $p = 0.49$). Schizophrenic males and females did not significantly differ in cortisol level (Mann-Whitney U = 259.5, p = 0.44) or cortisol/DHEAS ratio (Mann-Whitney U = 298.0, p = 0.09), whereas DHEAS was significantly higher in males than in females (Mann-Whitney U = 120.0, p = 0.008). Similarly, control males and females did not significantly differ in cortisol level (Mann-Whitney U = 154.0, p = 0.62) or cortisol/DHEAS ratio (Mann-Whitney U = 181.0, p = 0.74), whereas DHEAS was significantly higher in males than in females (Mann-Whitney U = 96.5, p = 0.025). Patients were significantly more likely to be smokers than controls; however, smokers and non-smokers did not significantly differ in the cortisol level, DHEAS level, or cortisol/DHEAS ratio for both patients (all $p > 0.1$) and controls (all $p > 0.3$). Patients were significantly more likely to have a family history of psychiatric disorders than controls, although the presence *vs* absence of such a history did not significantly impact on any of the three hormonal measures for both patients (all $p > 0.4$) and controls (all $p > 0.1$). In addition, all demographic and clinical variables were compared between males and females within each of the two diagnostic groups, and found no significant sex differences in any of the variables examined (all $p > 0.05$).

Relationships of Hormonal Measures with Clinical Variables and Symptoms

Clinical variables including age at onset of schizophrenia, antipsychotic dosage, duration of antipsychotic medication, and duration of hospitalizations were not significantly correlated with any of the three hormonal measures, i.e., cortisol level, DHEAS level, and cortisol/DHEAS ratio (all $p > 0.05$). In total, 5 patients were

Table 1. Demographic Characteristics and Clinical Variables of Schizophrenia Patients and Control Subjects

Variable	Schizophrenia Patients ($n = 43$)	Healthy Controls ($n = 37$)	Statistics	*P*
Sex, male/female (% female)	24/19 (44%)	20/17 (46%)	$\chi2(1) = 0.02$	0.87
Age, years	42.7 ± 11.9	41.1 ± 14.8	t = 0.54, df = 78	0.59
Smoking status, smokers/non-smokers	18/25	6/31	$\chi2(1) = 6.23$	0.013
Family history of psychiatric disorder, yes/no	18/25	5/32	$\chi2(1) = 7.80$	0.005
Age at onset, years	25.4 ± 9.2			
Duration of illness, years	16.1 ± 11.5			
Duration of antipsychotic medication, years	14.9 ± 11.5			
Chlorpromazine equivalents of antipsychotics, mg/day	634.2 ± 615.7			
Number of hospitalizations	3.6 ± 3.3			
Duration of total hospitalizations, months	12.9 ± 21.2			
Numbers of out-/in-patients	20/23			
PANSS total score	62.9 ± 21.0			
PANSS positive score	13.9 ± 5.2			
PANSS negative score	17.1 ± 7.8			
PANSS general psychopathology score	31.9 ± 10.4			

PANSS, Positive and Negative Syndrome Scale.

taking typical antipsychotic(s), 29 were taking atypical antipsychotic(s) and 9 were taking both types at the time of testing. No significant differences were seen between these three medication groups in any of the three hormonal measures (all $p > 0.6$). Duration of illness was significantly negatively correlated with DHEAS level ($\rho = -0.33$, $p = 0.033$), which was considered a reflection of a confounding effect of age because age was significantly correlated negatively with DHEAS level and positively with illness duration ($p < 0.001$). Number of hospitalizations was significantly correlated negatively with DHEAS level ($\rho = -0.36$, $p = 0.020$) and positively with cortisol/DHEAS ratio ($\rho = 0.33$, $p = 0.031$). Outpatients and inpatients did not differ in any of the three hormonal measures (all $p > 0.2$). Symptom dimensions as assessed with the PANSS, i.e., positive symptoms, negative symptoms, general psychopathology and total score of PANSS, were not significantly correlated with any of the hormonal measures (all $p > 0.2$). In healthy controls, no significant associations were seen between the three hormonal outcomes and any of the five symptom dimensions of the HSCL (all $p > 0.1$).

Comparisons of Hormonal Measures between Patients *vs* Controls

Results of cortisol and DHEAS levels and cortisol/DHEAS ratio (multiplied by 100) for schizophrenia patients and healthy controls are provided in Fig. (**1**). Patients showed significantly higher cortisol level and cortisol/DHEAS ratio, while no significant difference was seen in the DHEAS level. As for the suppression pattern, patients showed a greater ratio of non-suppression of cortisol than controls at a non-significant trend level ($\chi^2(1) = 3.35$, $p = 0.067$) while no significant differences were seen between the two groups in the ratio of enhanced-suppression ($\chi^2(1) = 1.55$, $p = 0.21$). Compared to controls, outpatients showed significantly higher cortisol level (Mann-Whitney $U = 503.0$, $p = 0.025$) but not cortisol/DHEAS ratio (Mann-Whitney $U = 460.0$, $p = 0.13$) while inpatients showed significantly higher cortisol level (Mann-Whitney $U = 598.0$, $p = 0.009$) and cortisol/DHEAS ratio (Mann-Whitney $U = 563.5$, $p = 0.036$).

Table **2** shows a comparison of findings from studies that examined both cortisol and DHEA and/or DHEAS levels in schizophrenia/first-episode psychosis. Of these nine studies, three [42-44] did not provide cortisol/DHEA(S) ratio. It is clear from this table that previous findings of these hormonal indices in schizophrenia have been variable, such that some studies reported an elevation of these hormonal indices in schizophrenia while others not.

DISCUSSION

The present study found that the cortisol level and cortisol/DHEAS ratio, in response to the 0.5mg DEX administration, were significantly higher in schizophrenia patients as compared to healthy controls. The DHEAS level was not significantly different between the two groups. These hormonal indices were not significantly associated with the antipsychotic dosage or symptom dimensions, while cortisol/DHEAS ratio was to some extent associated with the number of hospitalizations and outpatient/inpatient status.

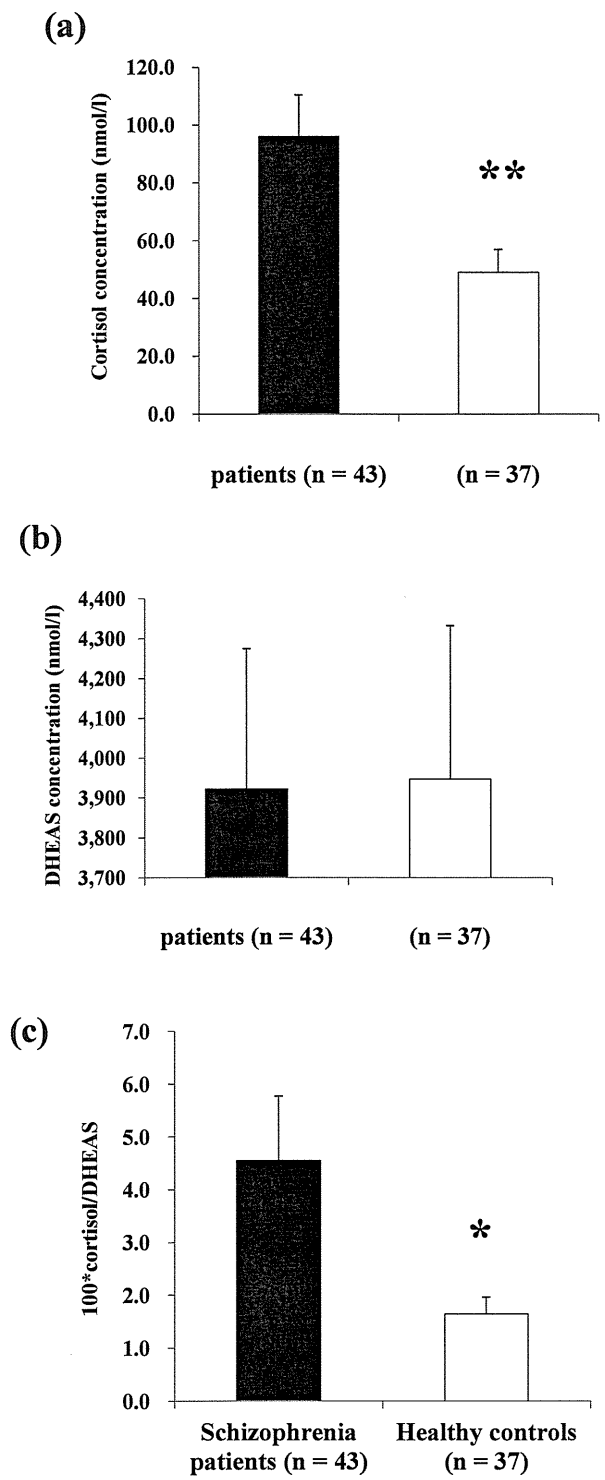

Fig. (1). Hormonal data, including cortisol level (**a**), DHEAS level (**b**) and 100*cortisol/DHEAS ratio (**c**), of schizophrenia patients (black bars) and healthy control subjects (white bars). *$p < 0.05$; **$p < 0.01$ (by Mann-Whitney U test). Error bars represent standard errors of the mean.

Table 2. Summary of Studies that Investigated Both Cortisol and DHEA(S) Levels in schizophrenia Patients and Healthy Controls

	Number of Sample		Matching Status	Patient Characteristics	Sampling Method	Hormonal Outcomes (Patients *vs* Controls)		
	Patients	Controls				Cortisol	DHEA(S)	Cortisol/DHEA(S)
The present study	43	37	age/sex	chronic	after DEX	↑↑	N.S.	↑↑
Shirayama *et al.* (2002)	14	13	males only[c]	moderate negative symptoms	basal	↑↑[e]	N.S.	N.A.
Ritsner *et al.* (2004)	40	15	age/sex	chronically ill, hospitalized	basal	N.S.	N.S.	↑↑
Goyal *et al.* (2004)	10	10	age/sex(males only)	N.A.	basal	N.S.	N.S.	N.A.
Strous *et al.* (2004)	37	25	age/sex	first-episode	basal	N.S.	↑↑	N.S.
Ritsner *et al.* (2007)[a]	43	20	age/sex	chronically ill, hospitalized	basal	↑↑	↑↑[f]	↑↑
Yilmaz *et al.* (2007)	66	28	age/sex(males only)	chronic	basal	↑↑	N.S.	N.A.
Gallagher *et al.* (2007)	20	20	age/sex	probably chronic	basal[d]	↑↑	↑↑	N.S.
Garner *et al.* (2011)[b]	39/20	25/15	none	first-episode	basal	N.S./N.S.	N.S./N.S.	N.S./N.S.

