2) Precise measurements of individual BNP species in plasma. Currently used BNP and NT-BNP assay kits have not been developed for measurements of individual molecules in complex BNP species in plasma, and include ambiguity in the reported values. To make these kits more reliable, the cross-reactivity of each BNP species in the assay kit should be evaluated with quantitated standard peptides of completely confirmed structures. Combination of the well characterized assay kits and several antibodies being developed, such as an antibody against a hinge region (proBNP[74-79]) of proBNP,[14] will pave the way for measurements of individual BNP species in plasma. The data thus obtained will provide insights into the relation between the complexity of plasma BNP species and heart disease.

BNP in the emergency department and ICU:

1) Positioning of BNP measurement in the emergency room (ER) and intensive care unit (ICU). BNP is an extremely valuable tool for diagnosing cardiac failure in various clinical settings. BNP values are useful for the following: the early diagnosis of HF in health screening, as well as for determining the differential diagnosis, severity, therapeutic effects and prognosis of patients with HF.

A rapid assay has recently become available that has further expanded the applicability of BNP, particularly in the emergency room (ER) and intensive care unit (ICU).

a) BNP reference values. The values of BNP increase when cardiac function decreases (Figure 4).[15-17] However, the type of changes in BNP values depends on the underlying heart disease. For example, values of ANP increase in mitral stenosis, which exerts stress on the atrium but not on the ventricle, whereas those of BNP increase only mildly. Also, BNP values obviously change with time from the onset of acute myocardial infarction (Figure 5).[18]

Issues regarding BNP reference values must be discussed because they change depending on the rationale for the measurement. The BNP value of healthy Japanese individuals is ≤ 18.4 pg/mL (approximately 20 pg/mL). For early detection of cardiac dysfunction in health screenings, a reference value can be set at about 40 pg/mL. However, the reference value needs to be substantially increased for applications in the ER where many patients are critically ill. When considering the relationship with prognosis, a value of about 200 pg/mL is appropriate (Figure 6).[19] An important issue is that ≤ 200 pg/mL does not mean the absence of HF; it merely indicates that HF is mild, and such reference values must not be misinterpreted.

BNP values are slightly affected by extracardiac factors. For example, BNP values tend to slightly increase in the elderly or in patients with renal failure, but tend to slightly decrease in obese individuals.

These facts taken together indicate that BNP values reflect not only the severity of HF, but also the type of underlying disease, the severity of renal dysfunction, and the effects of age and obesity.

b) In the ER. Rapid BNP assays are useful when patients transported to the ER have suspected HF. Combining chest X-ray images with standard blood test findings and BNP values renders a differential diagnosis of HF a relatively simple matter. The severity of HF when complicated by pneumonia was previously difficult to determine when BNP could not be measured. However, the introduction of the rapid BNP assay allows easier diagnosis of HF. Therefore, BNP can play a major role in the differential diagnosis and severity assessment of HF in the ER.

c) In the ICU. Measuring BNP values is also strongly recommended for patients in the ICU, particularly for understanding

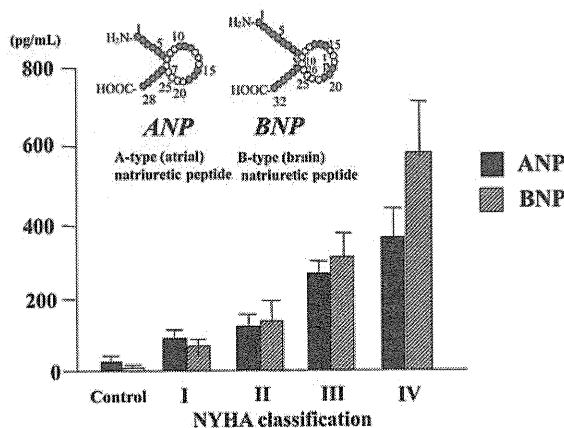

Figure 4. Plasma BNP levels in patients with heart failure. NYHA indicates New York Heart Association. Mukoyama M, Nakao K, *et al.* J Clin Invest 1991, Yasue H, Yoshimura M, *et al.* Circulation 1993, Yoshimura M, Yasue H, *et al.* Circulation 1994.

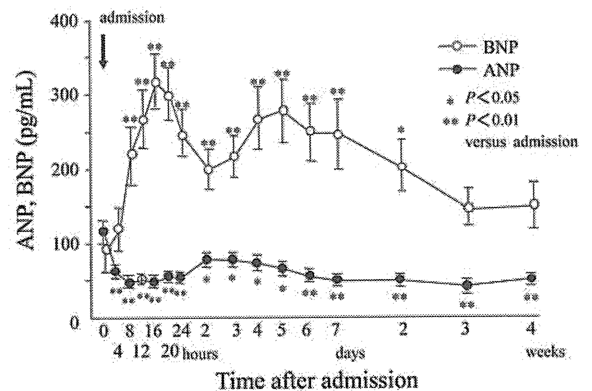

Figure 5. Time course of plasma levels of ANP and BNP in patients with AMI. Morita E, Yoshimura M, *et al.* Circulation 1993.

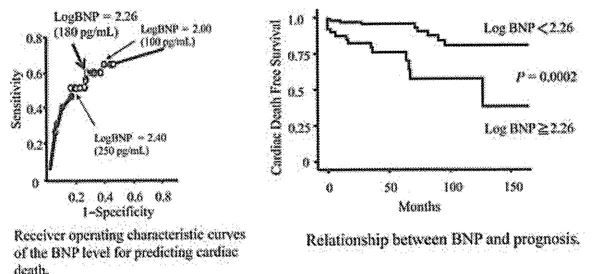

Figure 6. High survival rate in patients with low BNP value in acute myocardial infarction. BNP was measured after about 4 weeks after the onset of AMI. Suzuki S, Yoshimura M, *et al.* Circulation 2004.

Table III. How Good is the History and Physical ?

Variable	Sensitivity	Specificity	Accuracy
History of HF	62	94	80
Dyspnea	56	53	54
Orthopnea	47	88	72
Rales	56	80	70
S_3	20	99	66
JVD	39	94	72
Edema	67	68	68

the course of HF. It is important to know the chronological changes in BNP values after admission. Since therapy can lower BNP values relatively quickly, they should be measured every few days. However, BNP is not currently measured at this frequency because of its cost, but active BNP measurement is desirable in the future. Several clinical studies have clarified that persistently high BNP values are associated with a poor prognosis, and this can be improved by planning therapy based on BNP values. However, further investigations are warranted. Overall, BNP values are useful for assessing therapeutic effects and the prognosis of patients with HF in the ICU.

2) *Interpretation of BNP Levels in Acute Care.* When initially confronted with a symptomatic suspected HF patient, the physician has three tasks; namely to make an appropriate diagnosis, effect prompt treatment, and provide a disposition. Each task must be accomplished rapidly and accurately if optimal outcomes are to be realized, as erroneous diagnosis and treatment delays are associated with adverse outcomes.

While rapid treatment may be required to save the life of the patient, diagnostic accuracy is critical as dire consequences result from inappropriate treatment. In one study[20] of 499 patients transferred by ambulance and ultimately found to suffer HF, the probability of survival was 251% (95% CI 137-455, $P < 0.01$) higher if treatment was performed before transfer, rather than delayed an average of 36 minutes until hospital arrival. Conversely, mortality increased > 350% ($P < 0.05$) when non-HF dyspnea was treated with HF therapy instead of bronchodilators. Ultimately, there is a clear premium on diagnostic accuracy.

Unfortunately, the most rapidly available tools for diagnosis, namely the history and physical condition, are grossly inaccurate, as demonstrated in Table III. Even a finding such as the S3, which has excellent specificity, has such poor sensitivity that its value is minimized in a large portion of the HF population.

Natriuretic peptides (NPs) represent a rapid and accurate test to assist in the evaluation of patients presenting with acute dyspnea. In the Breathing Not Properly trial[21] of > 1,500 patients presenting to an emergency department with undifferentiated dyspnea, the physician's diagnostic accuracy by clinical judgment was only 74%, but improved to 81.6% when the results of BNP testing were considered.

Beyond accuracy, speed of treatment is also critical. In a study of hospitalized HF patients receiving vasoactive treatment,[22] patients receiving early therapy (mean, 1.1 hours) had a mortality of 4.3% compared to 10.9% ($P < 0.0001$) in those who received delayed (mean, 22 hours) therapy. The need for rapid diagnosis and treatment is further supported by analysis[23] of 14,900 patients stratified by BNP and time to loop diuretic quartiles. In this study, the greatest mortality was seen in those patients with the highest BNP levels and longest delay in treatment (Figure 7).

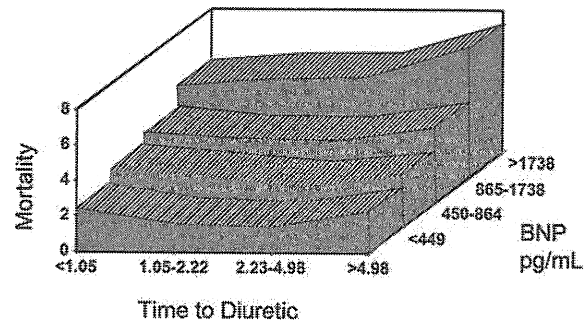

Figure 7. Mortality versus quartiles of diuretic time and BNP level.

Finally, BNP levels are directly associated with mortality. In an analysis of over 45,000 patients,[24] higher levels were reflective of greater acute mortality risk. The lowest inpatient mortality of 1.9% was seen when the BNP was < 430 pg/mL, and increased to 6% in patients with a BNP > of 1,730 pg/mL ($P < 0.0001$). As a mortality rate of 6% exceeds the contemporary mortality rate of myocardial infarction, this knowledge may help the physician to decide whether a patient is an ICU candidate as compared to requiring regular hospitalization.

3) *Consensus statements.* In patients presenting to their physicians with dyspnea, doctors should check the clinical history, perform a physical examination, chest X-ray and ECG, along with laboratory measurements that include BNP.

When using cut-off values in patients with acute dyspnea, apply two values: one to "rule out" (< 100 pg/mL in the United States, < 65 pg/mL in Japan) and one to "rule in" HF (> 400 pg/mL in the United States, > 254 pg/mL in Japan). The intermediate or grey zone (100-400 pg/mL in the United States, 65-254 pg/mL in Japan) area requires extra physician attention and ancillary testing. For early detection of cardiac dysfunction in health screenings, the reference value can be set at about 40 pg/mL in Japan and 66 pg/mL in the United States. However, the reference value needs to be substantially increased for applications in the ER where many patients are critically ill. When considering the relationship with prognosis, a value of about 200 pg/mL in Japan and 318 pg/mL in the United States is appropriate.

Clinical acumen and further testing are often necessary to make a correct final diagnosis. Even in patients with a high probability of diagnosis, the BNP level gives important information concerning risk stratification. This is especially important since there is a discrepancy in the perceived severity of CHF and BNP levels

Caveats in using NP levels: Natriuretic peptides, including BNP and NT-proBNP, have emerged over the years as invaluable tools both for the diagnostic approach and the prognostic assessment of patients with HF. However, there are several caveats related to NPs. Several conditions may lead either to false positive or to false negative results. Causes for NP elevation other than acute HF (HF) include a previous history of HF,

advanced age, renal dysfunction, acute coronary syndrome, pulmonary disease, pulmonary embolism, high-output states such as sepsis, cirrhosis or hyperthyroidism and atrial fibrillation. Pathogenetic mechanisms related with higher than expected levels of NP include myocardial wall stress from ventricular dysfunction, hypervolemia, hypertension, reduced renal clearance, subclinical ischemia, myocardial remodeling and fibrosis and maladaptive neurohormonal stimulation. On the other hand, NP levels may be lower than expected in the case of obesity, flash pulmonary edema, HF originating upstream to the left ventricle, as in the case of acute mitral regurgitation or mitral stenosis, cardiac tamponade and constrictive pericarditis. As outlined in the recent HF guidelines, there is a considerable grey zone for the BNP and NT-proBNP values ranging between 100 and 400 pg/mL and 400 and 2,000 pg/mL, respectively. Grey-zone values may result from the involvement of the right ventricle in cases with pulmonary disease, including chronic obstructive lung disease with cor pulmonale or pulmonary hypertension, right ventricular failure from long-standing left ventricular failure or right ventricular disease (infarction, valvular disease) and pulmonary embolism. Grey-zone values require a more detailed clinical and laboratory assessment, while knowledge of baseline NP levels would be of great help. In any case, NP should be interpreted as continuous variables. When using BNP in patients with acute dyspnea, two values should be applied: one to "rule out" HF (< 100 pg/mL) and one to "rule in" HF (> 400 pg/mL). When using NT-proBNP, instead, one rule-out value (< 300 pg/mL) and three different rule-in values based on age should be applied. In the case of renal dysfunction, given its frequent coexistence with heart disease, high NP levels should not be ignored, but the applied cut-offs for detecting HF may need to be raised when eGFR falls below 60 mL/minute. In contrast, in obese patients lower cut-off values need to be used and a rule-out BMP value of 50 pg/mL should be applied in patients with BMI (body mass index) > 35.