↑↑: significantly higher in schizophrenia patients
N.A.: not applicable
N.S.: not significant
[a]Samples were collected at baseline, after 2, and 4 weeks.
[b]Hormones were measured at baseline and after 12 weeks (baseline/after 12 weeks).
[c]Age not matched.
[d]Serial sampling from 13:00 h to 16:00 h.
[e]Not significant between schizophrenia patients with low negative symptoms and controls.
[f]DHEAS level was significantly higher in schizophrenia patients, while no significant difference was observed in DHEA level.

As described earlier, impaired negative feedback of HPA axis has been implicated in the pathophysiology of schizophrenia, yet findings on cortisol levels from DST studies were not consistent. As shown in Table **2**, studies that investigate both cortisol and DHEA(S) basal levels in schizophrenia patients have again yielded mixed findings, not only for cortisol and DHEA(S) levels but also for cortisol/DHEA(S) ratio. This controversy might be attributable, at least in part, to a number of differential demographic and/or clinical characteristics across studies, such as age, symptom severity, medication status, and comorbid psychiatric disorders, given that these variables are shown to affect cortisol and DHEA(S) levels. For instance, Ritsner *et al.* [45] first investigated cortisol/DHEA(S) ratio in schizophrenia patients in comparison to healthy controls and found this ratio to be significantly elevated in the patient group. This research group confirmed the elevated cortisol/DHEA(S) ratio in their subsequent study where hormonal levels were measured three times every two weeks [46]. In contrast, Gallagher *et al.* [30] did not find such a significant difference in the cortisol/DHEA ratio between schizophrenia patients and healthy controls. In addition, two studies that examined this ratio in individuals with first-episode psychosis did not observe significant differences in this ratio between these individuals and controls [9,47].

Against this background, we administered the DST to measure DHEAS as well as cortisol levels in chronic schizophrenia patients. The significantly elevated cortisol levels in patients than in controls, together with similar levels of DHEAS between the two groups, resulted in the significantly higher cortisol/DHEAS ratio in patients. Similar to the robust effect of oral DEX administration on cortisol suppression, the suppressive effect of DEX on DHEA(S) has been demonstrated [27,31]. Taken together, whereas our result of the elevated cortisol level in schizophrenia patients indicates overall impaired negative feedback inhibition of HPA axis in schizophrenia, the similar level of DHEAS in the two diagnostic groups might suggest that circulating DHEAS level is regulated by several other mechanisms as well as the negative feedback inhibition. However, the mechanism of this dissociation between cortisol and DHEA(S) levels is unclear. Nevertheless, our findings imply that HPA axis hyperactivity as indexed by the elevated cortisol level in schizophrenia is not compensated by the putative anti-glucocorticoid effect of DHEAS, thereby possibly leading to the persistent stress vulnerability in patients with chronic schizophrenia. It may also be worth noting that, among the five studies that have investigated cortisol/DHEAS ratio in patients with schizophrenia/psychosis, three studies including ours that examined chronically ill schizophrenia patients showed significantly higher cortisol/DHEAS ratio in patients than in controls [45,46] while two studies examining individuals with first-episode psychosis found no significant differences [9,47]. The cortisol/DHEAS ratio might thus vary depending on disease stages of schizophrenia, with this ratio becoming higher as the stage progresses.

With respect to the relationship between clinical variables and hormonal levels in schizophrenia patients, we found that, among a number of clinical variables, only those associated with hospital admission (i.e., the number of hospitalizations and the present status of in-/out-patient) were significantly related to altered HPA axis function; hospitalization was associated with higher cortisol/DHEAS ratio. It may be that although an alteration in HPA axis function is present independent of clinical states of schizophrenia patients, which is in line with a previous finding [46], acutely ill inpatients would exhibit even greater alteration. To draw any conclusion, further studies, longitudinal follow-up studies in particular, are required.

Another important issue that should be taken into consideration is the protocol for DST, i.e., the dose of DEX used for the pretreatment as well as the time of hormonal

assays after DEX administration. As for the dose of DEX, most of the earlier studies in schizophrenia have used 1.0 mg of DEX to assess negative feedback of HPA axis [1]. However, we herein administered a lower dose of DEX (i.e., 0.5 mg) to make the sensitivity high for both incomplete and enhanced suppression of cortisol, taking into account a growing body of evidence indicating that HPA axis abnormalities consist of hyper- and hypo-cortisolism. This approach, that is the DST using a low dose DEX, has successfully been employed in the studies of patients with PTSD to detect their enhanced negative feedback of cortisol [48,49]. Although we did not find any excess of enhanced suppression in schizophrenia patients as compared to controls, the fact that we were able to detect the altered HPA axis function in schizophrenia would point to the usefulness of DST with a low dose DEX for this population as well. Regarding the time of hormonal measurements, we decided to draw blood samples at 10:00 h because previous DST studies that have looked at enhanced suppression of cortisol using low-dose DEX draw blood in the morning [48,49]. However, such differences in the dose of DEX and the time of hormonal measurements should be taken into account when comparing the present results with previous ones.

Several limitations need to be commented upon. First, as we did not include baseline measurements of cortisol and DHEAS levels, the extent to which each participant suppressed his/her cortisol and DHEAS in response to the 0.5 mg of DEX cannot be determined. Second, as we sampled blood for hormone measurements only at a single point, the diurnal variation of the hormone levels was unknown. Third, since all patients were receiving antipsychotics, such medication may have influenced HPA axis function as has been demonstrated [50,51]. Although we did not find any significant correlations between antipsychotic dosage and hormonal measures, we have to acknowledge that our data on the chlorpromazine equivalents are themselves limited in that the inpatients were in the midst of their acute treatment and thus the prescription of antipsychotics tended to be frequently changed around the time of the hormone measurement. It should also be noted that chlorpromazine equivalents are an approximate indicator of D2 receptor antagonist activity and most antipsychotics have multiple effects that could have variable influence on the HPA axis function. Fourth, HPA axis abnormalities have been reported in a variety of psychiatric disorders, particularly in mood disorders, it is not known whether the altered cortisol level and cortisol/DHEAS ratio observed here represent schizophrenia-specific HPA axis dysfunction or rather common HPA axis alteration in relation to stressful conditions in general. Finally, we did not collect data on the menstrual cycle or history of childhood trauma, both of which are shown to moderate HPA axis function.

To sum, the present study found that HPA axis function in schizophrenia is altered, as indicated by the elevated cortisol level and cortisol/DHEAS ratio in response to the low-dose DEX administration. In addition to the cortisol level, cortisol/DHEAS ratio may reflect some aspect of HPA axis abnormalities in schizophrenia. Future studies that examine these hormones both before and after the DEX administration are needed to disentangle the baseline and feedback components of the HPA axis alteration in schizophrenia.

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CONFLICT OF INTEREST

None declared.

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Growth factors stimulate expression of neuronal and glial miR-132

Tadahiro Numakawa[a,b,*], Noriko Yamamoto[a], Shuichi Chiba[a], Misty Richards[c], Yoshiko Ooshima[a], Soichiro Kishi[d], Kazuo Hashido[d], Naoki Adachi[a,b], Hiroshi Kunugi[a,b]

[a] Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan
[b] Core Research for Evolutional Science and Technology Program (CREST), Japan Science and Technology Agency (JST), Saitama, Japan
[c] Albany Medical College, Albany, NY 12208, USA
[d] Administrative Section of Radiation Protection, National Institute of Neuroscience, NCNP, Tokyo, Japan

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ABSTRACT

Brain-specific microRNAs (miRs) and brain-derived neurotrophic factor (BDNF) are both involved in synaptic function. We previously reported that upregulation of miR-132 is involved in BDNF-increased synaptic proteins, including glutamate receptors (NR2A, NR2B, and GluR1) in mature cortical neurons [7]. However, the potential role of other growth factors in miR-132 induction has not been clarified. Here, we examined the effect of growth factors including basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), glial cell line-derived neurotrophic factor (GDNF), and epidermal growth factor (EGF), on expression of miR-132 and glutamate receptors in immature cortical neurons. We found that BDNF and bFGF upregulated levels of miR-132 in cortical cultures, though bFGF failed to increase glutamate receptors such as NR2A, NR2B, and GluR1. IGF-1, GDNF, and EGF did not have a positive influence on miR-132 and glutamate receptors in neuronal cultures. Furthermore, bFGF significantly upregulated miR-132 in cultured astroglial cells, while other growth factors failed to elicit such a response. It is possible that the growth factor-stimulated neuronal and glial action of miR-132 plays a critical role in brain function.

1. Introduction

Multiple cellular mechanisms including differentiation, death, and metabolism are influenced by miRs via regulating expression of target genes [1,2,19]. It is possible that brain-specific miRs play an important role in synaptic plasticity. Deletion of the Dicer1 gene in mouse forebrain caused diminished levels of brain-specific miRs including miR-124, -132, -137, -138, -29a, and -29c, resulting in increased hippocampal synaptic activity and more robust translation of BDNF [8]. Importantly, Schratt et al. showed that miR-134 helps to regulate hippocampal spine size [21]. They reported that miR-134 negatively regulates spine size via inhibiting the translation of Limk1 mRNA (LIM kinase 1), and that BDNF relieves suppression of Limk1 caused by miR-134 [21].

It is well known that BDNF, which is an important neurotrophin, contributes to regulation of synaptic plasticity in addition to neuronal survival and differentiation via stimulating the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phospholipase Cγ (PLCγ), and phosphatidylinositol 3-kinase (PI3K) intracellular signaling pathways [5,14,15]. Recent studies revealed an interaction between brain-specific miRs and BDNF. Interestingly, Vo et al. showed that miR-132, one of the brain-specific miRs, is involved in the biological effect of BDNF. They found that marked upregulation of miR-132 was induced by BDNF in cultured cortical neurons. Overexpression of miR-132 increased the outgrowth of primary neurites, while an application of antisense for miR-132 decreased neurite outgrowth [23]. We also reported that BDNF stimulates miR-132 expression, contributing to greater BDNF-mediated upregulation of glutamate receptors including NR2A, NR2B, and GluR1 in cultured cortical neurons [7]. Importantly, we confirmed that the MAPK/ERK pathway is essential for BDNF-increased miR-132 levels and maintenance of glutamate receptor expression [7,9,22]. Taken together, it is possible that miR-132 plays a role in BDNF functions. However, the possible contribution of other growth factors in the regulation of miRs is not well known in the central nervous system (CNS).

Here, we examined the effect of growth factors including BDNF, bFGF, IGF-1, GDNF, and EGF on the expression of miR-132 in both neuronal and astroglial cultures. We found that BDNF and bFGF upregulated miR-132 in cortical cultures, though IGF-1, GDNF, and EGF did not. Furthermore, we found a significant miR-132 increase stimulated by bFGF, but not by other factors, in glial cultures.

* Corresponding author at: Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan. Tel.: +81 42 341 2711x5132; fax: +81 42 346 1744.
E-mail address: numakawa@ncnp.go.jp (T. Numakawa).

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2. Materials and methods

2.1. Cortical neurons and astroglial cultures

Cortical neuronal cultures were prepared as previously reported [16]. Dissociated cortical neurons obtained from postnatal 1- to 2-day-old rats were plated at a final density of $5 \times 10^5/cm^2$ on polyethyleneimine-coated plates or dishes (BD Falcon, CA, USA). Neural cultures from the cerebral cortex of rats were maintained with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 5% fetal bovine serum (FBS) and 5% heated-inactivated horse serum. Astroglial cultures were also prepared from rat cerebral cortex [4]. Dissociated glial cells were plated on non-coating dishes. The pure astroglial cells, judging from immunostaining with anti-glial fibrillary acidic protein (GFAP) antibody (1:1000, CHEMICON, CA, USA), were grown on dishes in medium containing 10% FBS. Cells were used for assay after reaching 70–80% confluence.

2.2. Growth factor treatment

To stimulate cortical neurons, growth factors including BDNF, bFGF, IGF-1, GDNF, and EGF, were applied at 4 days in vitro (4 DIV). Twenty-four hours later, neuronal cells were harvested for sample collection for PCR and immunoblotting. When the glial cell contribution in cortical neuronal cultures was checked, arabinosylcytosine (1.0 μM, SIGMA, MO, USA) was applied at 24 h after cell plating to block proliferation of glial cells. We found no change in BDNF-stimulated intracellular signaling with or without pretreatment with arabinosylcytosine as previously reported [12]. Growth factors (BDNF, bFGF, IGF-1, GDNF, and EGF) were also added to pure astroglial cultures. Treatment with growth factors was maintained for 24 h before sample collection for PCR and immunoblotting. U0126 (10 μM) was applied 20 min before growth factor addition. Regeneron Pharmaceutical Co., Takeda Chemical Industries Ltd., and Sumitomo Co. Ltd. donated the BDNF and bFGF. IGF-1, GDNF, and EGF were purchased from R&D Systems (MN, USA), Wako Pure Chemical Industries Ltd. (Osaka, Japan) and from Invitrogen (CA, USA), respectively.

2.3. RNA isolation and quantification of mature miR-132

Total RNA, including miRs, was extracted from cultured cells using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) at room temperature according to the manufacturer's instructions. The total RNA was eluted with 50 μl of elution buffer provided by the manufacturer. 100 ng of total RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and miR-specific stem-loop primers (part of TaqMan miRNA assay kit; Applied Biosystems). The expression levels of miR-132 were quantified by real-time PCR using specific primers with the 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions.

Expression of miR-9 and -124a were similarly determined using specific primers. There is no current consensus on the use of an internal control for real-time PCR analysis of miR. Therefore, we used a fixed amount of starting total RNA (100 ng) and inserted this into the RT reaction (5 μl) in each assay for technical consistency. All values were normalized to the same amount of miR-16 as an endogenous control. Data analysis was performed by SDS 2.2 real-time PCR data analysis software (Applied Biosystems).

2.4. Immunoblotting

Lysate collection and western blotting were prepared as described [13]. Briefly, neuronal or glial cultures were lysed in SDS lysis buffer (1% SDS, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride). To load an equivalent amount of protein for each immunoblotting, protein concentration in each sample was quantified using a BCA Protein Assay Kit (PIERCE, IL, USA). Primary antibodies were used at the following dilutions: anti-Akt (1:1000, Cell Signaling, MA, USA), anti-pAkt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-pERK (1:1000, Cell Signaling), anti-NR2A (1:500, SIGMA), anti-NR2B (1:500, SIGMA), anti-GluR1 (1:1000, CHEMICON, CA, USA), and anti-βactin (1:5000, SIGMA) antibodies. The immunoreactivity was quantified by using Lane & Spot Analyzer software (ATTO Corporation, Tokyo, Japan).

2.5. Statistical analysis

Data shown are presented as mean ± standard deviation (SD). Statistical significance was evaluated using a one-way ANOVA followed by Scheffe's post hoc test in SPSS ver11 (SPSS Japan, Tokyo, Japan). Probability values less than 5% were considered statistically significant.

3. Results

We used cultured cortical neurons to investigate the effect of several growth factors (including BDNF, bFGF, IGF-1, GDNF, and EGF) on induction of miR-132 expression. BDNF application was performed at 4 DIV and a significant increase in expression of miR-132 was observed 24 h later (Fig. 1A). bFGF also increased miR-132 expression, though the induced levels were lower compared with the levels achieved by BDNF. On the other hand, other factors including IGF-1, GDNF, and EGF all failed to upregulate miR-132 in neuronal cultures. In the induction of miR-132 expression, the dose-dependency of BDNF's effect was determined (Fig. 1B). A dose-dependent effect of bFGF was also observed (Supplementary Fig. S1). As shown in Fig. 1C, we investigated the effect of growth factors on miR-9 and miR-124a as the control, and no change in these miRs expression by growth factors were observed. In the present study, the mature form of miRs was quantified.

Previously, we reported that BDNF upregulated miR-132 and that the upregulation of miR-132 was important for a BDNF-mediated increase in postsynaptic protein levels. Therefore, in the present study, a change of glutamate receptor expression after exposure to growth factors was investigated. As expected, 24 h-BDNF treatment induced upregulation of NR2A, NR2B, and GluR1, which are glutamate receptor subunits, in cortical cultures (Fig. 2A and B). Interestingly, bFGF did not increase the levels of these glutamate receptors. Though IGF-1 demonstrated an increased trend in the expression of these postsynaptic proteins (especially on GluR1), the IGF-1 effect did not reach statistical significance (Fig. 2A and B). Both GDNF and EGF had no influence on levels of NR2A, NR2B, and GluR1 in cultured cortical neurons (Fig. 2A and B).

Next, we examined the effect of growth factors on miR-132 expression in pure astroglial cells. In astroglial cultures, BDNF had no effect (Fig. 3A). On the other hand, bFGF positively influenced astroglial miR-132 expression. All other growth factors such as IGF-1, GDNF, and EGF did not have any effect on glial miR-132 levels (Fig. 3A). The dose-dependent effect of bFGF on glial miR-132 expression was determined (Fig. 3B). In astroglial cultures, no influence on miR-9 and -124a levels by all growth factors was observed (Fig. 3C).

In the present study, we compared endogenous miR-132 levels between cortical neurons and pure astrocytes. Neuronal expression of miR-132 was approximately three times higher than that of astrocytes (Supplementary Fig. S2A). As expected, mRNA

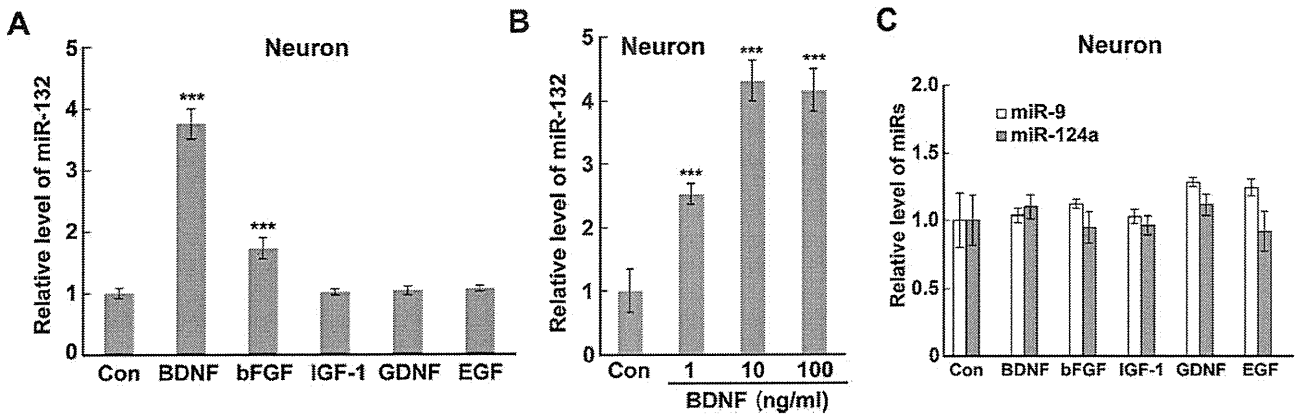

Fig. 1. Effect of growth factors (including BDNF, bFGF, IGF-1, GDNF, and EGF) on induction of miR-132 in cultured cortical neurons. (A) Following growth factor application for 24 h, levels of miR-132 were determined by real-time PCR. All values were normalized to miR-16 levels. Growth factors were applied at the final concentration of 100 ng/ml, respectively. Data represent mean ± SD ($n = 4$, n indicates the number of dishes for each experimental condition). ***$P < 0.001$. Reproducibility was confirmed with separated cortical cultures. (B) Dose-dependency of BDNF effect on miR-132 upregulation ($n = 4$). ***$P < 0.001$. (C) Levels of miR-9 and -124a after growth factor stimulation (100 ng/ml, 24 h) were shown ($n = 3$).