Consensus statements.

a) Grey zone. The grey zone (100-400 pg/mL in the United States, 65-254 pg/mL in Japan, for BNP, or 400 to 2,000 for NTproBNP) represents 25% of dyspneic patients, 75% of whom will have CHF as the ultimate diagnosis. The presence of concomitant pulmonary disease is present in many of these patients.

The overall prognosis for dyspneic patients with BNP levels in the grey zone is good and may help risk stratify patients for admission or discharge.

For BNP levels of 100-400 pg/mL in the United States, 65-254 pg/mL in Japan, the following must be considered:

Stable underlying dysfunction

Right ventricular failure from pulmonary hypertension

Acute pulmonary embolism

Renal failure (SCr usually > 2.5 mg/dL)

Patients may present with HF with healthy BNP levels or with below expected levels. This can occur in the following situations:

Flash pulmonary edema (< 1-2 hours)

HF up-stream from the left ventricle (ie, acute mitral regurgitation from papillary muscle rupture)

Obese patients (BMI > 35 kg/m^2)

b) Renal dysfunction. Modest alterations in BNP levels occur with renal insufficiency (eGFR below 60 mL/minute), with a likely recalibration of the cutoff value to approximately 200 pg/mL in the United States and 128 pg/mL in Japan for patients presenting with dyspnea in the ED.

As HF is the cause of dyspnea in patients with renal dysfunction, BNP can be of great value in diagnosing acute HF in patients with renal insufficiency.

c) Obesity. Since obese patients (BMI > 35 kg/m^2) express lower levels of BNP for any given severity of HF, caution should be exercised in interpreting BNP levels in such patients.

In patients presenting with acute dyspnea, obese patients (BMI > 35) should have a rule out value of 50 pg/mL in the United States and 33 pg/mL in Japan. Most obese patients with acute HF will, however, have BNP levels over 100 pg/mL in the United States and 65 pg/mL in Japan.

There seems to be a linear relationship between BMI and BNP. Patients who are obese or very obese should have their BNP multiplied by 2.0-3.0 to obtain a BNP level of similar severity with those of healthy weight.

BNP levels are still highly prognostic in obese patients.

Cost-effectiveness of BNP testing: The use of plasma BNP levels, in combination with other clinical information, provides information that seems to be helpful in the diagnosis, prognosis, and management of HF as well as screening for left ventricular dysfunction. However, there are very few data on the cost-effectiveness of BNP testing. The BASEL study is an important study in patients with acute HF.[25] A prospective, randomized, controlled study of 452 patients who presented to the emergency department with acute dyspnea: 225 patients were randomly assigned to a diagnostic strategy involving the measurement of BNP levels with the use of a rapid bedside assay, and 227 were assessed in a standard manner. In the BASEL study, final diagnosis of HF was about 50%. Time to the initiation of the appropriate therapy was about 30 minutes, which is significantly shorter in the BNP group compared to in the control group. The mean total cost of treatment was significantly lower in the BNP group than in the control group. The BASEL study indicated that rapid-BNP testing in the management of acute HF reduced the total cost of treatment by 26% (P = 0.0006).

To compare the cost-effectiveness of BNP and echocardiography for predicting outcome in patients with CHF at discharge, 116 patients hospitalized with CHF underwent simultaneous BNP and Doppler echocardiographic examinations once ready for discharge.[26] In this study, in patients admitted to hospitals with CHF, predischarge BNP was more cost-effective than comprehensive Doppler echocardiographic examination for the prediction of future cardiac death or rehospitalization for CHF ($P < 0.001$).

In the United States, less than $50,000 per Quality Adjusted Life of Years (QALY) is evaluated as cost-effective. Screening populations with 1% prevalence of reduced EF (mean at age 60) with BNP followed by echocardiography seem to be cost-effective.[27] In Europe, less than $60,000 per QALY is evaluated as cost-effective. In Japan, consensus has not been reached as to the limit of cost-effectiveness. There is no data regarding the prevalence of low ejection fractions in the general population in Japan, but there is likely a lower prevalence compared to the United States and Europe. Further studies are needed to assess the cost-effectiveness of BNP testing for establishing international guidelines on the management of HF.

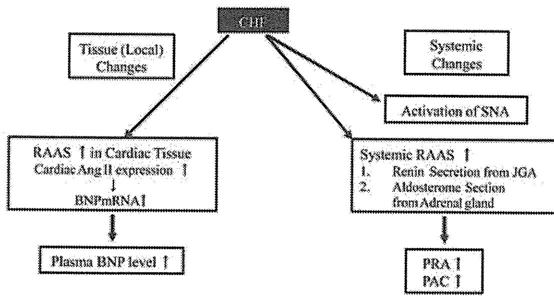

Figure 8. Biomarkers indicating systemic or cardiac RAAS activity. RAAS indicates renin angiotensin aldosterone system; SNA, sympathetic nervous system; JGA, juxta glomerular apparatus; PRA, plasma renin activity; and PAC, plasma aldosterone concentration.

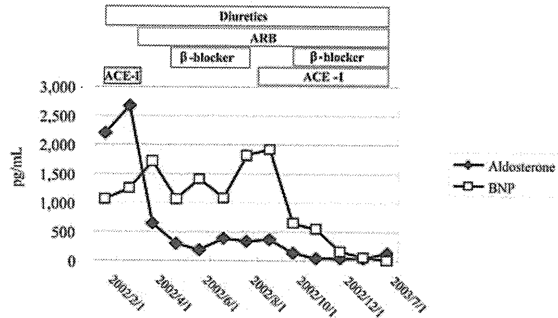

Figure 9. Change in plasma BNP and aldosterone levels during CHF treatment.

Rationale of BNP-guide management for in-patients/out-patients with HF: BNP is a cardiac hormone secreted mainly from the ventricle, and its expression is augmented by ventricular wall stretch. The plasma BNP level is elevated in accordance with the severity of HF.[28,29] With the biological characteristics of BNP expression, BNP is widely used as a biomarker to diagnose HF and predict its prognosis. Now BNP is recognized as the most faithful surrogate marker for hard endpoints in clinical trials in HF. Recent studies have reported that HF therapy guided by BNP-relating peptide improves outcome compared with conventional therapy.[30-32] However, the investigators have not reached consensus on the target value of the plasma BNP or N-terminal proBNP (NT-proBNP) in the management of HF.

1) Circulating BNP is elevated with the activation of the ventricular renin-angiotensin system (RAS). In HF, the systemic and local RAS as well as the sympathetic nervous system are activated to maintain blood supply to vital organs and to maintain cardiac performance by changing loading conditions in the acute phase and by remodeling the ventricle in the chronic phase. RAS activation is a compensatory mechanism, but RAS is over-activated probably because of genetic programming, and consequently leads to a vicious cycle of HF in humans. Nowadays, inhibition of over-activated RAS by an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker is essential in the treatment of HF. From the clinical point of view, how do physicians recognize whether the inhibition of the systemic and local RAS is sufficient?

In the case of the systemic RAS, renin is the rate-limiting enzyme for its activation, and plasma renin activity and the plasma concentration of end product, aldosterone, indicate its activation (Figure 8). However, these plasma markers do not indicate the activation of the cardiac RAS. In the ventricular tissues, activation of cardiac local RAS up-regulates BNP gene expression in cardiomyocytes directly or indirectly, that is, through endothelin released from cardiac fibroblasts. Thus, elevation of plasma BNP levels indicates the activation of cardiac RAS, because BNP is exclusively synthesized in the heart (Figure 8). In other words, elevation of plasma BNP level suggests that the dosage of RAS blockers is not sufficient to block over-activated RAS in the heart.

2) Representative case of HF treated with RAS blockers (Figure 9). A 40-year old male patient with acute decompensated heart failure (ADHF) due to dilated cardiomyopathy was admitted to our hospital because of severe dyspnea and severely reduced cardiac performance. He had a plasma BNP level of 1,070 pg/mL and aldosterone of 2,160 pg/mL without RAS blocking agents. Echocardiography revealed diffuse severe hypokinesis with an ejection fraction of 16%. Medical treatment consisting of 75 mg of alacepril and 40 mg of furosemide was begun, but alacepril was changed to 50 mg of losartan because of adverse effects on liver function. As shown in Figure 9, the treatment with ARB promptly decreased plasma aldosterone concentration but did not affect plasma BNP levels. The addition of carvedilol at a dose of 2.5 mg was not tolerated. At that time, chest X-rays and echocardiogram indicated left- and right-sided heart failure, with prerenal failure with serum creatinine of 6.7 mg/dL. Finally, 2.5 mg of enalapril was added to the regimen and the dose was increased to 5 mg, and carvedilol was initiated, followed by reduction of the plasma BNP level below the level of 100 pg/mL. Keeping the plasma BNP level below 100 pg/mL for more than 6 months brought reverse remodeling of the ventricle and the patient's symptoms were improved.

Before the addition of enalapril, the plasma aldosterone level was decreased from over 2,000 pg/mL to around 200 pg/mL, but the plasma BNP level was not changed, suggesting that systemic RAS was substantially inactivated by the ARB treatment but cardiac RAS is not changed. After the addition of enalapril and carvedilol, both the plasma BNP and aldosterone concentrations were decreased to near normal ranges, and cardiac performance was restored to an ejection fraction of about 40% and cardiomegaly disappeared. This finding suggests that reverse remodeling was only achieved by sufficient inhibition of cardiac local RAS by sufficient doses of RAS blockers.

3) The lower the better. Circulating BNP level is a surrogate marker of cardiac RAS activation, indicating that physicians should try to reduce and keep the plasma BNP level as low as possible. Based on prospective studies, a plasma BNP level below 200 pg/mL may be a cutoff for better prognosis. Figure 10 shows three different trajectories of BNP release. The patient with the lowest BNP at discharge usually has the best prognosis.

4) Consensus statements. Not everyone who is clinically euvolemic is without congestion.

Clinical euvolemia with a high BNP level means that in some patients congestion is still present. If BNP can be low-

ered in these patients without causing renal insufficiency or hypotension, true optivolemia may be reached.

While in a given patient the BNP level does not always correlate to wedge pressure, in a patient admitted with acute HF, a high BNP level (generally over 600 pg/mL in the United States, 378 pg/mL in Japan) and high filling pressures secondary to volume overload, a treatment-induced decrease in wedge pressure will almost always be associated with a rapid drop in BNP levels as long as the patient is maintaining adequate urine output (> 30 mL/hour).

At least three BNP levels should be measured during hospitalization: admission and at discharge when optivolemic. Further levels might be drawn in an attempt to support clinical improvement or lack thereof.

While a drop in BNP level of 30% is important, it is not the magnitude of the drop as much as it is the final BNP level that relates to optivolemic status and prognosis.

Outpatient titration: The correlation between the drop in BNP level and the patient's improvement in symptoms (and subsequent outcome) during hospitalization suggests that BNP-guided "tailored therapy" in an outpatient setting might be effective. Since NP levels reflect end-diastolic wall stress, which is elevated by both increased filling pressures and by LV dilation,[33] measuring serial levels over time may provide a way, in conjunction with our clinical acumen, to monitor the effects of drug therapy on LV remodeling.[34]

In a pilot study of 69 patients with HF and LV dysfunction who were randomized to receive therapy guided by natriuretic peptide levels or standard care, BNP-guided treatment, targeting a BNP level < 200 pg/mL significantly reduced total cardiovascular events, and delayed time to first event.[35] The STARS-BNP trial, which used a target BNP < 100 pg/mL, also showed a reduction in HF deaths and hospitalizations for HF when BNP levels were used to tailor outpatient therapy.[36]

Perhaps BNP-guided therapy is successful simply because it serves as a reminder to physicians to give evidence-based treatment. But in the STARS trial most patients were already on adequate doses of guideline-derived therapy. Hence it may be that BNP levels offer further information that can help decide whether to up-titrate therapy or perhaps even withhold further therapy.