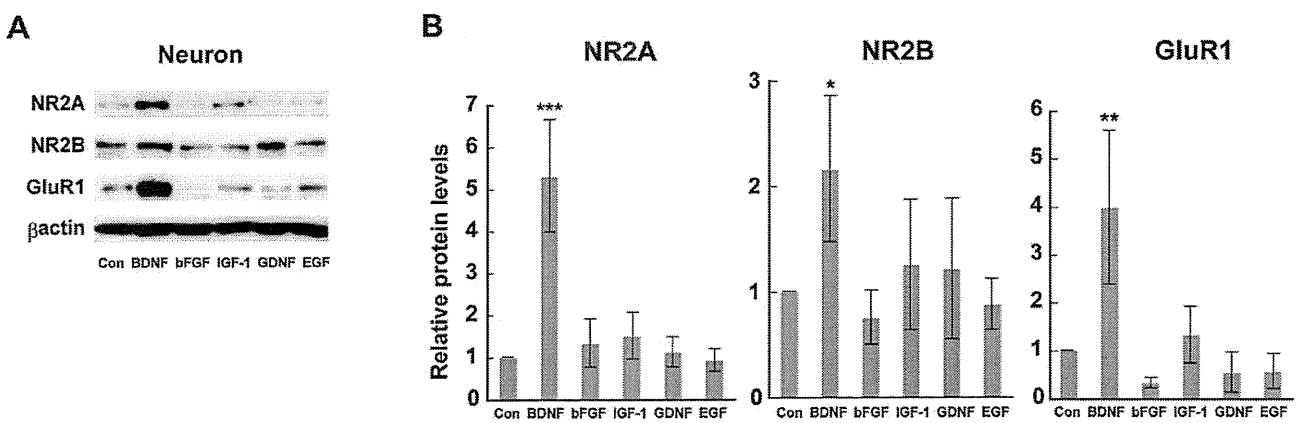

Fig. 2. Effect of growth factors (BDNF, bFGF, IGF-1, GDNF, and EGF) on expression of glutamate receptor subunits in cultured cortical neurons. (A) Levels of NR2A, NR2B, and GluR1 were determined by immunoblotting after growth factor application for 24 h. Each growth factor was applied at 100 ng/ml. βactin is a control. (B) Quantification was carried out. Normalization to a level in control (Con, without growth factors) was performed. Data represent mean ± SD (NR2A, $n = 7$, NR2B, $n = 6$, GluR1, $n = 5$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

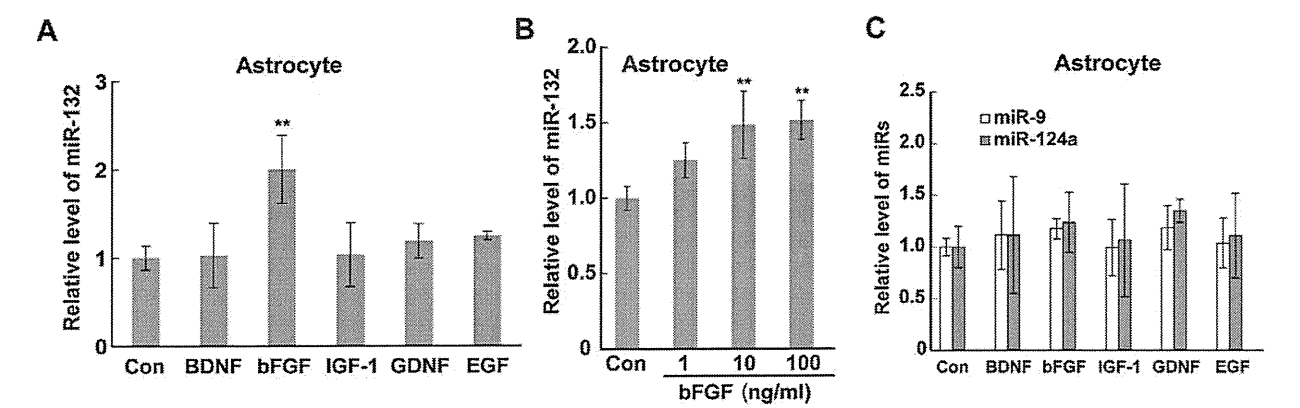

Fig. 3. Effect of growth factors on miR-132 expression in cultured astroglial cells. (A) Astroglial expression of miR-132 was determined by real-time PCR. Growth factors, including BDNF, bFGF, IGF-1, GDNF, and EGF, were applied at 100 ng/ml for 24 h, respectively. Data represent mean ± SD ($n = 5$, n indicates the number of dishes for each experimental condition). **$P < 0.01$. (B) Dose-dependency of bFGF effect on glial miR-132 ($n = 4$). **$P < 0.01$. (C) Growth factors did not change levels of glial miR-9 and -124a. Growth factors were applied at 100 ng/ml for 24 h, respectively ($n = 3$).

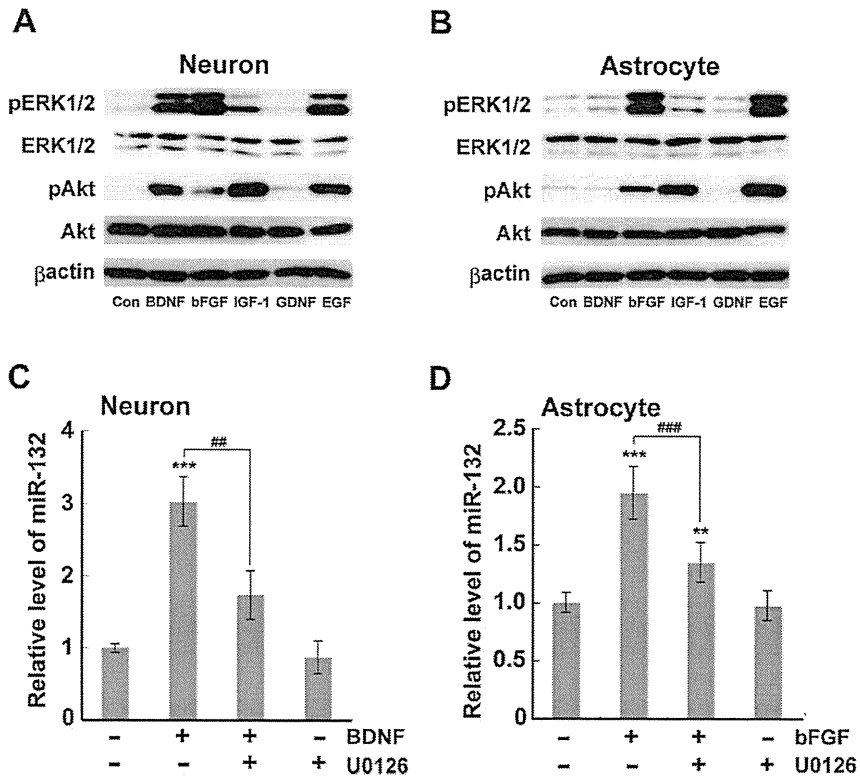

Fig. 4. MAPK/ERK signaling is involved in upregulation of miR-132. Activation of intracellular signaling including MAPK/ERK and PI3K/Akt pathways after growth factor (BDNF, bFGF, IGF-1, GDNF, and EGF) stimulation was examined in neuronal or glial cultures. (A) Activated ERK1/2 (phosphorylated ERK1/2, pERK1/2), activated Akt (phosphorylated Akt, pAkt), total ERK1/2, and total Akt in cortical neurons. Growth factors were applied at 100 ng/ml for 20 min. (B) Levels of pERK1/2, pAkt, total ERK1/2, and total Akt in astroglial cultures. Treatment with growth factors was carried out at 100 ng/ml for 20 min. (C) U0126, an inhibitor for ERK1/2 signaling, inhibited BDNF-induced miR-132 expression in neurons. BDNF (100 ng/ml) was applied for 24 h ($n=3$). $^{***}P<0.001$, $^{\#\#}P<0.01$. (D) U0126 inhibited bFGF-induced miR-132 expression in astrocytes. bFGF (100 ng/ml) was applied for 24 h ($n=5$). $^{***}P<0.001$, $^{**}P<0.01$, $^{\#\#\#}P<0.001$.

expression of microtubule-associated protein 2 (MAP2, neuronal marker) in our cortical cultures was robust, while very low levels of GFAP mRNA were confirmed (Supplementary Fig. S2B and C). Astroglial cultures expressed high levels of GFAP mRNA (Supplementary Fig. S2C).

As shown in Fig. 4A, we examined whether BDNF, bFGF, IGF-1, GDNF, and EGF elicited intracellular signaling cascades, including MAPK/ERK and PI3K/Akt pathways, in neuronal cultures, as BDNF-stimulated increases in miR-132 expression in mature cortical neurons is via activation of MAPK/ERK [7]. BDNF, bFGF, IGF-1, and EGF stimulated both MAPK/ERK and PI3K/Akt pathways, while GDNF did not (Fig. 4A). In pure glial cultures, BDNF could not stimulate these intracellular signaling cascades (Fig. 4B). Importantly, bFGF, IGF-1, and EGF activated MAPK/ERK and PI3K/Akt pathways in glial cultures, whereas GDNF did not have any effect on these signaling cascades (Fig. 4B). As shown in Fig. 4C and D, BDNF and bFGF induced miR-132 expression in neuronal and glial cultures, respectively, while both of these responses were suppressed by U0126, an inhibitor for ERK signaling. bFGF-induced miR-132 expression in neuronal cultures was also blocked by U0126 (Supplementary Fig. S3). Recently, it was reported that the expression of pri/pre-miR-132 is altered to a larger extent than mature miR-132 after growth factor stimulation [20]. Pre-miRs are processed and approximately 22nt mature miRs are generated following cleavage by Dicer in the cytoplasm [1]. Thus, we examined possible alterations in pre-miR-132 expression with Northern blotting, but failed to detect pre-miR-132, although expression of mature miR-132 was significant (Supplementary Fig. S4).

4. Discussion

In the present study, BDNF induced upregulation of miR-132 and increased expression of glutamate receptors in cultured cortical neurons, though astroglia miR-132 expression was not influenced. Others including IGF-1, GDNF and EGF did not change neuronal miR-132 and glutamate receptor levels. Interestingly, bFGF increased levels of neuronal and glial miR-132, but did not impact the expression of glutamate receptors in neuronal cultures.

Upregulation of miR-132 is involved in neuronal BDNF functioning in the CNS. Vo et al. showed that BDNF induces upregulation of miR-132 and that miR plays a critical role in the outgrowth of primary neurites in cultured cortical neurons [23]. We previously reported that 24 h-treatment with BDNF was able to induce miR-132 expression in 10–11 DIV matured cortical neurons. Furthermore, BDNF-mediated miR-132 expression enhanced the overall expression of glutamate receptors including NR2A, NR2B, and GluR1 in mature neurons [7]. In the present study, we found that 4 DIV of immature cortical neurons also responded to BDNF in a significant manner. We confirmed that BDNF upregulated these glutamate receptors in immature neurons, suggesting that BDNF plays a role in synaptic development, in addition to regulation of synaptic plasticity in a matured cortical system. Interestingly, induction of miR-132 appeared when bFGF was used to stimulate cortical neurons, while IGF-1, GDNF, and EGF had no effect. Many studies reported that IGF-1, GDNF, and EGF exert a biological effect on the neuronal population [6,18,24]. Though experimental conditions such as neuronal maturity, brain region, or neuron

types might contribute to the difference in neuronal response, it is possible that BDNF has a strong influence on the induction of miR-132 and miR-132-mediated neuronal function.

In addition to BDNF, bFGF induced miR-132 upregulation in cultured cortical neurons. To our knowledge, this is the first report concerning bFGF-mediated upregulation of miRs. Unexpectedly, alterations in synaptic protein expression were not caused by bFGF in our system. Previously, we reported that acute application of bFGF stimulated glutamate release, an excitatory neurotransmitter, via activation of the ERK pathway, which is different from that (PLCγ pathway) in BDNF-induced glutamate release in cultured cortical neurons [13,16,17]. Furthermore, with regard to miR-132 induction by BDNF in matured cortical neurons, activation of ERK1/2 is essential [7]. In the present study, we found the possibility that both BDNF- and bFGF-mediated miR-132 in immature cortical neurons were regulated through activation of ERK1/2. Though chronic BDNF enhanced expression of NR2A, NR2B, and GluR1, long-term treatment with bFGF did not impact levels of these glutamate receptors, implying a novel role of bFGF-mediated miR-132 expression in neurons. Although EGF also stimulated pERK1/2 in neuronal cultures, in addition to astroglial cultures, significant upregulation of miR-132 (in both neuronal and astroglial cultures) was not observed. Distribution of activated ERK1/2 in subcellular levels might be different among BDNF-, bFGF-, and EGF-dependent stimulation. Furthermore, in addition to ERK1/2, other signaling molecules that are not stimulated by EGF may contribute to miR-132 induction.

Interestingly, bFGF upregulated astroglial miR-132 expression, while others, including BDNF, had no effect. In neurons, we previously demonstrated an important role of ERK1/2 signaling in both miR-132 induction by BDNF and synaptic protein maintenance in neurons [7,9,22]. Here, bFGF caused an increase in ERK1/2 activation and miR-132 expression in astroglial cultures. Considering that expression of glutamate receptors may present in lower levels compared to that of neurons, the role of bFGF-mediated glial miR-132 is interesting and further studies are needed.

It is well known that miRs play a role in cellular function including cell death, differentiation, and metabolism via regulating expression of target genes [1,2,19]. Importantly, current evidence suggests that brain-specific miRs regulate neuronal function [21,23], and alteration in expression and/or function of miRs is involved in brain diseases such as schizophrenia [10], panic disorder [11], and neurodegeneration-related diseases [3]. Furthermore, it is well established that BDNF affects the pathophysiology of brain diseases [14,15], though the contribution of bFGF in this paradigm is still unclear. Taken together, further examination of the neuronal and glial functioning of miR-132 stimulated by growth factors is very important.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2011.10.025.

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Review

MicroRNA function and neurotrophin BDNF

Tadahiro Numakawa [a,b,*], Misty Richards [c], Naoki Adachi [a,b], Soichiro Kishi [d], Hiroshi Kunugi [a,b], Kazuo Hashido [d]

[a] Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo 187-8502, Japan
[b] Core Research for Evolutional Science and Technology Program (CREST), Japan Science and Technology Agency (JST), Saitama 332-0012, Japan
[c] Albany Medical College, Albany, NY 12208, USA
[d] Administrative Section of Radiation Protection, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Tokyo, Japan

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ABSTRACT

MicroRNAs (miRs), endogenous small RNAs, regulate gene expression through repression of translational activity after binding to target mRNAs. miRs are involved in various cellular processes including differentiation, metabolism, and apoptosis. Furthermore, possible involvement of miRs in neuronal function have been proposed. For example, miR-132 is closely related to neuronal outgrowth while miR-134 plays a role in postsynaptic regulation, suggesting that brain-specific miRs are critical for synaptic plasticity. On the other hand, numerous studies indicate that BDNF (brain-derived neurotrophic factor), one of the neurotrophins, is essential for a variety of neuronal aspects such as cell differentiation, survival, and synaptic plasticity in the central nervous system (CNS). Interestingly, recent studies, including ours, suggest that BDNF exerts its beneficial effects on CNS neurons via up-regulation of miR-132. Here, we present a broad overview of the current knowledge concerning the association between neurotrophins and various miRs.

1. Introduction

miRs regulate the expression of target genes and ultimately affect translation. Through this process, multiple mechanisms are impacted, including differentiation, cell death, and cell metabolism in plants and animals (Bartel, 2004; Chapman and Carrington, 2007; Pillai et al., 2007). Recent evidence suggests that brain-specific miRs may be involved in the regulation of neuronal function. For instance, miR-132 is important for neurite outgrowth, while miR-134 negatively regulates the spine size in neurons (Vo et al., 2005; Schratt et al., 2006). Furthermore, change in expression and/or function of some miRs may be associated with several brain diseases such as schizophrenia (Mellios et al., 2009), panic disorder (Muiños-Gimeno et al., 2010), and Rett syndrome (Wu et al., 2010), etc. Additionally, regulation of neurodegeneration-related toxic molecules by miRs has been suggested (Eacker et al., 2009). Taking these findings together, an extensive examination of the basic functioning of miRs and their relationship with brain disease pathophysiology is very important.

Many studies discuss the involvement of BDNF, a critical neurotrophic factor, in brain function. On a cellular level, it is well-established that BDNF influences proliferation, maturation, survival, and synaptic function via activation of intracellular signaling cascades including mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), phospholipase Cγ (PLCγ), and phosphatidylinositol 3-kinase (PI3K) pathways (Huang and Reichardt, 2003; Minichiello, 2009; Russo et al., 2009; Numakawa et al., 2010a). As expected, alteration in expression/function of BDNF is involved in the pathophysiology of several brain diseases including neurodegenerative diseases and mental disorders (Karege et al., 2005; Numakawa et al., 2011). As both miRs and BDNF affect neuronal processes, the crosstalk between these molecules is very interesting. In the present review, we focus on the current relationship between miRs and BDNF in neurons, in addition to giving a broad overview of the basic functioning of miRs.

2. Biogenesis of microRNA and its basic function

The first miR, *lin-4*, was initially discovered as a direct target when researchers were performing genetic analysis of defects in post-embryonic development of *Caenorhabditis elegans* (Chalfie et al., 1981; Ambros, 1989). *Lin-4* encodes a 22-nucleotide non-coding RNA that binds to the 3′UTR of *lin-14* mRNA, which serves to regulate protein translation (Wightman et al., 1991; Lee et al., 1993). The second miR to be identified was *let-7* (Reinhart et al., 2000). The *let-7* miR encodes a 21-nucleotide RNA that binds to the 3′UTR of *lin-41* and *hbl-1* (*lin-57*) in order to inhibit translation (Slack et al., 2000; Abrahante et al., 2003; Lin et al., 2003; Vella

* Corresponding author at: Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1, Ogawa-Higashi, Kodaira, Tokyo 187-8502, Japan. Tel.: +81 42 341 2711x5132; fax: +81 42 346 1744.
E-mail address: numakawa@ncnp.go.jp (T. Numakawa).

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et al., 2004). Both *let-7* and its target *lin-41* are evolutionarily conserved throughout a wide range of animal species, including vertebrate, ascidian, hemichordate, mollusc, annelid and arthropod, suggesting a general role of small RNA molecules in developmental regulation. As a result of the above findings, extensive analysis and identification of new miRs were triggered (Pasquinelli et al., 2000). Since this time, several hundred miRs have been identified in various organisms such as viruses, worms, flies, fish, frogs, mammals and plants (Lagos-Quintana et al., 2001, 2003; Lau et al., 2001; Lee and Ambros, 2001; Llave et al., 2002; Reinhart et al., 2002; Lim et al., 2003; Pfeffer et al., 2005; Watanabe et al., 2005). The miR database now lists over 15,000 miR gene loci in over 140 species, and over 17,000 distinct mature miR sequences (Kozomara and Griffiths-Jones, 2011).

Generally, miR genes are transcribed by RNA polymerase II as primary miRs (pri-miRs are hundreds to thousands of nucleotide-length transcripts) containing cap structures and poly (A) tails (Cai et al., 2004; Lee et al., 2004; Parizotto et al., 2004, and see Fig. 1). At this point, known transcription factors engage in the transcriptional control of miR genes. For instance, the myogenic transcription factors, such as Myogenin and MyoD, bind to regions upstream of miR-1 and miR-133 loci and are likely to regulate their expression during myogenesis (Rao et al., 2006). HBL-1, a protein product of *hbl-1* and one of *let-7*'s targets, is responsible for inhibiting the transcription of *let-7* temporarily in seam cells, vulval precursor cells, and the hypodermal syncytium 7. This negative feedback mechanism between *let-7* and HBL-1 has been identified and characterized by researchers (Roush and Slack, 2009). Epigenetic control also contributes to miR gene expression. For example, the miR-203 gene locus is hypermethylated in several hematopoietic tumors, including chronic myelogenous leukemia and some acute lymphoblastic leukemias (Bueno et al., 2008).