Other drugs for HF appear to decrease BNP levels.[37-42] It appears that ACE inhibitors, angiotensin receptor blocker agents, spironolactone, and perhaps beta blockers drive BNP levels down, although it is unclear whether this is a true marker of clinical improvement. In the Valsartan Heart Failure Trial (Val-HeFT), changes in BNP over time induced by pharmacologic therapy were shown for the first time to correlate with morbidity and mortality.[42] Patients with the greatest percentage decrease in BNP and norepinephrine (NE) from baseline had the lowest morbidity and mortality, whereas patients with the greatest percentage increase in BNP and NE were at greatest risk. The authors found BNP to be more predictive of morbidity and mortality than NE, or, in a separate analysis, than aldosterone.

Consensus statements. NP levels drawn early after discharge may confirm the adequacy of outpatient therapy. Early rises in NP levels following hospital discharge are often a manifestation of inadequate diuretic therapy.

There is considerable day-to-day variation in BNP levels. A 50% increase in BNP levels over baseline in the appropriate clinical setting often represents decompensation, especially with weight gain and either edema or dyspnea.

Treatment with ACE inhibitors, beta-blockers and, angiotensin receptor blockers and aldosterone antagonists result in decreases in optivolemic BNP levels over the long term.

Randomized controlled trials using NP-guided therapy have demonstrated a significant reduction in the primary combined endpoint of death and re-hospitalization in patients less than 76 years of age.

BNP in general screening:

1) The main issue. HF is a progressive disease that is clearly recognized to benefit from early diagnosis and therapeutic intervention. Thus, recognition during the asymptomatic stages can lead to early initiation of treatment with expectations of improved survival and quality-of-life. Asymptomatic LV dysfunction is thought to be present in approximately 3% - 5% of the general population. The issue at present is that despite the fact that BNP is widely recognized to be the 'gold standard' biomarker for detection of left ventricular dysfunction and of cardiac dysfunction overall, a cut-off level or clinical decision limit that will allow for accurate detection of patients that are still in asymptomatic stages that will benefit from early recognition and treatment are still lacking or have not reached a consensus.

2) What the current guidelines show (Table IV). The Japanese JCS Guidelines (2005/2006) state that the cut-off value of BNP for screening LV dysfunction remains unclear. However, the guidelines recognize that BNP has high negative predictive value for CHF. The ACC/AHA Guidelines (2009) state that BNP represents a potential tool for screening LV dysfunction. The ESC Guidelines (2008) do not make a specific statement on this issue.

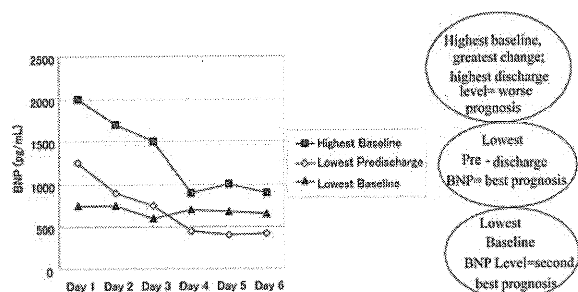

Figure 10. BNP trends during hospitalization and subsequent prognosis upon.

Table IV. Descriptions on Use of BNP as a Tool for Screening LV Dysfunction in JCS, ACC/AHA and ESC Guidelines

	Description
JCS 2005/2006	The cut-off value of BNP for screening LV dysfunction remains unclear. However, BNP has high negative predictive value for CHF.
ACC/AHA 2009	BNP represents a potential tool for screening LV dysfunction.
ESC 2008	No description

Table V. Summary of Studies on LVD and BNP Cut-off Values in the General Population

Number (*n*)	Cohort	BNP (pg/mL)	Criteria	Sensitivity (%)	Specificity (%)	Ref.
249	General	18.4	LVD	-	-	(1)
1252	General	17.9	LVSD	77	87	(2)
155	70-84 years	64.7	LVSD	92	65	(3)
480	Men	47.0	LVD + α^{*1}	69	91	(4)
173	Men > 65 years	47.0	LVD + α^{*1}	89	84	(4)
248	Men risk (+)[*2]	46.0	LVD + α^{*1}	76	88	(4)
513	Women	85.0	LVD + α^{*1}	50	95	(4)
205	Women > 65 years	85.0	LVD + α^{*1}	75	90	(4)
244	Women risk (+)[*2]	84.0	LVD + α^{*1}	75	92	(4)
4527	Men	32.3	CHF	83	77	(5)
8939	Women	62.4	CHF	64	94	(5)

[*1] indicates VHD, HCM, HHD, IHD and AF, [*2] indicates HTN and/or DM. Ref.(1) indicates Jpn Heart J 2000; (2), Lancet 1998; (3), BMJ 2000; (4), J Card Fail 2005; and (5), Int J Cardiol 2009.

Table VI. Description of BNP and HFNEF in JCS, ACC/AHA and ESC Guidelines

	Description
JCS 2005/2006	In patients with normal systolic function, elevated BNP levels and diastolic filling abnormalities might help to reinforce the diagnosis of diastolic dysfunction.
ACC/AHA 2009	BNP in association with echocardiographic filling patterns can improve diagnostic accuracy.
ESC 2008	BNP and NT-proBNP rise in response to myocardial wall stress. Usually, lower levels are observed in patients with preserved LV systolic function.

3) New data supporting stronger indications (Table V). Although initially the normal cut-off value of 18.4 pg/mL was used as both the normal cut-off as well as the clinical decision limit, it was recognized early on that approximately 15% of seemingly healthy asymptomatic subjects show levels that exceed this level. Subsequent studies conducted to determine a cut-off for asymptomatic patients with heart disease show that in general that a clinical decision limit ranging from 40 pg/mL to 80 pg/mL might be applicable (see Table V showing various clinical decision limits as determined through various studies). However, these studies have also outlined that age, sex and body mass (obesity) are complicating factors that affect BNP levels and how to take these factors into account still remains an issue.

4) Consensus points. BNP is clearly recognized to be beneficial for detecting cardiac dysfunction including from still asymptomatic stages.

However, establishing a definitive cut-off level to allow reasonable discrimination of patients is still an ongoing subject of discussion.

BNP and diastolic dysfunction:

1) The main issue. Diastolic dysfunction is recognized to be a cause of HF. In fact, diastolic dysfunction may be present in almost one-half of patients with HF. In the presence of preserved or systolic function, this type of HF is referred to as HF with preserved/normal ejection fraction (HF-NEF). As HF is recognized to be present in approximately 10% of elderly people and is a leading global medical problem, recognition is of the utmost importance. Diastolic dysfunction is often associated with hypertension and increased myocardial wall thickness (stiffness), but at present, optimal therapeutic interventions are still not clear. It is noteworthy that HF-NEF patients represent a rather heterogeneous population. To better improve care and attention to this condition, recognition remains prerequisite but this remains a challenge as echocardiographic measures are still the only reliable method of detection. BNP is clearly recognized to be elevated in diastolic dysfunction, but how it can be used remains unclear.

2) What the current guidelines show (Table VI). The Japanese JCS Guidelines (2005/2006) state that in patients with normal systolic function, elevated BNP levels and diastolic filling abnormalities might help to reinforce the diagnosis of diastolic dysfunction. The ACC/AHA Guidelines (2009) state that BNP in association with echocardiographic filling patterns can improve diagnostic accuracy. The ESC Guidelines (2008) state that BNP and NT-BNP rise in response to myocardial wall stress, and that lower levels are usually observed in patients with preserved LV systolic function.

3) New data supporting stronger indications. The data at present show that BNP is clearly elevated in HF-NEF but to lesser values than in systolic function. However, BNP cannot discriminate between the two conditions. Furthermore, recent studies have shown that factors that affect diastolic function and myocardial stiffness such as increased wall thickness, presence of hypertension and atrial fibrillation as well as ischemia are factors that need to be accounted for.

4) Consensus statements. In patients presenting with acute HF with preserved-LV function, BNP levels are elevated, although usually not as high as in patients with systolic dysfunction (800 pg/mL versus 400 pg/mL in the United States, 506 pg/mL versus 254 pg/mL in Japan).

BNP should not be used by itself to differentiate systolic from diastolic dysfunction in the emergency department.

In patients with normal systolic function, elevated BNP levels along with diastolic filling abnormalities might help to reinforce the diagnosis of diastolic dysfunction.

In the future, drug-trials for treating patients with diastolic dysfunction might include BNP levels as entrance criteria and as endpoints for treatment success.

BNP concentrations above age-adjusted cut-off points may identify elderly patients with diastolic dysfunction

BNP measurement in the guidelines (Japan, Europe, and USA):

1) The main issue. BNP is clearly recognized to be beneficial for diagnosing and treating HF in not only the Japanese Guidelines (JCS) but also those of the United States (ACC/AHA) and Europe (ESC). However, there are different recommendations for the use of BNP on the global scale, and harmonization between international societies is still in the discussion phase.

2) What the current guidelines show

a) Heart failure (Table VII). The JCS published two guidelines on HF, namely Guidelines for Treatment of Chronic Heart Failure (CHF) in 2005 and Guidelines for Treatment of Acute Heart Failure in 2006 by different committees and members. In the JCS Guidelines of CHF, BNP was classified as Class I

Table VII. Comparison Among Guidelines for Use of BNP in Management and Treatment of Heart Failure

	JCS 2005 (1)/2006 (2)	ACC/AHA 2009(3)	ESC 2008 (4)
To diagnose chronic HF	Class I (1)	Class IIa*	Class I
To diagnose acute HF	Not available (2)		
To adjust drug therapy	Class II (1)	Class IIb	Class II

*Class I in hospitalized patients.
(1) Guidelines for Treatment of Chronic Heart Failure (JCS 2005) (2) Guidelines for Treatment of Acute Heart Failure (JCS 2006)
(3) 2009 focused update incorporated into the ACC/AHA 2005 Guidelines for the Diagnosis and Management of Heart Failure in Adults
(4) ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008

in the diagnosis of CHF. However, the JCS Guidelines of Acute HF state that BNP measurements are not always necessary on the basis that the significance of BNP is unclear under this condition because BNP levels are higher than several hundred pg/mL in patients with acute HF. However, it was noted that BNP is useful for monitoring and in the diagnosis of patients with preserved systolic function (diastolic dysfunction).

- CLASS I (For diagnosis of CHF)

The efficacy of measurements of BNP in the diagnosis of CHF has been reported. Plasma concentrations of ANP and BNP correspond to hemodynamics. BNP reflects better end-diastolic pressure of LV than ANP, and therefore BNP is superior to ANP for the diagnosis of CHF, especially in the diagnosis of 1) the presence, 2) severity, and 3) prognosis of CHF.

- Not available (For diagnosis of acute HF)

BNP measurements are not always necessary. The significance of BNP is unclear because BNP levels are higher than several hundred pg/mL in patients with acute HF.

- CLASS II (For therapy of CHF)

Measurements of serum concentration of BNP are useful for predicting the efficacy of beta-blocker therapy. However, no agreement regarding the optimal value has been obtained.

In general, the ACC/AHA and ESC Guidelines recognize the usefulness of BNP in the diagnosis of HF regardless of being in chronic or acute phases. The ACC/AHA Guidelines describe the significance of BNP in the diagnosis of HF as Class IIa. Moreover, in the ACC/AHA Guidelines, BNP measurement was upgraded to Class I in hospitalized patients with HF in 2009. The ESC Guidelines state that plasma concentrations of natriuretic peptides are useful biomarkers in the diagnosis of HF (Class I).

b) STEMI. For BNP in STEMI, the JCS Guidelines (2008) state that BNP is a class IIa recommendation, and that BNP reflects infarction size and is a predictor of adverse cardiac events.

c) In ACS/NSTEMI. The JCS Guidelines (2008) do not have a recommendation for BNP although they state that higher levels of plasma BNP may indicate poor prognosis in ACS patients.

For pulmonary hypertension, the JCS Guidelines (2007) do not have a recommendation for BNP although they state that higher or increased levels of BNP may be associated with increased mortality rates in patients with PPH.

For perioperative cardiovascular evaluation and management for noncardiac surgery, the JCS Guidelines (2008) do not have a recommendation for BNP although they state that BNP may predict cardiac morbidity and mortality after major surgery.

For valvular heart disease, the JCS Guidelines (2008) do not have a recommendation for BNP although they state that BNP may predict symptom-free survival and postoperative outcome in severe aortic stenosis.

3) New data supporting stronger indications. The JCS Guidelines for Heart Failure (chronic in 2005 and acute in 2006) need to be updated.