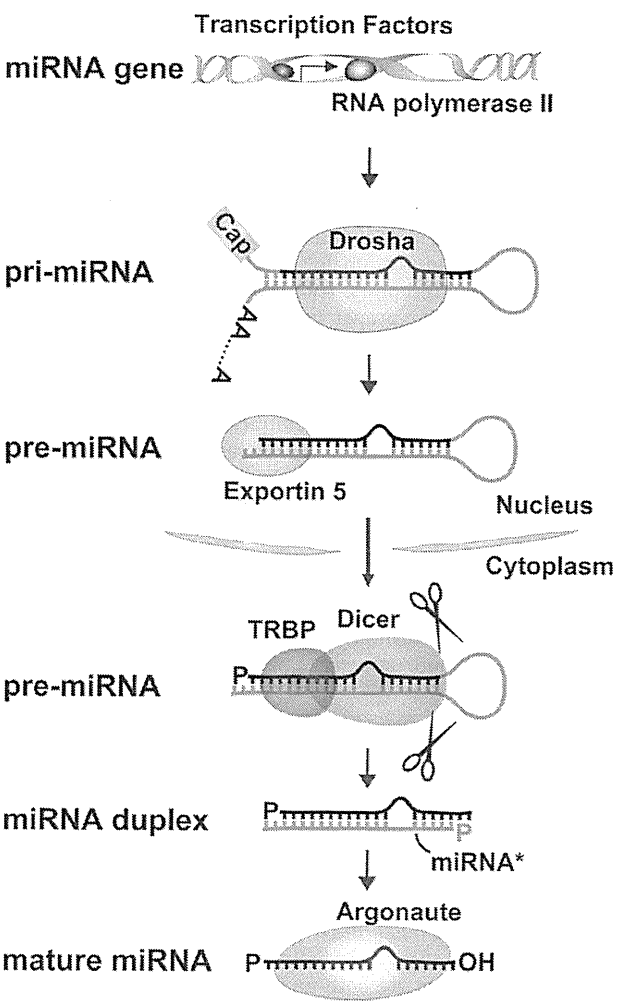

Fig. 1. MicroRNA biogenesis. An miRNA (miR) gene is generally transcribed by RNA polymerase II as primary miR (pri-miR). At this point, some transcription factors engage in the transcriptional control of miR genes. In the nucleus, the RNase III endonuclease Drosha cleaves the pri-miR to produce an approximately 70 nucleotide stem loop structure as precursor miR (pre-miR). Exportin5 transports the pre-miR into the cytoplasm, where it is cleaved by another RNase III endonuclease, Dicer, together with the trans activator RNA (TAR)-binding protein (TRBP), generating approximately 22 nucleotide miRNA/miRNA* duplex. One strand of the duplex remains Argonaute as a mature miR, while the other strand (miR*) is degraded.

Following this, Drosha, an RNase III enzyme, cleaves the pri-miRs into approximately 70 nucleotide stem-loop structures as precursor miRs (pre-miRs) (Lee et al., 2003, and see Fig. 1). In plants, instead of Drosha protein, Dicer-like protein 1 (DCL1) acts to convert pri-miRs to pre-miR duplexes (Papp et al., 2003; Xie et al., 2004). Then, the excised approximately 70 nucleotide fold-back double-strand RNA (dsRNA) precursors are exported from the nucleus to the cytoplasm by the protein Exportin5 (Yi et al., 2003). In the cytoplasm, the pre-miRs are cleaved by the RNase III enzyme, Dicer, generating approximately 22 nucleotide siRNA-like complexes (miR/miR*) loaded onto Argonaute (Ago) protein (Hutvágner et al., 2001; Maniataki and Mourelatos, 2005, and see Fig. 1). Specifically, the stem of pre-miR has an imperfectly double-stranded structure, and the region of RNA duplex is essentially the same as siRNA. The mature miR is partially paired to the miRNA* and the small RNA resides on the opposite side of the pre-miR stem. One strand of the duplex remains in Ago as a mature miR, while the other strand (miR*) is degraded (Schwarz et al., 2003; Du and Zamore, 2005, and see Fig. 1).

Following Dicer cleavage, the resultant approximately 22 nucleotide RNA duplex is loaded onto Ago protein so as to generate an effecter complex of the RNA-induced silencing complex (RISC) recruited by trans activator RNA (TAR)-binding protein (TRBP) and PACT (Protein activator of PKR; dsRNA activated protein kinase) (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). GW182 proteins are also crucial for the miR-mediated gene silencing in animal cells. GW182 family proteins interact directly with Ago. The middle and C-terminal regions act as an autonomous domain in repressive function and the function is independent of both the interaction with Ago and of P-body (Processing body) localization (Ding et al., 2005; Eulalio et al., 2009). Translationally repressed mRNAs are collected in discrete cytoplasmic foci as P-bodies (Pillai et al., 2005; Eulalio et al., 2007; Parker and Sheth, 2007).

Many studies indicate that miRs repress post-transcriptional activity. In *C. elegans*, *lin-4* negatively regulates *lin-14* via repressing its translation without inhibiting *lin-14* mRNA biogenesis and translation initiation (Olsen and Ambros, 1999). Bantam binds to the 3′UTR of the pro-apoptotic gene *hid* to exert a negative effect on its translation in *Drosophila* (Brennecke et al., 2003). Most animal miRs inhibit the translation of target mRNAs (Fig. 2). In plants, most miRs mediate the stabilization of target mRNAs (Hake, 2003, and see Fig. 2). Plant miRs pair with mRNA almost perfectly and their complementary sites are located throughout the transcribed region of the target, not limited to the 3′UTR region. The Epstein-Barr virus (human herpesvirus) has been shown to encode miRs, though viral miRs do not have close homologs in other viral or host genomes. Viral miRs are expressed individually or in clusters from either polymerase or promoters, and regulate both viral and host genes (Pfeffer et al., 2004). In the fly and animal, miRs pair with their targets through limited 2–7 nucleotides of its 5′ region (called the seed region), to prohibit translation and direct degradation of

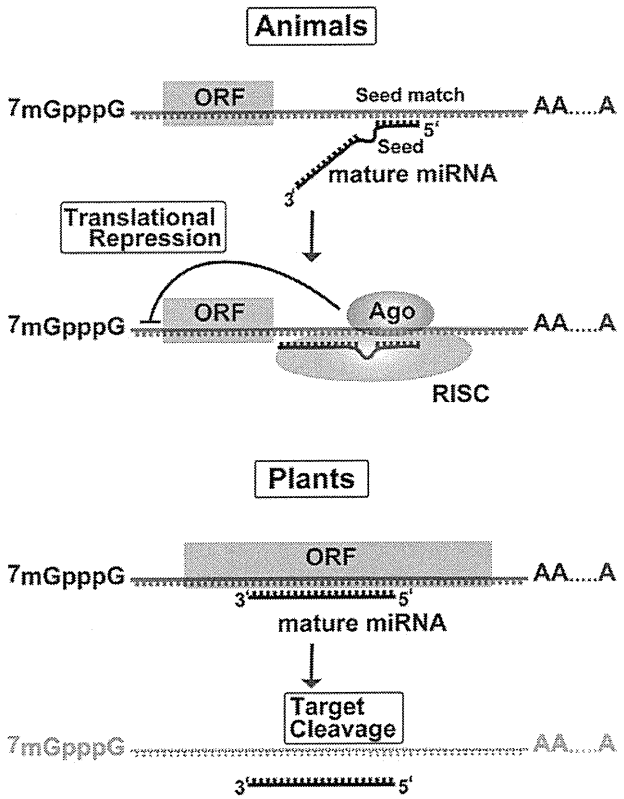

Fig. 2. MiR targeting in animal- and plant-cells. Most animal miRs recognize partially complementary binding sites, which are generally located in 3′UTRs. In the fly and animal, miRs pair with their targets through limited 2–7 nucleotides of its 5′ region (called the seed region), to prohibit translation and direct degradation of target mRNAs. Plant miRs pair with target mRNA almost perfectly and their complementary sites are located through the transcribed region of the target mRNA, not limited to the 3′UTR region, and direct endonucleolytic mRNA cleavage within the base-paired region.

target mRNAs (Fig. 2). Considering that the seed region is so short, miRs are predicted to regulate a huge number of genes. In fact, human miRs may regulate as many as one-third of all protein-coding genes (Lewis et al., 2003, 2005; Xie et al., 2005).

3. miR expression in brain

miRs play a role in various cellular processes, including apoptosis and metabolism in both plants and animals. Recently, miRs that affect neuronal function in the brain have been reported. Characterization of the expression profiles of 119 miRs in mouse and human organs indicate that these miRs are phylogenetically conserved, organ-enriched and organ-specific. Interestingly, 13 of these miRs are enriched in the brain. Moreover, eight brain miRs are induced during neuronal differentiation of human and mouse EC cells after retinoic acid application, suggesting that there may be a conserved role in mammalian neuronal development (Sempere et al., 2004). Indeed, several studies indicate a relationship between miR and neuronal development. For example, miR-124a, which is perfectly conserved at the nucleotide level from worms to humans, is expressed throughout the embryonic and adult CNS (Lagos-Quintana et al., 2002). miR-124 plays an important role in neuronal differentiation and function, as overexpression of miR-124 in differentiating mouse P19 cells promotes neurite outgrowth, while blockade of miR-124 function decreases neurite outgrowth and levels of acetylated α-tubulin (Yu et al., 2008). Interestingly, miR-18 and -124a reduce glucocorticoid receptor (GR)-mediated events in addition to decreasing GR protein levels by binding to the 3′ untranslated region of GR (Vreugdenhil et al., 2009). Glucocorticoid is a stress hormone and putatively involved in the pathophysiology of mental disorders such as depression (see Sections below). Furthermore, expression of miR-9 is also found in the CNS and the knockdown of the miR results in inhibition of neurogenesis along the anterior–posterior axis (Bonev et al., 2011). miR-134 is also localized in the dendritic compartment of hippocampal neurons (Schratt et al., 2006). In midbrain dopamine neurons, miR-133b specifically expresses and regulates neural maturation and function via a negative feedback circuit that includes Pitx3, a paired-like homeodomain transcription factor (Kim et al., 2007). Recently, it was shown that overexpression of miR-137 promotes the proliferation of adult neuronal stem cells, whereas a reduction of miR-137 results in enhancement in differentiation (Szulwach et al., 2010). Taken together, all these studies indicate that brain-specific miRs have multiple roles in neuronal function. In the following section, we focus specifically on the relationship between miR function and the neurotrophin BDNF, which is well known as a critical mediator in synaptic plasticity.

4. miRs and BDNF

4.1. BDNF and synaptic function

In addition to BDNF, a member of the neurotrophin family, nerve growth factor (NGF), neurotrophin-3 (NT-3), and NT-4/5 are found mainly in mammals. As high-affinity tropomyosin-related kinase (Trk) receptors, TrkA (for NGF), TrkB, and TrkC (for NT-3) are essential for proper neurotrophin functioning. In the brain, it is well known that expression of both BDNF and its receptor TrkB is very strong, as this ligand–receptor complex is responsible for multiple neuronal functions. Via stimulation of TrkB, BDNF initiates activation of various intracellular signaling cascades such as MAPK/ERK, PLCγ, and PI3K pathways. BDNF/TrkB signaling is essential for cell differentiation and survival, neurite outgrowth, and synaptic function (Huang and Reichardt, 2003; Reichardt, 2006; Minichiello, 2009; Russo et al., 2009; Numakawa et al., 2010a). BDNF is critical in glutamatergic functioning, contributing to the maintenance of both NMDA and AMPA receptors as well as glutamate-mediated synaptic plasticity (Caldeira et al., 2007a, 2007b; Yoshii and Constantine-Paton, 2010). We also demonstrated a BDNF-dependent increase in levels of glutamate receptor subunits including NR2A, NR2B, and GluR1, in addition to presynaptic synapsin I and synaptotagmin (Matsumoto et al., 2006; Kumamaru et al., 2008, and see Fig. 3). In the upregulation of synaptic proteins, activation of MAPK/ERK signaling was required (Matsumoto et al., 2006; Kumamaru et al., 2008). A recent study showed that unsupervised learning (*in vivo*) and theta burst stimulation (with hippocampal slices) increases the number of postsynaptic densities co-localized with activated TrkB in the hippocampus. The potentiation of the co-localization was inhibited by a scavenging extracellular BDNF or NMDA receptor antagonist (Chen et al., 2010), suggesting that ligand BDNF and stimulation of glutamate receptors are both involved in the learning process. In general, it is believed that an increase in intracellular Ca^{2+} concentration contributes to the activity-dependent synaptic secretion of BDNF. An increase in Ca^{2+} via voltage-gated Ca^{2+} channels (VDCC), internal Ca^{2+} stores, and NMDA receptors was, in fact, demonstrated (Hartmann et al., 2001; Kuczewski et al., 2009; Lessmann and Brigadski, 2009). Taken together, an intimate relationship exists between BDNF and synaptic function. A detailed investigation of the mechanisms underlying BDNF-mediated changes in synaptic plasticity is paramount, and the possible

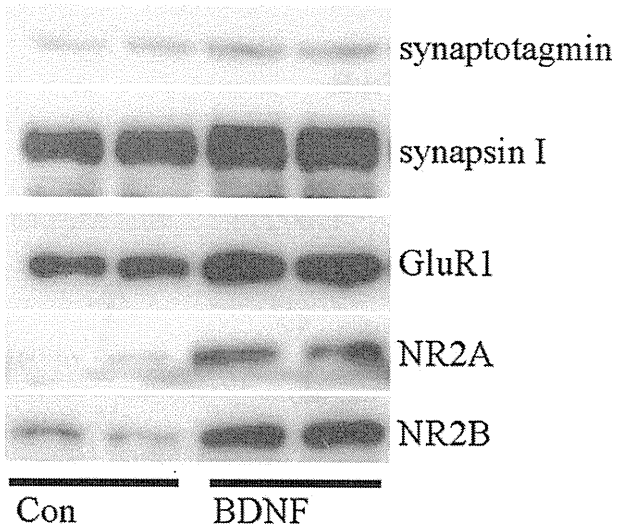

Fig. 3. BDNF increased expression of synaptic proteins including synaptotagmin, synapsin I, GluR1, NR2A, and NR2B. Cultured cortical neurons were prepared from postnatal 2 day-old rats. Six-hours after cell plating, BDNF (100 ng/ml) was added. After an additional 5-day culture, the cells were lysed for immunoblotting.

association of these mechanisms with brain-specific miRs is a topic of increased interest.

4.2. Neuronal function of miRs and BDNF

Brain-specific miRs play a role in synaptic function. In addition, BDNF is also a key player in synaptic plasticity in CNS neurons. Indeed, the interaction between BDNF and brain-specific miRs is an area of recent focus. Remarkably, after the loss of brain-specific miRs, enhanced learning and memory is observed in mice (Konopka et al., 2010). After deletion of the Dicer1 gene in mouse forebrain, a whole set of brain miRs (including miR-124, -132, -137, -138, -29a, and -29c) was significantly decreased. In Dicer1 mutant mice, the efficacy at hippocampal synapses and translation of BDNF was higher than in controls (Konopka et al., 2010). As mentioned above, Schratt et al. reported involvement of miR-134 in BDNF-regulated dendritic spine size in hippocampal neurons (Schratt et al., 2006). The miR-134 is localized in dendrites, and negatively regulates the spine size via repressing the translation of Limk1 mRNA (LIM kinase 1), which is known to regulate dendritic structures (Bamburg, 1999; Meng et al., 2004). They demonstrate that BDNF relieves suppression of Limk1 translation caused by miR-134 (Schratt et al., 2006). The current study shows that miR-134 regulates memory and neuronal plasticity (Gao et al., 2010). It is possible that the mammalian Sir2 homolog SIRT1 promotes synaptic plasticity via mediating expression of CREB by miR-134, as mutant mice lacking SIRT1 catalytic activity showed reduction in both CREB and BDNF proteins and upregulation of miR-134. A knockdown of miR-134 reversed the hippocampal long-term potentiation in SIRT1 mutant mice (Gao et al., 2010). In the cell differentiation of SH-SY5Y cells, a human neuroblastoma cell line, possible involvement of miR-125b has been reported (Le et al., 2009). miR-125b was significantly upregulated after all-trans-retinoic acid and BDNF application, and ectopic expression of miR increased differentiated SH-SY5Y cells with neurite outgrowth (Le et al., 2009). miR-375 also plays a role in regulation of neurite differentiation. Though exposure to BDNF increased neurite outgrowth in human neuroblastoma BE(2)-M17 cells, the miR-375 overexpression diminished BDNF action. Interestingly, BDNF-promoted neurite outgrowth was inhibited by silencing HuD (neuronal RNA-binding protein) or overexpressing miR-375, suggesting that miR suppresses neurite outgrowth via decreasing the HuD levels. Indeed, reporter analysis revealed that miR-375 decreased HuD expression through targeting the HuD 3′UTR (Abdelmohsen et al., 2010).

miR-132 contributes to the action of BDNF. In cultured cortical neurons, BDNF induces upregulation of miR-132 (Vo et al., 2005). Interestingly, overexpression of miR-132 increases the outgrowth of primary neurites, and transfection of an antisense RNA for miR-132 results in a substantial decrease in neurite outgrowth (Vo et al., 2005). Recently, we also reported that BDNF induced a marked increase in miR-132 levels in cultured cortical neurons (Kawashima et al., 2010, and see Fig. 4). miR-132 upregulation may be a direct result of the BDNF in neurons, as the other miRs such as miR-9, -124, -128a, -128b, -134, -138, and -16 (as a control) were unchanged. While BDNF increased the expression of synaptic proteins including NR2A, NR2B and GluR1 (Fig. 3), transfection of double strand-miR-132 also upregulated these glutamate receptors (Kawashima et al., 2010, and see Fig. 4). Antisense RNA to inhibit endogenous miR-132 function decreased the number of BDNF-increased glutamate receptors. Interestingly, we found that blockade of the MAPK/ERK pathway suppressed BDNF-increased miR-132 levels (Fig. 4). As described above, we previously showed an important role of ERK signaling in the maintenance of glutamate receptor expression (Matsumoto et al., 2006; Kumamaru et al., 2008; Tuerxun et al., 2010). Taken together, it is possible that an increase of miR-132 via ERK activation is required for upregulation of glutamate receptors after BDNF stimulation. Furthermore, Remenyi et al. reported upregulation of miR-212/132 by BDNF stimulation in cultured cortical neurons (Remenyi et al., 2010). In their system, miRs were regulated by the ERK pathway, specifically via downstream MSK1 (mitogen- and stress-activated kinase 1) and CREB signaling.

Importantly, we found that the BDNF-increased ERK1/2 activation, miR-132 expression, and postsynaptic proteins were all diminished following chronic treatment with glucocorticoids, suggesting that treatment with chronic glucocorticoids caused suppression in BDNF-dependent neuronal function via reducing miR-132 expression (Kawashima et al., 2010, and see Fig. 5). Interestingly, increased blood levels of glucocorticoids (cortisol in humans, and corticosterone in rodents), which are stress hormones released from the adrenal glands, are putatively associated with the pathophysiology of mental disorders including depression (McEwen, 2005; Kunugi et al., 2010). Prolonged exposure to glucocorticoids as a result of stressful conditions may cause depression via damaging vital neuronal functions, as many studies suggest that downregulation of expression/function of BDNF is also closely related to the onset of depression (Altar, 1999; Nestler et al., 2002; Knable et al., 2004; Gervasoni et al., 2005; Karege et al., 2005; Numakawa et al., 2010b). We recently found a functional interaction of glucocorticoids with BDNF in cortical neurons. In cortical cultures, GR expression and an interaction between the GR and TrkB were decreased after exposure to glucocorticoids. The BDNF-evoked release of glutamate was also reduced because the GR-TrkB interaction is important for glutamate release induced by BDNF (Numakawa et al., 2009). Considering that BDNF affects levels of miRs, and glucocorticoids impact the level/function of BDNF negatively, further investigation into the relationship between glucocorticoids and miRs expression is warranted. It was reported that miR-18 and -124a decreased GR-mediated events in addition to reducing GR expression levels (Vreugdenhil et al., 2009). Both miRs suppressed levels of glucocorticoid-induced leucine zipper expression induced by DEX, a GR-selective glucocorticoid, and the reporter assays indeed revealed that miR-124a putatively bound to the 3′UTR of GR (Vreugdenhil et al., 2009). As the expression of miR-124a is restricted to the brain (Lagos-Quintana et al., 2002), further study of the neuronal role of miR-124a is needed.