4) Consensus statements. BNP is recognized to be beneficial for diagnosing and treating HF in addition to various heart conditions not only in the Japanese guidelines (JCS) but also those of the United States (ACC/AHA) and Europe (ESC). Harmonization and consensus between countries is an outstanding issue.

Role of BNP in acute coronary syndrome (diagnosis of the role of TnT, H-FABP and BNPs in acute myocardial infarction):

1) Cardiac biomarkers for acute coronary syndrome. At present, three groups of cardiac biomarkers are applied for detection of myocardial damage and the early diagnosis of ACS as shown in Figure 11. Earlier investigation revealed that measurements of cardiac troponin T detected the presence of minor myocardial damage in patients with unstable angina. CK and CKMB were not significantly elevated, and those with minor myocardial damage showed higher risk for cardiac events (acute myocardial infarction, cardiac death, or necessity of emergency coronary revascularization) in the acute phase compared with those without minor myocardial damage.[43,44) Thereafter, myocardial infarction was redefined based on clinical presentation, ECG findings and the elevation of cardiac troponins instead of conventional CK or CKMB measurements.[45)

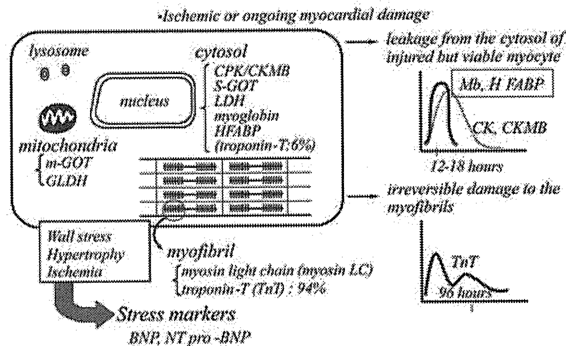

Figure 11. Detection of evolving myocardial damage difference in release kinetics of cardiac markers.

2) H-FABP rapid panel test for earlier diagnosis of myocardial infarction. Japanese investigators developed a whole blood rapid panel test for heart-type fatty acid-binding protein (H-FABP) and demonstrated its clinical utility for early diagnosis of myocardial infarction.[46) The diagnostic sensitivity and negative predictive value of the H-FABP test were superior compared with those in the rapid troponin T test.

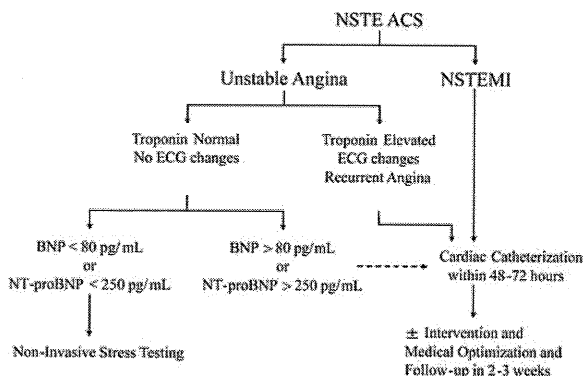

Figure 12. Proposed algorithm.

Risk factors:
- Age ≥ 65 years
- ≥ 3 risk factors for CAD
- Prior coronary stenosis ≥ 50%
- ST-segment deviation on ECG
- ≥ 2 anginal events in last 24 hours
- Use of ASA in last 7 days
- Elevated serum cardiac markers CK-MB or troponin

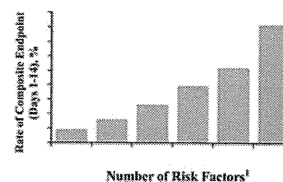

Figure 13. TIMI risk score.
Each risk factor is assigned 1 point, and the total represents a given patient's TIMI Risk Score[1].
Event rates (all-cause mortality, MI, or urgent revascularization) increase with each 1-point increase in score ($P < 0.001$ by chi square test for trend)[1].
[1] Antman EM, *et al.* JAMA. 2000; 284: 835-42.
Copyright© (2000) American Medical Association. All right reserved.

3) Profile of BNP elevation in ACS. In patients with acute STEMI, the magnitude and plasma profile of BNP elevation are associated with the size of the infarct area and subsequent LV dysfunction. Following the STEMI, plasma levels of BNP increase rapidly and peak after approximately 12 to 24 hours. Furthermore, differences in the profiles of NT-proBNP elevation between STE-ACS and NSTE-ACS were demonstrated in 165 consecutive patients admitted to the CCU.[47] Conventional myocardial necrosis markers, CKMB and TnT levels, on admission were significantly higher in the STE-ACS than those in NSTE-ACS. However conversely, NT-proBNP on admission was significantly higher in the NSTE-ACS compared with the STE-ACS especially in the earlier phase.[47] When the correlations between TnT and NT-proBNP were analyzed in STE-ACS and STE-ACS groups, the differences revealed augmented elevation of NT-proBNP in the NSTE-ACS patients as compared with prominent elevation of troponin T in the STE-ACS, indicating larger ischemic insult despite the smaller myocardial necrosis in NSTE-ACS as compared with STE-ACS.[48]
4) Timing of measurements. Data from the FRISK trial[48] showed that NT-proBNP levels are highest on admission, within 24 hours of onset, decreased markedly in the first 24 hours and then gradually over the following 6 months. Interestingly, the predictive ability of NT-proBNP appears to increase with time, suggesting that persistent elevation is a particularly strong marker of adverse outcome. In a substudy of PRISM,[49] the addition of a second NT-proBNP value at 72 hours following the admission appeared to improve risk prediction concerning the endpoint of death or recurrent MI at 30 days. Regardless of the NT-proBNP value on admission, an NT-proBNP value > 250 pg/mL (assumable BNP > 80 pg/mL) at 72 hours indicated a marked increased risk.[49]

These studies clearly demonstrate that both circulating BNP and NT-pro-BNP levels obtained in the acute phase or in the subacute phase are strongly associated with short-term and long-term cardiovascular mortality, independently of conventional risk factors, extent of myocardial necrosis and of coronary artery disease, HF, and LV dysfunction. Importantly, BNP and NT-proBNP identify patients without clinical signs of HF and with preserved LV function who are at high risk for death or HF events. It is meaningful that the association between BNPs and recurrent MI is generally weak, and in most studies nonexistent after adjustment for potential confounders. BNPs are closely associated with the incidence of HF, suggesting that the ability of BNPs to predict death in ACS is mainly explained by its ability to predict HF. BNPs provide complementary prognostic information to troponins; where troponins are superior to BNPs in predicting ischemic events.
5) Proposed algorithm. A proposed algorithm for measurement of BNPs in NSTE-ACS is shown in Figure 12. However, more clinical studies are necessary to evaluate the role of natriuretic peptides in the management of ACS.
High sensitivity troponin in acute care: Chest pain is common to a large number of presentations. The main concern is potential acute coronary syndrome (ACS). This possibility requires the clinician to make a determination as to the probability of its presence, and it is the sorting that represents the diagnostic challenge. One of the earliest available risk stratification tools is the ECG. Although obtained in minutes, and having excellent specificity, it is very insensitive. In fact Pope, *et al*[50] reported finding an ECG diagnosis of STEMI in only 2% of suspected ACS patients.

Thus, scoring systems were created to improve risk stratification. One of the most common scoring systems is the TIMI (thrombolysis in myocardial infarction) risk score.[51] This assigns points based on coronary artery disease risk factors, and 2 acute event markers (Figure 13). While high scores are associated with adverse outcomes, low scores do not predict safety. Furthermore, since TIMI scores do not diagnose an acute event, in the emergency department where disposition decisions are based on the presence or absence of an event, and not necessarily the underlying disease risk, scoring systems have limited utility for disposition decisions.

In current practice, serum cardiac markers are the dominant objective risk stratification tool. Contemporary troponin assays can identify the presence of myocardial necrosis within 4-6 hours of presentation. Unfortunately, in the time immediately after chest pain onset, troponin assays are commonly negative. This initial insensitivity is both a function of the underlying pathology (as it relates to the timing of troponin release from necrotic heart cells) as well the inability of many of our current troponin assays to detect very low levels of this protein. Newer troponin assays, with better lower level detection offer the promise of earlier diagnosis.

It is important to detect low levels of troponin, as even slight increases above the 99th percentile value confer high risk for short-term adverse events. Also if detectable, lower

Table VIII. If It Moves, It Is Bad

Marker	Comparator	OR for 30 day MACE	95% CI
↓ing Tn	versus stable Troponin	2.25	1.42-3.55
↑ing Tn		3.04	1.94-4.75
↓ing CKMB	versus stable CKMB	0.67	0.48-0.95
↑ing CKMB		0.96	0.57-1.60

Logistic regression models showing the odds ratios for predicting ACS. MACE indicates MI, revascularization (PCI or CABG), or positive testing (> 70% stenosis at catheterization, [+] MPI or non-invasive stress testing) within 30 days of index visit.

levels that change over time also suggest increased risk. In a study of 2,188 patients undergoing serial serum marker testing (Table VIII), very small changes in markers, never exceeding the manufacturer's recommended cutpoint, were associated with increased 30 day adverse outcomes.[52)]

A recent study of high sensitivity troponin[53)] suggests that they have improved overall diagnostic accuracy. In one study of 718 emergency department (ED) patients with suspected AMI, the performance of the hsTn far exceeded that of the current standard, with negative predictive values (NPV) in the high 90s (Figure 14). While not of sufficient exclusionary power for immediate discharge from the ED, its accuracy is a significant improvement over existing markers. A second analysis of 1,813 suspected MI patients[54)] found that a high sensitivity troponin had superior performance compared to current troponin assays, myoglobin, or CKMB (Figure 15).

Although the newer troponin offers improved accuracy, this will complicate the interpretation of results. Improvements in sensitivity are realized with concurrent decreases in specificity. Consequently, while a Tn > 99th percentile is associated with increased short-term adverse outcomes, it is not necessarily the case that the underlying pathology is MI resulting from epicardial coronary artery occlusion. Thus greater diagnostic acumen is required.

A multiple cutpoint strategy for marker interpretation may help solve this conflict. It has been suggested[55)] that patients with a troponin level between the upper limit of the reference interval and the decision limit for AMI should be labeled as having "myocardial injury." In this fashion all troponin elevations > 99th percentile would be considered in a high risk group, requiring additional therapy and evaluation to determine the etiology of their myocardial injury, while those with levels exceeding the MI cutpoint would receive immediate MI care.

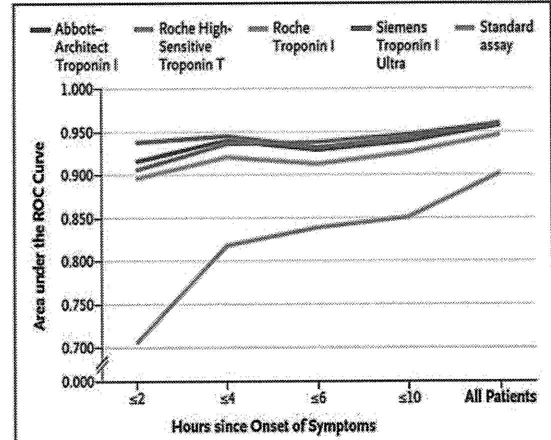

Figure 14. Diagnostic accuracy of cardiac troponin assays at presentation according to time since onset of chest pain.
Reichlin T. N Engl J Med 2009; 361: 858-67.
Copyright© 2009 Massachusetts Medical Society. All Right Reserved.

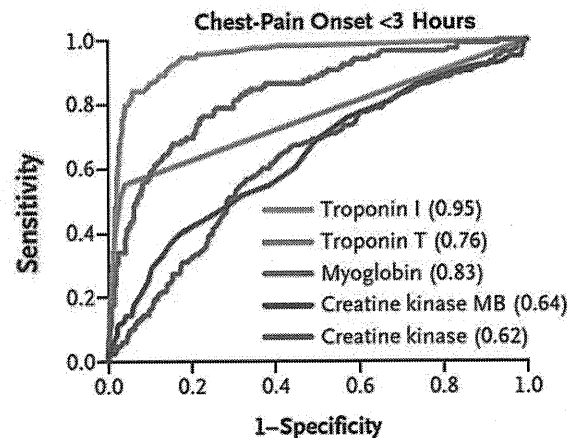

Figure 15. hsTn versus standard markers.
Keller T, *et al.* N Engl J Med 2009; 361: 868-77.
Copyright© 2009 Massachusetts Medical Society. All Right Reserved.