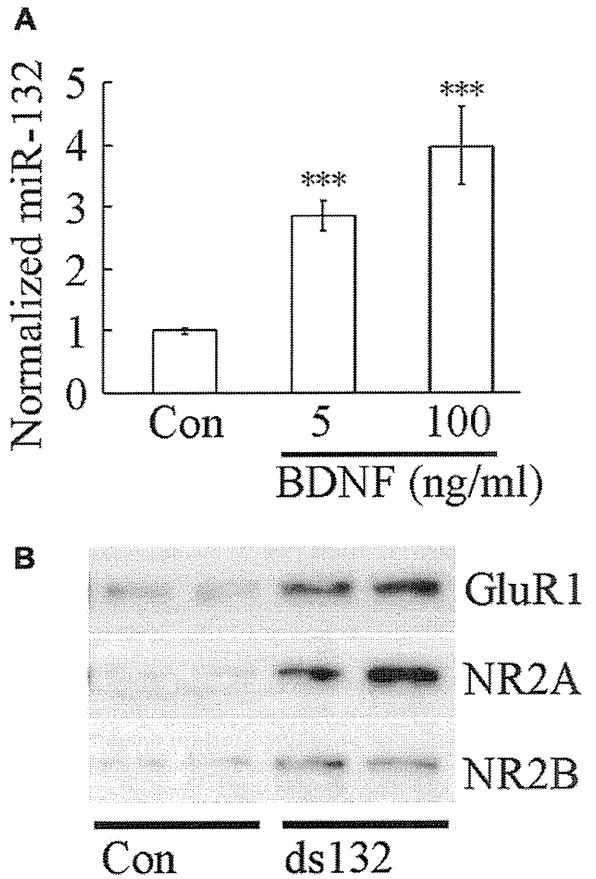

Fig. 4. BDNF induced increase in the levels of miR-132 in cultured cortical neurons. (A) BDNF increased the miR-132 levels in a dose-dependent manner. BDNF (5, or 100 ng/ml) was applied at 4 days *in vitro* (4DIV). Twenty-hours later, quantitative analysis of miR-132 was carried out by RT-PCR. Data represent mean ± S.D. ($n = 4$). ***$P < 0.001$. (B) Ds-miR-132 (double-stranded synthesized mature microRNA) transfection induced upregulation of glutamate receptors (GluR1, NR2A, and NR2B). The transfection was carried out at 9DIV. After an additional 2-day culture, the cortical neurons were harvested. (C) BDNF-induced upregulation of miR-132 is through the MAPK/ERK signaling cascade. U0126 (10 μM) is an inhibitor of the MAPK/ERK pathway. BDNF (100 ng/ml) was applied at 4DIV. Twenty-hours later, levels of miR-132 were determined by RT-PCR. Data represent mean ± S.D. ($n = 4$). ***$P < 0.001$.

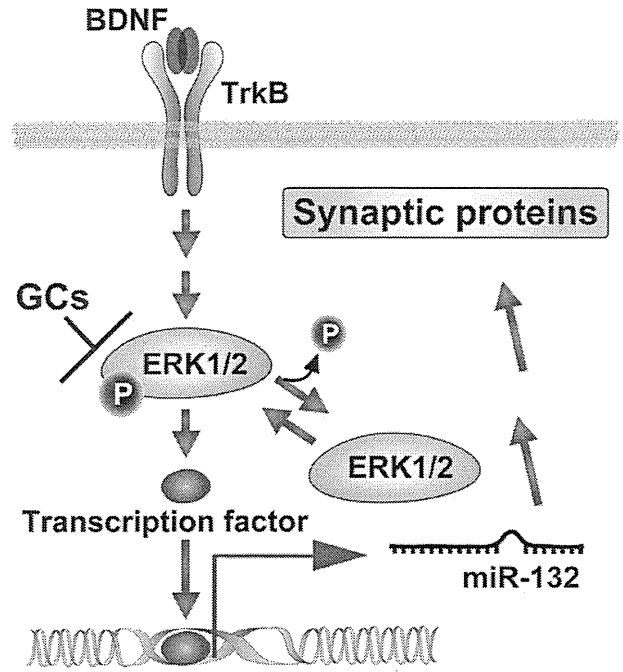

Fig. 5. BDNF increases levels of synaptic proteins via upregulation of miR-132. In cortical neurons, BDNF induces miR-132 expression, which is required for BDNF-increased postsynaptic proteins. The BDNF-induced miR-132 was inhibited by glucocorticoids (GCs). As GCs reduced the ERK1/2 activation, and both GCs and an inhibitor for the ERK1/2 pathway repressed the miR-132 expression, it is possible that BDNF upregulates synaptic proteins via stimulating the ERK/miR-132 system.

4.3. Regulation of BDNF levels by miRs

As shown above, some miRs are involved in BDNF functions including morphological change and regulation of neurotransmission. On the other hand, several recent reports indicate that BDNF expression is directly regulated by miRs. Mellios et al. showed that miR-30a-5p and miR-195 target the 3′UTR of BDNF (Mellios et al., 2008). In rat forebrain neurons, they observed that miR-30a-5p overexpression induced marked decreases in BDNF protein. In a postmortem study using the prefrontal cortex of schizophrenia and control subjects, the association among BDNF, GABAergic transcript [NPY(neuropeptide Y), SST (somatostatin), and PV (parvalbumin) mRNAs], and miRs (miR-195 and miR-30a-5p) was examined by the same group (Mellios et al., 2009). They showed deficits in NPY and PV mRNAs in the schizophrenic group, and levels of BDNF protein were positively correlated with NPY and SST levels. Importantly, BDNF levels showed a strong inverse association with miR-195 levels. NPY, SST, and PV are all well known GABAergic neuronal markers, and abnormalities in GABA-mediated neuronal function are putatively involved in the pathogenesis of schizophrenia (Hashimoto et al., 2008; Lewis et al., 2008). A plethora of evidence indicates that BDNF regulates the GABA system (Seil, 2003; Gottmann et al., 2009), therefore a possible association between miR-195 and schizophrenia is plausible. Though not in the CNS system, miR-15a-1 and miR-18a were reported to play a critical role in zebrafish inner ear development, and this is especially interesting given that BDNF mRNA is one of the miR-15a targets (Friedman et al., 2009). Furthermore, the possible involvement of miR-210 in BDNF regulation was also reported. HEK-293 cells transfected with plasmid encoding miR-210 showed downregulation of BDNF, while, in contrast, BDNF upregulation after transfection of anti-miR-210 oligonucleotide was observed (Fasanaro et al., 2009).

5. miRs and brain diseases

The cross relationship between BDNF and miRs in neurons suggests that miRs may be involved in brain disorders including mental illness. Interestingly, the prefrontal cortex of subjects with schizophrenia demonstrate downregulation of GABAergic genes (NPY and SST mRNAs). It is possible that miR-195 regulates BDNF levels, which may implicate miR-195 as being a key player in the disease-related deficits in the GABAergic genes (Mellios et al., 2009). Case-control studies (with panic disorder and control subjects) show that human miR-22, -138-2, -148a, and -488 are associated with panic disorder. Interestingly, reporter assays reveal that the BDNF gene is potentially repressed by miR-22 (Muiños-Gimeno et al., 2010). Remarkably, it was reported that methyl CpG binding protein 2, MeCP2, regulated striatal BDNF levels and cocaine addiction via homeostatic interaction with miR-212. In their system, a knockdown of striatal MeCP2 decreased the cocaine-seeking behavior of rats. Importantly, miR-212 increased after MeCP2 knockdown, while miR-212 inhibited MeCP2 expression. Considering that blockade of endogenous BDNF signaling in the dorsal striatum decreased cocaine intake, it is possible that the homeostatic interaction of MeCP2 with miR-212 is involved in cocaine addiction via regulation of striatal BDNF levels (Im et al., 2010).

Generally, it is recognized that the loss-of-function mutations in the MeCP2 gene contribute to Rett syndrome (RTT) (Amir et al., 1999). In MeCP2-null mice, reduced levels of BDNF and an increase in amplitude of spontaneous miniature and evoked EPSCs in nTS (nucleus tractus solitarius) neurons were observed. Such synaptic dysfunction in MeCP2-null mice was reversed by exogenous BDNF application (Kline et al., 2010). Recently, Wu et al. identified miRs whose level is changed in MeCP2-null mice before and after the onset of severe neurological symptoms (Wu et al., 2010). They showed that aberrantly increased miRs (including miR-30a/d, miR-381, miR-495) in the absence of MeCP2 may induce downregulation of BDNF in RTT brains. Furthermore, evidence suggests that miRs may influence levels of toxic molecules related to neurodegeneration (Eacker et al., 2009). In neurodegenerative diseases, including Alzheimer's disease (AD), a possible contribution of miRs have been suggested (Hébert and De Strooper, 2009). For example, a decrease in miR-29a/b-1 clusters in sporadic AD showed a correlation with upregulation of BACE1 (β-site APP-cleaving enzyme 1)/β-secretase, the AD related molecules (Hébert et al., 2008). Furthermore, Kim et al. reported miR-133b to be concentrated in the midbrain compared with measurements in the cerebellum and cerebral cortex, while also finding that the midbrain miR-133b levels were decreased in Parkinson's disease patient samples (Kim et al., 2007). Further studies are needed to elucidate the role of miRs expression as a biomarker for brain diseases.

6. Concluding remarks

Brain-specific miRs and BDNF are both involved in neuronal function. Dysfunction of either of these regulatory substances may result in the onset of brain diseases. Indeed, a close relationship between the downregulation of BDNF and the pathophysiology of brain diseases has been suggested (Numakawa et al., 2010b, 2011). Recently, new insight into miRs action has been shown. Some miRs are frequently dysfunctional in cancer, and are present in human plasma in a remarkably stable form. The detection method for measurement of tumor-derived miRs in serum or plasma has been well established (Mitchell et al., 2008). It was revealed that some miRs are released through exosome-dependent exocytosis. These secreted miRs are transferable and functional in the recipient cells (Kosaka et al., 2010), suggesting that specific miRs are promising biomarkers for disorders including cancer. Importantly, the miR-124 level is altered in the serum of patients following brain injury (Laterza et al., 2009). Therefore, in addition to measuring the amount of BDNF, evaluating levels of specific miRs in blood may be diagnostically beneficial in approaching brain diseases, though further study is necessary.

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