References

1. Fischer Y, Filzmaier K, Stiegler H, *et al.* Evaluation of a new, rapid bedside test for quantitative determination of B-type natriuretic peptide. Clin Chem 2001; 47: 591-4.
2. Nishikimi T, Maeda N, Matsuoka H. The role of natriuretic peptides in cardioprotection. Cardiovasc Res 2006; 69: 318-28. (Review)
3. Minamino N, Horio H, Nishikimi T. Natriuretic peptides in the cardiovascular system. In: The Handbook of Biologically Active Peptides. Ed. Kastin, Academic Press, 2006. P.1217-25.
4. Waldo SW, Beede J, Isakson S, *et al.* Pro-B-type natriuretic peptide levels in acute decompensated heart failure. J Am Coll Cardiol 2008; 51: 1874-82.
5. Seferian KR, Tamm NN, Semenov AG, *et al.* The brain natriuretic peptide (BNP) precursor is the major immunoreactive form of BNP in patients with heart failure. Clin Chem 2007; 53: 866-73.
6. Liang F, O'Rear J, Schellenberger U, *et al.* Evidence for functional heterogeneity of circulating B-type natriuretic peptide. J Am Coll Cardiol 2007; 49: 1071-8.
7. Schellenberger U, O'Rear J, Guzzetta A, Jue RA, Protter AA, Pollitt NS. The precursor to B-type natriuretic peptide is an O-linked

glycoprotein. Arch Biochem Biophys 2006; 451: 160-6.
8. Luckenbill KN, Christenson RH, Jaffe AS, *et al.* Cross-reactivity of BNP, NT-proBNP, and proBNP in commercial BNP and NT-proBNP assays: preliminary observations from the IFCC Committee for Standardization of Markers of Cardiac Damage. Clin Chem 2008; 54: 619-21.
9. Nishikimi T, Minamino N, Ikeda M, *et al.* Diversity of molecular forms of plasma brain natriuretic peptide in heart failure--different proBNP-108 to BNP-32 ratios in atrial and ventricular overload. Heart 2010; 96: 432-9.
10. Seferian KR, Tamm NN, Semenov AG, *et al.* Immunodetection of glycosylated NT-proBNP circulating in human blood. Clin Chem 2008; 54: 866-73.
11. Sawada Y, Suda M, Yokoyama H, *et al.* Stretch-induced hypertrophic growth of cardiocytes and processing of brain-type natriuretic peptide are controlled by proprotein-processing endoprotease furin. J Biol Chem 1997; 272: 20545-54.
12. Semenov AG, Postnikov AB, Tamm NN, *et al.* Processing of pro-brain natriuretic peptide is suppressed by O-glycosylation in the region close to the cleavage site. Clin Chem 2009; 55: 489-98.
13. Hammerer-Lercher A, Halfinger B, Sarg B, *et al.* Analysis of circulating forms of proBNP and NT-proBNP in patients with severe heart failure. Clin Chem 2008; 54: 858-65.
14. Giuliani I, Rieunier F, Larue C, *et al.* Assay for measurement of intact B-type natriuretic peptide prohormone in blood. Clin Chem 2006; 52: 1054-61.
15. Mukoyama M, Nakao K, Hosoda K, *et al.* Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. J Clin Invest 1991; 87: 1402-12.
16. Yoshimura M, Yasue H, Okumura K, *et al.* Different secretion patterns of atrial natriuretic peptide and brain natriuretic peptide in patients with congestive heart failure. Circulation 1993; 87: 464-9.
17. Yasue H, Yoshimura M, Sumida H, *et al.* Localization and mechanism of secretion of B-type natriuretic peptide in comparison with those of A-type natriuretic peptide in normal subjects and patients with heart failure. Circulation 1994; 90: 195-203.
18. Morita E, Yasue H, Yoshimura M, *et al.* Increased plasma levels of brain natriuretic peptide in patients with acute myocardial infarction. Circulation 1993; 88: 82-91.
19. Suzuki S, Yoshimura M, Nakayama M, *et al.* Plasma level of B-type natriuretic peptide as a prognostic marker after acute myocardial infarction: a long-term follow-up analysis. Circulation 2004; 110: 1387-91.
20. Wuerz RC, Meador SA. Effects of prehospital medications on mortality and length of stay in congestive heart failure. Ann Emerg Med 1992; 21: 669-74.
21. Maisel AS, Krishnaswamy P, Nowak RM, *et al.* Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. N Engl J Med 2002; 347: 161-7.
22. Peacock WF, Emerman C, Costanza MR, Diercks DB, Lopatin M, Fonarow GC. Early vasoactive drugs improve heart failure outcomes. Congest Heart Fail 2009; 15: 256-64.
23. Maisel AS, Peacock WF, McMullin N, *et al.* Timing of immunoreactive B-type natriuretic peptide levels and treatment delay in acute decompensated heart failure: an ADHERE (Acute Decompensated Heart Failure National Registry) analysis. J Am Coll Cardiol 2008; 52: 534-40.
24. Fonarow GC, Peacock WF, Phillips CO, Givertz MM, Lopatin M, ADHERE Scientific Advisory Committee and Investigators. Admission B-type natriuretic peptide levels and in-hospital mortality in acute decompensated heart failure. J Am Coll Cardiol 2007; 49: 1943-50.
25. Mueller C, Scholer A, Laule-Kilian K, *et al.* Use of B-type natriuretic peptide in the evaluation and management of acute dyspnea. N Engl J Med 2004; 350: 647-54.
26. Dokainish H, Zoghbi WA, Ambriz E, Lakkis NM, Quinones MA, Nagueh SF. Comparative cost-effectiveness of B-type natriuretic peptide and echocardiography for predicting outcome in patients with congestive heart failure. Am J Cardiol 2006; 97: 400-3.
27. Heidenreich PA, Gubens MA, Fonarow GC, Konstam MA, Stevenson LW, Shekelle PG. Cost-effectiveness of screening with B-type natriuretic peptide to identify patients with reduced left ventricular ejection fraction. J Am Coll Cardiol 2004; 43: 1019-26.
28. Saito Y, Nakao K, Itoh H, *et al.* Brain natriuretic peptide is a novel cardiac hormone. Biochem Biophys Res Commun 1989; 158: 360-8.
29. Mukoyama M, Nakao K, Hosoda K, *et al.* Brain natriuretic peptide as a novel cardiac hormone in humans. Evidence for an exquisite dual natriuretic peptide system, atrial natriuretic peptide and brain natriuretic peptide. J Clin Invest 1991; 87: 1402-12.
30. Nishii M, Inomata T, Takehana H, *et al.* Prognostic utility of B-type natriuretic peptide assessment in stable low-risk outpatients with nonischemic cardiomyopathy after decompensated heart failure. J Am Coll Cardiol 2008; 51: 2329-35.
31. Richards M and Troughton RW. NT-proBNP in heart failure: therapy decisions and monitoring. Eur J Heart Fail 2004; 6: 351-4. (Review)
32. Pfisterer M, Buser P, Rickli H, *et al.* BNP-guided vs symptom-guided heart failure therapy: the Trial of Intensified vs Standard Medical Therapy in Elderly Patients With Congestive Heart Failure (TIME-CHF) randomized trial. JAMA 2009; 301: 383-92.
33. Iwanaga Y, Nishi I, Furuichi S, *et al.* B-type natriuretic peptide strongly reflects diastolic wall stress in patients with chronic heart failure: comparison between systolic and diastolic heart failure. J Am Coll Cardiol 2006; 47: 742-8.
34. Nohria A, Givertz MM. B-type natriuretic peptide and the stressed heart. J Am Coll Cardiol 2006; 47: 749-51.
35. Troughton RW, Frampton CM, Yandle TG, Espiner EA, Nicholls MG, Richards AM. Treatment of heart failure guided by plasma aminoterminal brain natriuretic peptide (N-BNP) concentrations. Lancet 2000; 355: 1126-30.
36. Jourdain P, Jondeau G, Funck F, *et al.* Plasma brain natriuretic peptide-guided therapy to improve outcome in heart failure: the STARS-BNP Multicenter Study. J Am Coll Cardiol 2007; 49: 1733-9.
37. Lainchbury JG, Troughton RW, Frampton CM, *et al.* NTproBNP-guided drug treatment for chronic heart failure: design and methods in the "BATTLESCARRED" trial. Eur J Heart Fail 2006; 8: 532-8.
38. Benedict CR, Francis GS, Shelton B, *et al.* Effect of long-term enalapril therapy on neurohormones in patients with left ventricular dysfunction. Am J Cardiol 1995; 75: 1151-7.
39. Yoshimura M, Mizuno Y, Nakayama M, *et al.* B-type natriuretic peptide as a marker of the effects of enalapril in patients with heart failure. Am J Med 2002; 112: 716-20.
40. Richards AM, Doughty R, Nicholls MG, *et al.* Neurohumoral prediction of benefit from carvedilol in ischaemic left ventricular dysfunction. Circulation 1999; 99: 786-92.
41. Tsutamoto T, Wada A, Maeda K, *et al.* Effect of spironolactone on plasma brain natriuretic peptide and left ventricular remodeling in patients with congestive heart failure. J Am Coll Cardiol 2001; 37: 1228-33.
42. Anand IS, Fisher LD, Chiang YT, *et al.* Changes in brain natriuretic peptide and norepinephrine over time and mortality and morbidity in the Valsartan Heart Failure Trial (Val-HeFT). Circulation 2003; 107: 1278-83.
43. Seino Y, Tomita Y, Takano T, Hayakawa H. Early identification of cardiac events with serum troponin T in patients with unstable angina. Lancet 1993; 342: 1236-7.
44. Seino Y, Tomita Y, Hoshino K, Setsuta K, Takano T, Hayakawa H. Pathophysiological analysis of troponin T release kinetics in evolving ischemic myocardial injury. Jpn Circ J 1996; 60: 265-76.
45. Thygesen K, Alpert JS, White HD; Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction. Universal definition of myocardial infarction. Eur Heart J 2007; 28: 2525-38.
46. Seino Y, Ogata K, Takano T, *et al.* Use of a whole blood rapid panel test for heart-type fatty acid-binding protein in patients with acute chest pain: comparison with rapid troponin T and myoglobin

tests. Am J Med 2003; 115: 185-90.
47. Ogawa A, Seino Y, Yamashita T, Ogata K, Takano T. Difference in elevation of N-terminal pro-BNP and conventional cardiac markers between patients with ST elevation vs non-ST elevation acute coronary syndrome. Circ J 2006; 70: 1372-8.
48. Lindahl B, Lindbäck J, Jernberg T, *et al.* Serial analyses of N-terminal pro-B-type natriuretic peptide in patients with non-ST-segment elevation acute coronary syndromes: a Fragmin and fast Revascularization during In Stability in Coronary artery disease (FRISC)-II substudy. J Am Coll Cardiol 2005; 45: 533-41.
49. Heeschen C, Hamm CW, Mitrovic V, Lantelme NH, White HD; Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Investigators. N-terminal pro-B-type natriuretic peptide levels for dynamic risk stratification of patients with acute coronary syndromes. Circulation 2004; 110: 3206-12.
50. Pope JH, Aufderheide TP, Ruthazer R, *et al.* Missed diagnoses of acute cardiac ischemia in the emergency department. N Engl J Med 2000; 342: 1163-70.
51. Antman EM, Cohen M, Bernink PJ, *et al.* The TIMI risk score for unstable angina/non-ST elevation MI: A method for prognostication and therapeutic decision making. JAMA 2000; 284: 835-42.
52. McMullin N, Lindsell CJ, Lei L, *et al.* Outcomes associated with small changes in normal range cardiac markers. Am J Emerg Med 2011; 29: 162-7.
53. Reichlin T, Hochholzer W, Bassetti S, *et al.* Early diagnosis of myocardial infarction with sensitive cardiac troponin assays. N Engl J Med 2009; 361: 858-67.
54. Keller T, Zeller T, Peetz D, *et al.* Sensitive troponin I assay in early diagnosis of acute myocardial infarction. N Engl J Med 2009; 361: 868-77.
55. Wu Ah, Apple FS, Gibler WB, Jesse RL, Warshaw MM, Valdes R Jr. National Academy of Clinical Biochemistry Standards of Laboratory Practice: recommendations for the use of cardiac markers in coronary artery diseases. Clin Chem 1999; 45: 1104-21.

Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2011 | 118 | 34–44 doi: 10.1111/j.1471-4159.2011.07282.x

ORIGINAL ARTICLE

NMDA receptor regulates migration of newly generated neurons in the adult hippocampus via *Disrupted-In-Schizophrenia 1* (*DISC1*)

Takashi Namba,*,†,‡ Guo-li Ming,§,¶ Hongjun Song,§,¶ Chikako Waga,* Atsushi Enomoto,** Kozo Kaibuchi,†,‡ Shinichi Kohsaka* and Shigeo Uchino*

*Department of Neurochemistry, National Institute of Neuroscience, Tokyo, Japan
†Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Japan
‡CREST, Japan Science and Technology Agency, Kawaguchi, Japan
§Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD, USA
¶Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, USA
**Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Abstract
In the mammalian brain, new neurons are continuously generated throughout life in the dentate gyrus (DG) of the hippocampus. Previous studies have established that newborn neurons migrate a short distance to be integrated into a pre-existing neuronal circuit in the hippocampus. How the migration of newborn neurons is governed by extracellular signals, however, has not been fully understood. Here, we report that NMDA receptor (NMDA-R)-mediated signaling is essential for the proper migration and positioning of newborn neurons in the DG. An intraperitoneal injection of the NMDA-R antagonists, memantine, or 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) into adult male mice caused the aberrant positioning of newborn neurons, resulting in the overextension of their migration in the DG. Interestingly, we revealed that the administration of NMDA-R antagonists leads to a decrease in the expression of *Disrupted-In-Schizophrenia 1* (*DISC1*), a candidate susceptibility gene for major psychiatric disorders such as schizophrenia, which is also known as a critical regulator of neuronal migration in the DG. Furthermore, the overextended migration of newborn neurons induced by the NMDA-R antagonists was significantly rescued by exogenous expression of *DISC1*. Collectively, these results suggest that the NMDA-R signaling pathway governs the migration of newborn neurons via the regulation of *DISC1* expression in the DG.
Keywords: *DISC1*, hippocampus, migration, neurogenesis, NMDA receptor.
J. Neurochem. (2011) **118**, 34–44.

The generation of new neurons, known as neurogenesis, persists throughout life in the DG of the hippocampus (Seki and Arai 1993, 1995; Kuhn *et al.* 1996; Ming and Song 2005; Namba *et al.* 2005). Recent studies have shown that newly generated neurons are associated with synaptic plasticity and cognitive functions, such as learning and memory, as well as some psychiatric diseases, including depression and schizophrenia (Ming and Song 2005; Wojtowicz *et al.* 2008; DeCarolis and Eisch 2010).

Neurogenesis is a complex, multi-step process that involves progenitor cell proliferation, neuronal differentiation, migration, and the integration of newly generated neurons into pre-existing neuronal circuits; these steps are thought to be properly regulated by several factors. In the adult hippocampus, cumulative evidence has shown that

Received December 24, 2010; revised manuscript received April 12, 2011; accepted April 13, 2011.
Address correspondence and reprint requests to Dr Shinichi Kohsaka, Department of Neurochemistry, National Institute of Neuroscience, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. E-mail: kohsaka@ncnp.go.jp and Dr. Kozo Kaibuchi, Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, 65 Tsurumai, Showa, Nagoya 466-8550, Japan. E-mail: kaibuchi@med.nagoya-u.ac.jp

Abbreviations used: BrdU, 5-bromo-2-deoxyuridine; BSA, bovine serum albumin; B.W., body weight; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; DG, dentate gyrus; *DISC1*, *Disrupted-In-Schizophrenia 1*; GCL, granule cell layer; GFP, green fluorescent protein; i.p., intraperitoneally; LIS1, lissencephaly-1; MK-801, (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine hydrogen maleate; NDEL, NudE-like; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SDS, sodium dodecyl sulfate; SGZ, subgranular zone.

© 2011 The Authors
Journal of Neurochemistry © 2011 International Society for Neurochemistry,

neurotransmitters, including glutamate and GABA, affect neurogenesis. The administration of NMDA-R antagonists, such as (5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5-10-imine hydrogen maleate (MK-801), CGP37849, and memantine, stimulates progenitor cell proliferation at least 2 days after the drug administration, which may lead to subsequent neuronal production within the following 7–9 days (Cameron *et al.* 1995; Nacher *et al.* 2001; Nacher and McEwen 2006; Maekawa *et al.* 2009; Namba *et al.* 2009). Although the activation of NMDA-R or GABA-R signaling by a specific agonist accelerates neuronal differentiation of progenitor cells, the administration of NMDA-R or GABA-R antagonist inhibits *in vitro* or *in vivo* neuronal differentiation (Kitayama *et al.* 2004; Tozuka *et al.* 2005). In addition, the integration of newly generated neurons into adult hippocampal circuits is also regulated by NMDA-R signaling (Tashiro *et al.* 2006). Thus, the neurotransmitters have crucial modulatory effects on multiple steps of adult neurogenesis, but the effect of neurotransmitters on neuronal migration in the adult hippocampus remains to be addressed. Since the inhibition of NMDA-R or GABA-R signaling in the embryonic neocortex results in unusual neuronal migration (Behar *et al.* 1999; Hirasawa *et al.* 2003; Manent and Represa 2007; Denter *et al.* 2010; Uchino *et al.* 2010), neurotransmitters appear to serve as important extracellular signals for neuronal migration (Heng *et al.* 2007).

Recently, several lines of study have identified various types of intracellular signals involved in neuronal migration. For example, *DISC1* is thought to regulate neuronal migration in the postnatal hippocampus, since the knockdown of *DISC1* expression leads to an abnormal overextension of the migration of newly generated neurons (Duan *et al.* 2007; Kvajo *et al.* 2008; Enomoto *et al.* 2009; Kim *et al.* 2009). *DISC1* was originally identified as a candidate susceptibility gene for major psychiatric disorders based on studies of a chromosomal translocation found in a large Scottish family with a high frequency of schizophrenia, bipolar disorder, and major depression (Millar *et al.* 2000; Chubb *et al.* 2008) and is involved in cAMP signaling, neurite elongation, and cargo trafficking in addition to neuronal migration (Kamiya *et al.* 2005; Millar *et al.* 2005; Shinoda *et al.* 2007; Taya *et al.* 2007). Furthermore, DISC1 cooperates with its binding partners, including lissencephaly-1 (LIS1), NudE-like (NDEL), and Girdin, to regulate neuronal migration (Ozeki *et al.* 2003; Brandon *et al.* 2004; Taya *et al.* 2007; Enomoto *et al.* 2009; Kim *et al.* 2009). *LIS1* was initially identified as a responsible gene for type-1 lissencephaly (Reiner *et al.* 1993; Hattori *et al.* 1994), and its mutation leads to defects in neuronal migration during embryonic and postnatal neurogenesis (Hirotsune *et al.* 1998; Wang and Baraban 2007). NDEL1 was identified as a LIS1-binding protein (Niethammer *et al.* 2000; Sasaki *et al.* 2000). The NDEL1/LIS1/DISC1 complex regulates cytoplasmic dynein and controls proper neuronal migration in embryonic and adult brains (Kamiya *et al.* 2005; Duan *et al.* 2007). Girdin, an actin-binding protein, serves as an Akt substrate and stabilizes actin stress fibers to prevent cell migration (Enomoto *et al.* 2009). Recent studies have demonstrated that Girdin cooperates with DISC1 and regulates the migration of newly generated neurons in the postnatal hippocampus (Enomoto *et al.* 2009; Kim *et al.* 2009). Thus, DISC1 and its interacting protein complex play important roles in neuronal migration during hippocampal neurogenesis.

In this study, we found that the administration of an NMDA-R antagonist in adult mice leads to the overextension of the migration of newly generated neurons in the hippocampus and reduced the expression of *DISC1* in the DG. The overextended migration caused by the NMDA-R antagonist was partially rescued by the lentiviral-mediated exogenous expression of DISC1. These findings suggest that NMDA-R signaling regulates neuronal migration by controlling *DISC1* expression.

Materials and methods

The animals used in this study were 3-month-old male and embryonic day (E) 15.5 pregnant C57BL6/J mice (Clea Japan Inc., Tokyo, Japan). All experiments in the result section were performed in 3-month-old male mice. All experimental procedures were approved by The Animal Care and Use Committee of the National Institute of Neuroscience and Nagoya University.

Animals and drug administration

Mice were injected intraperitoneally (i.p.) with MK-801 [1 mg/kg body weight (B.W.); Wako, Osaka, Japan], CPP (10 mg/kg B.W.; Tocris Cookson, Bristol, UK), memantine (10, 30, or 50 mg/kg B.W.; Sigma, St. Louis, MO, USA), methamphetamine (2 mg/kg B.W.; Dainippon Pharmaceuticals Ltd, Osaka, Japan), or D-cycloserine (30mg/kg B.W.; Sigma). Control mice were injected i.p. with the same volume of 0.9% saline (Ohtsuka Pharmaceuticals, Tokyo, Japan). For the 5-bromo-2-deoxyuridine (BrdU; Sigma)-labeling experiment, the mice were injected i.p. with 75 mg/kg B.W. of BrdU from day 9 to day 7 before the memantine injection. The mice were then killed at 2 days after the memantine, MK-801, or CPP-injection.

Tissue preparation

After the mice were deeply anesthetized with sodium pentobarbital (Kyoritsu Pharmaceuticals, Tokyo, Japan), the mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). The brains were immersion-fixed at 4°C overnight in the same fixative. After washing in PBS, the brains were successively equilibrated in 10% and 20% sucrose in PBS. Finally, the brains were embedded in Tissue-Tek optimal cutting temperature compound (Sakura, Tokyo, Japan), and frozen in liquid nitrogen (Seki *et al.* 2007).

© 2011 The Authors

Immunohistochemistry

Immunohistochemistry was performed using a floating method as described previously (Namba *et al.* 2009). Briefly, the frozen brains were sliced into 40-μm sections using a cryostat (CM-3000; Leica, Nussloch, Germany). After washing in PBS, the sections were incubated at 4°C overnight with rabbit polyclonal anti-green fluorescent protein (GFP) antibody (1:200; Medical & Biological Laboratories, Nagoya, Japan), goat polyclonal anti-doublecortin (Dcx) antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Neuronal nuclei (NeuN) antibody (1:200; Millipore, Temecula, CA, USA), and/or rabbit polyclonal anti-NDEL1 antibody (1:200; Abcam, Cambridge, MA, USA) in PBS containing 1% bovine serum albumin (BSA), then incubated at room temperature (20–25°C) for 1–2 h with Alexa488-conjugated anti-rabbit IgG (1:400; Invitrogen, Carlsbad, CA, USA), Cy5-conjugated anti-mouse IgG (1:200; Jackson, West Grove, PA, USA) and/or Cy3 or Cy5-conjugated anti-goat IgG antibody (1:200; Jackson) in PBS containing 1% BSA. After washing in PBS, the sections were mounted on a glass slide and examined for fluorescent signals using a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan). For immunestaining with anti-BrdU antibody, the sections were incubated in 2N HCl at 37°C for 35 min, and then washed in PBS. The sections were then incubated with rat monoclonal anti-BrdU antibody (1:400; MorphoSys UK Ltd., Oxford, UK) at 4°C overnight in PBS containing 1% BSA. After washing in PBS, they were then incubated for 1–2 h in PBS containing 1% BSA plus Cy3-conjugated anti-rat IgG antibody (1:200; Jackson) between 20–25°C.

Reverse transcription-PCR

Total RNA extraction, cDNA synthesis, and real-time PCR analysis were performed as described previously (Namba *et al.* 2010) using RNeasy Plus Mini kit (QIAGEN, Germantown, ML, USA), Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA) and the SYBR green labeling system (SYBR Premix Ex Taq 2; Takara, Shiga, Japan), and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), respectively. The primers and sizes of the PCR products are listed in Table 1. The thermocycler conditions were as follows: 5 s at 95°C, 10 s at 55°C, and 30 s at 72°C for 40 cycles. The data were analyzed using the delta–delta C_t method with glyceraldehydes-3-phosphate dehydrogenase as an internal control (Kodomari *et al.* 2009; Namba *et al.* 2010).

Immunoblot analysis

Lysate was prepared from the dissected dentate gyri at 2 days after the injection of memantine or saline as described previously (Namba *et al.* 2010). Briefly, the dissected dentate gyri were homogenized in lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)]. After removing the nuclei and debris by centrifugation (2000 *g*, 4°C, 10 min), the protein concentration of the supernatant was determined using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA).

The proteins were subjected to immunoblotting with rabbit polyclonal anti-pan Ca^{2+}/calmodulin-dependent kinase II (CaMKII) antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA) or rabbit polyclonal anti-phospho CaMKII antibody (1:1000; Cell Signaling Technology). Blots were visualized using a chemiluminescence detection system (ECL-prime; Amersham Biosciences, Piscataway, NJ, USA). The membrane was re-probed with anti-β actin antibody (1:1000; Sigma) after incubation with Stripping buffer [62.5 mM Tris-HCl (pH 7.5), 2% SDS, 100 mM 2-mercaptoethanol] at 60°C for 30 min.

Infection of lentivirus into the dentate gyrus

Lentivirus carrying GFP-tagged mouse *DISC1* cDNA (pFUW-GFP-mDISC1) (Duan *et al.* 2007) was prepared using the ViraPower Lentiviral expression system (Invitrogen) according to the manufacturer's instructions. The lentivirus-GFP-*DISC1* (1 μL/injection) was stereotaxically injected into the dentate gyrus (anteroposterior, 2.5 mm; lateral, 2.0 mm; ventral, 3.0 mm from bregma, respectively) as described previously (Seki *et al.* 2007; Namba *et al.* 2010).

Statistical analysis

To analyze the localization of Dcx+ or BrdU+ cells, more than 100 cells from two to five sections per mouse were analyzed. To avoid double counting, adjacent sections were not used for the cell counting. All of the counting was performed under the confocal laser-scanning microscope (FV1000) with 60× objective. Data were evaluated using a one-way ANOVA followed by a *post hoc* Scheffe F-test. All the values were expressed as the mean ± SEM, and p-values < 0.05 were considered significant.

Results

Administration of NMDA-R antagonist causes the overextended migration of newly generated neurons in the adult hippocampus

In the adult hippocampus, neuronal progenitor cells arising from the subgranular zone (SGZ) migrate into the granule

Table 1 Primer sequences for PCR

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplification size (bp)	Accession Nos
DISC1-1	CAGTGGGTTTTGGCAAGAAT	CCAAGGGAGAGTTGGATGAA	210	NM_174854.2
DISC1-2	GAGCTCACGGAGGAGATTTG	ACCTTCCAACACTTCCATGC	216	NM_174854.2
GAPDH	GTCATCATCTCCGCCCCTTCTGC	GATGCCTGCTTCACCACCTTCTTG	443	NM_008084
Girdin	GTGTCATTGCAGGAGCAGAA	GACTTCAGGGTGCCATGTTT	287	NM_176841.3
Lis1	GATGACAAGACCCTCCGTGT	GAGCTCAAATGGGGTAACCA	240	NM_013625.3
NDEL1	GGACTCTGCGCGATATCAAT	TTCCACATCCAGTGATCGAG	126	NM_023668.2

© 2011 The Authors

cell layer (GCL) and differentiate in mature neurons. Since most newly generated neurons express Dcx (a marker for immature neuron), Dcx+ cells are mainly located in the SGZ and inner part of the GCL. To investigate the effect of NMDA-R antagonist on the migration of newly generated neurons, we first analyzed the localization of Dcx+ immature neurons. Three-month-old male mice were i.p. injected with saline as a control or three types of NMDA-R antagonist: uncompetitive (memantine, 50 mg/kg B.W.), non-competitive (MK-801, 1 mg/kg B.W.), or competitive (CPP, 10 mg/kg B.W.). Two days later, their brains were fixed with 4% PFA for immunohistochemistry using anti-Dcx antibody (Fig. 1a–e). To perform the statistical analysis, we divided the GCL into three parts, that is, the outer third of the GCL, the middle third of the GCL, and the inner third of the GCL and the SGZ, and then counted the Dcx+ cells localized in each layer. In the control mice, most of the Dcx+ cells were located in the inner third of the GCL (97.0 ± 0.6%, n = 3), whereas a few Dcx+ cells were found in the middle and outer third of the GCL (outer, 0.0 ± 0.0%; middle, 3.0 ± 0.6%; n = 3; Fig. 1b and f). In contrast, the percentages of the Dcx+ cells in the outer and middle thirds of the GCL were significantly higher in the memantine-injected mice (outer, 3.7 ± 0.3%; middle, 12.3 ± 0.3%; n = 3). Conversely, the percentage of Dcx+ cells in the SGZ and inner third of the GCL was significantly reduced (84.0 ± 1.8%, n = 3; Fig. 1c and f). Similar results were obtained from the mice injected with the other antagonists, CPP and MK-801 (CPP: outer, 1.7 ± 0.3%; middle, 11.7 ± 2.2%; inner, 86.7 ± 1.9%; MK-801: outer, 1.3 ± 0.3%; middle, 8.7 ± 1.8%; inner, 90.0 ± 1.7%; n = 3; Fig. 1d–f). On the other hand, the total number of Dcx+ cells was approximately equal among the control and drug-injected mice (Figure S1).

To investigate the dose-dependent effect of NMDA-R antagonist on the localization of the Dcx+ cells, we used memantine because it showed most prominent effect on neuronal positioning (Fig. 1f). Mice were injected with 10, 30, or 50 mg/kg B.W. of memantine and were killed 2 days after the injection (Fig. 1g). At the 10 mg/kg B.W. dose, no significant differences in the localization of the Dcx+ cells, compared with the control mice, were observed (outer, 0.3 ± 0.3%; middle, 5.0 ± 0.6%; inner, 94.7 ± 0.7%; n = 3).

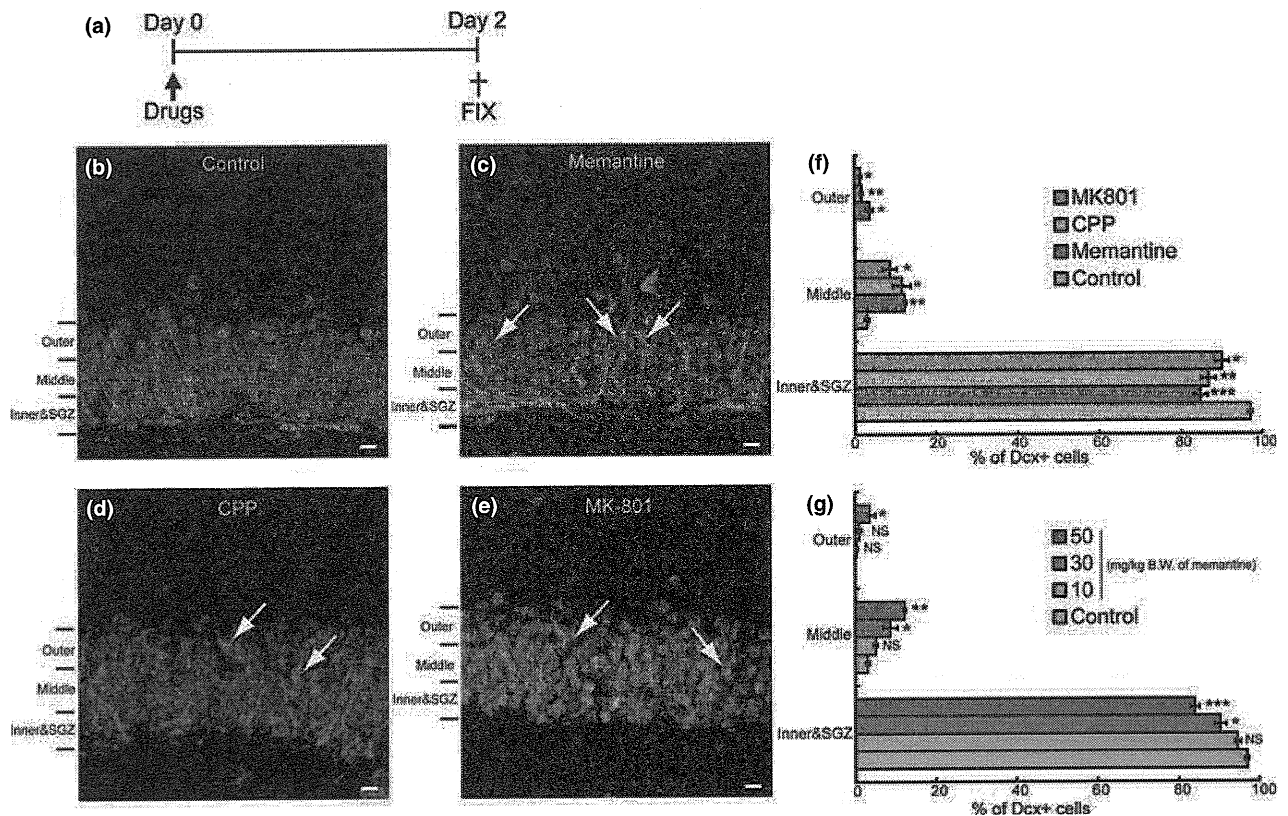

Fig. 1 Administration of NMDA-R antagonist results in the aberrant positioning of Dcx+ immature neurons. (a) Schematic illustration of the experimental design. (b–e) Representative immunohistochemical images of the Dcx+ cells (red) and the NeuN+ cells (white) in control mice (b) and mice injected with memantine (c), CPP (d), or MK-801 (e). (f) Quantitative analysis of the location of the Dcx+ cells in the GCL. (g) Dose-dependent effect of memantine on the positioning of the Dcx+ cells. *p < 0.05, **p < 0.01, ***p < 0.001, NS, not significant. Scale bars = 10 μm.

© 2011 The Authors

In contrast, at dose of 30 and 50 mg/kg B.W., the percentage of Dcx+ cells in the middle third of the GCL increased significantly, whereas those in the SGZ and the inner third of the GCL decreased significantly (30 mg/kg: outer, 1.0 ± 0.6%; middle, 8.7 ± 1.9%; inner, 90.3 ± 1.5%; $n = 3$; 50 mg/kg: outer, 3.7 ± 0.3%; middle, 12.3 ± 0.3%; inner, 84.0 ± 1.8%; $n = 3$), indicating that the effect of memantine on the localization of the Dcx+ cells was in a dose-dependent manner.

Since previous studies have shown that some NMDA-R antagonists stimulate dopamine release and act as a dopamine receptor agonist (Imperato *et al.* 1990; Spanagel *et al.* 1994), we examined the involvement of dopaminergic signal in cellular migration. Here, we used methamphetamine as a dopamine receptor agonist at a low dose (2 mg/kg B.W.; Adachi *et al.* 2001; Shoblock *et al.* 2003), because the high dose of methamphetamine (e.g. more than 20 mg/kg B.W.) induced neurotoxicity (Fukumura *et al.* 1998). Mice were injected with methamphetamine (2 mg/kg B.W.) and fixed 2 days after the injection. In contrast to the NMDA-R antagonist, methamphetamine did not affect the localization of the Dcx+ cells (Fig. 2), suggesting that a dopaminergic signal was not involved in the migration of newly generated neurons in the adult hippocampus.

Previous evidence demonstrated that the aberrant activation of NMDA-R elicits epileptic seizure in rats (Mares and Velisek 1992). We therefore examined the effect of D-cycloserine, an NMDA-R agonist (Hood *et al.* 1989), on the localization of the Dcx+ cells. Mice were injected with D-cycloserine (30 mg/kg B.W.) (Yang *et al.* 2010) and their brains were fixed 2 days later. D-Cycloserine did not affect the localization of the Dcx+ cells (Fig. 3) suggesting that activation of NMDA-R did not affect the migration of newly generated neurons in the adult hippocampus.

To examine whether the aberrant localization of the Dcx+ cells was caused by the overextended migration of newly generated cells, we next performed a BrdU pulse-labeling experiment. The mice were injected with BrdU from day 9 to day 7 before the memantine-injection; 2 days later, their brains were fixed with 4% PFA for immunohistochemistry with anti-BrdU antibody (Fig. 4a–c). In the memantine-injected mice, the percentages of BrdU+ cells in the outer and middle thirds of the GCL increased significantly, whereas those in the SGZ and the inner third of the GCL decreased significantly ($n = 3$; Fig. 4b–d). We further examined the effect of memantine on neuronal differentiation and maturation using an anti-NeuN antibody (a mature neuron marker) and an anti-Dcx antibody (an immature neuron marker). The administration of NMDA-R antagonist did not affect the rate of neuronal differentiation and maturation ($n = 3$; Fig. 5) suggesting that the aberrant localization of the BrdU+ cells was not caused by the abnormal neuronal differentiation and maturation of newly generated cells. These results show that the administration of an NMDA-R antagonist causes the overextended migration of newly generated neurons in the adult hippocampus.

Administration of NMDA-R antagonist reduces the expression of *DISC1* mRNA in the adult dentate gyrus

Since recent studies have shown that DISC1 plays an important role in the proper migration of newly generated neurons in the adult hippocampus (Duan *et al.* 2007), we next examined the expression of *DISC1* in drug-injected mice using a quantitative RT-PCR method. Three-month-old

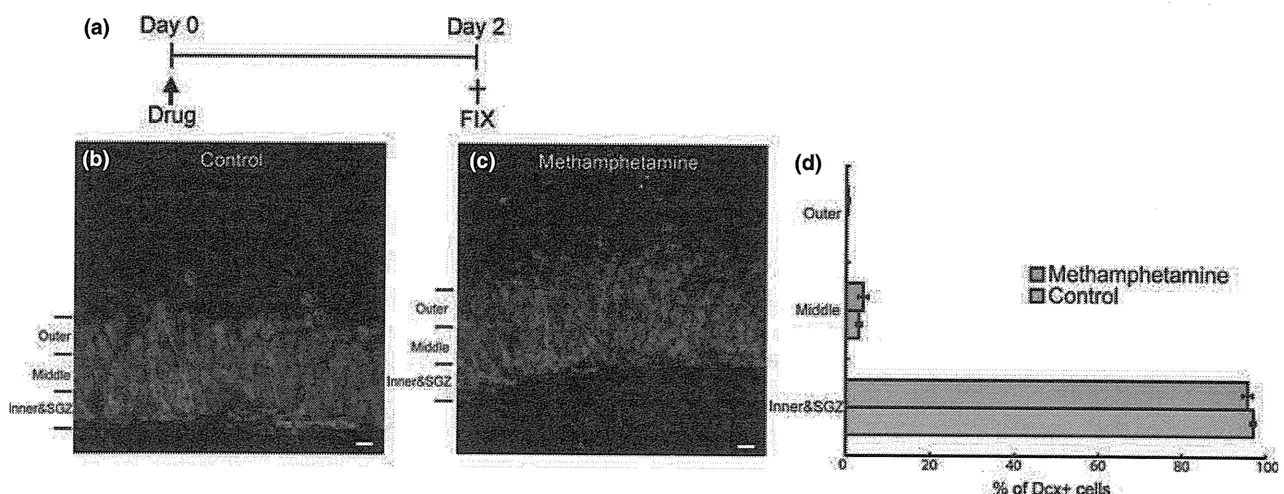

Fig. 2 Administration of methamphetamine does not affect the positioning of Dcx+ immature neurons. (a) Schematic illustration of the experimental design. (b and c) Representative immunohistochemical images of the Dcx+ cells (red) and the NeuN+ cells (white) in the control (b) or methamphetamine-injected mice (c). (d) Quantitative analysis of the location of the BrdU+ cells in the GCL. $n = 3$. Scale bars = 10 μm.

© 2011 The Authors

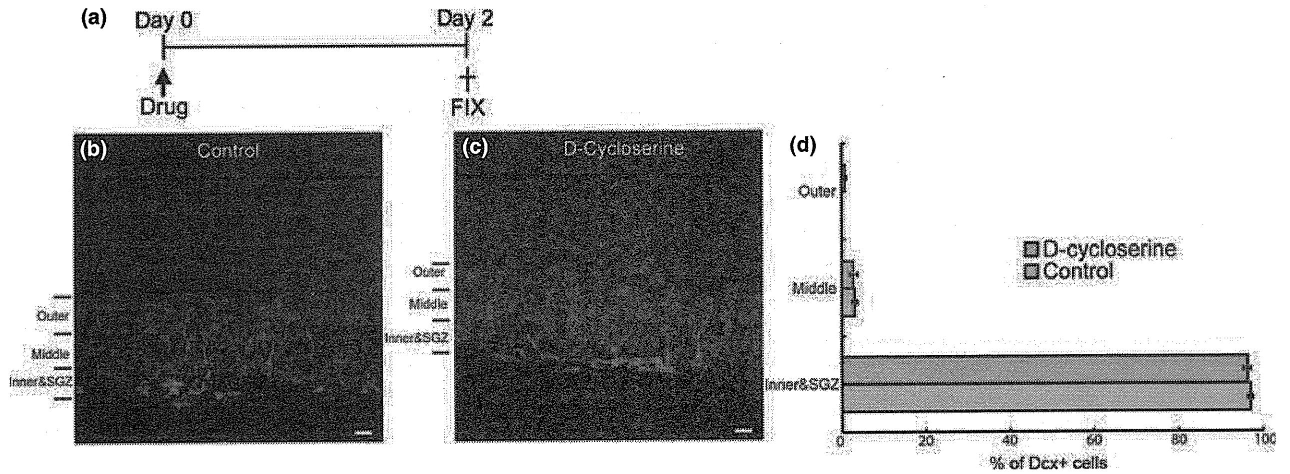

Fig. 3 Administration of D-cycloserine does not affect the positioning of Dcx+ immature neurons. (a) Schematic illustration of the experimental design. (b and c) Representative immunohistochemical images of the Dcx+ cells (red) and the NeuN+ cells (white) in the control (b) or methamphetamine-injected mice (c). (d) Quantitative analysis of the location of the BrdU+ cells in the GCL. $n = 3$. Scale bars = 10 μm.

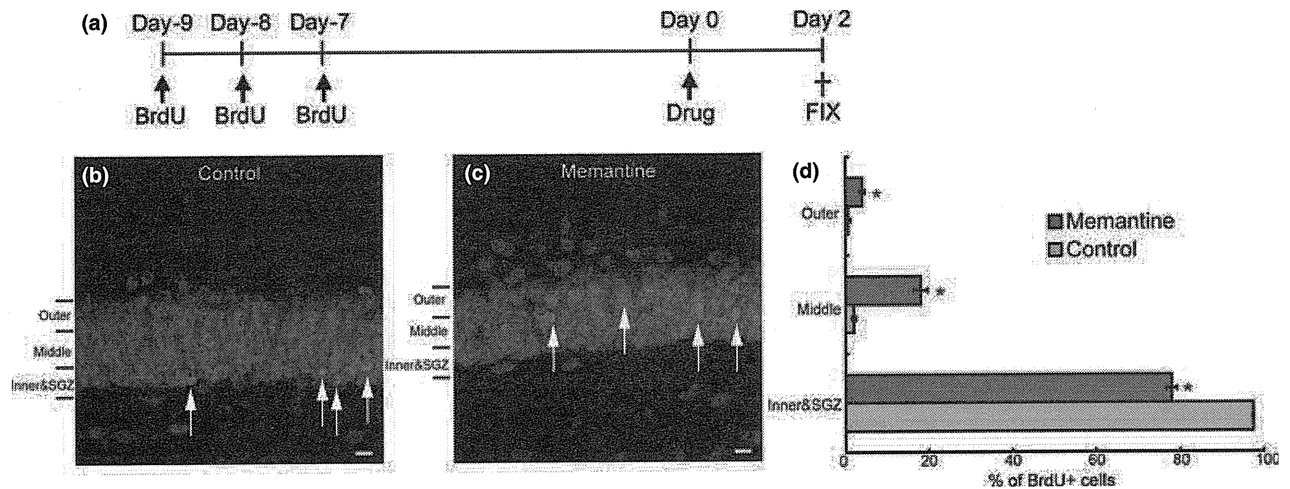

Fig. 4 Administration of NMDA-R antagonist causes overextended migration of newly generated neurons. (a) Schematic illustration of the experimental design. (b and c) Representative immunostaining images of the BrdU+ cells (red) and the NeuN+ cells (white) in the control (b) or memantine-injected mice (c). Arrows indicate the BrdU+ cells. Scale bars = 10 μm. (d) Quantitative analysis of the location of the BrdU+ cells in the GCL. $*p < 0.05$.

male mice were injected with saline as a control, memantine (50 mg/kg B.W.), CPP (10 mg/kg B.W.), methamphetamine (2 mg/kg B.W.), or D-cycloserine (30 mg/kg B.W.); 1 day later, the total RNA was prepared from the DG. So far, two mouse *DISC1* mRNA isoforms have been identified: one isoform consists of 13 exons and the other lacks exon 9. We then designed two specific primer sets for RT-PCR. One primer set (*DISC1-1*) was used for the amplification of exon 2 and the other (*DISC1-2*) was used for the amplification of exons 9–10. A significant reduction in *DISC1* mRNA expression was observed in the memantine-injected and CPP-injected mice but not in the methamphetamine-injected mice and D-cycloserine-injected mice using both primers sets ($n = 3$; Fig. 6a and b). We further examined the dose-dependent effect of memantine on *DISC1* mRNA expression, and found that *DISC1* mRNA expression was reduced in a dose-dependent manner ($n = 3$; Fig. 6c). These results indicate that the administration of the NMDA-R antagonist reduced *DISC1* mRNA expression in the adult DG.

Since previous studies have shown that NDEL1, Girdin, and LIS1, which are molecules that interact with DISC1, are also important for the migration of hippocampal granule cells (Duan *et al.* 2007; Wang and Baraban 2007; Enomoto *et al.* 2009; Kim *et al.* 2009), we then investigated the expression of *NDEL1*, *Girdin*, and *LIS1* mRNAs in memantine (50 mg/kg B.W.)-injected mice using a quantitative RT-PCR method.

© 2011 The Authors

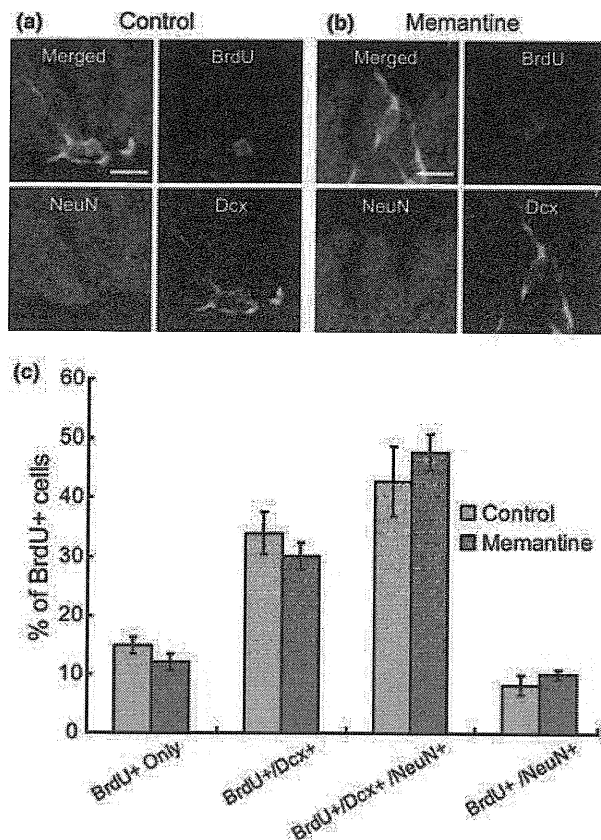

Fig. 5 Administration of NMDA-R antagonist does not affect the rate of neuronal differentiation and maturation. (a and b) Representative immunostaining images of BrdU+ cells (red), NeuN+ cells (white), and Dcx+ cells (blue) in the control (a) or memantine-injected mice (b). Scale bars = 10 μm. (c) Quantitative analysis of the percentage of BrdU+ cells, BrdU+/Dcx+ cells, BrdU+/Dcx+/NeuN+ cells, and BrdU+/NeuN+ cells.

The result showed that the expression levels of each of these three genes were almost comparable between control and memantine-injected mice ($n = 3$; Fig. 6d) suggesting that NMDA-R-mediated regulation of neuronal migration does not involve the transcription of these genes.

Exogenous expression of *DISC1* partially rescued the overextended migration of Dcx+ cells caused by the administration of NMDA-R antagonist

To investigate whether the overextended migration of newly generated neurons in NMDA-R antagonist-injected mice is attributable to the reduction in *DISC1* expression, we next performed *in vivo* rescue experiments using DISC1-expressing lentivirus. We injected lentivirus that encodes for DISC1 fused with GFP (GFP-DISC1) into the DG of 3-month-old mice at 7 days following saline or memantine (50 mg/kg B.W.) injection and prepared brain sections 2 days after the drug injection (Fig. 7a). In the saline-injected mice, no differences in the localization of the Dcx+ cells were seen between the lentivirus-infected (GFP+) (Fig. 7b and c) and non-infected (GFP-) cells (Fig. 7b and d) suggesting that exogenous expression of GFP-DISC1 itself had no effect on the migration of the newly generated cells, consistent with the previous study (Duan *et al.* 2007). In addition, the exogenous expression of GFP-DISC1 appeared not to affect the expression of NDEL1 (Figure S2). In the memantine-injected mice, the aberrant positioning of Dcx+ cells was partially rescued by the exogenous expression of GFP-DISC1 (Fig. 7e and f), whereas the non-infected (GFP-) cells remained in the outer or middle third of the GCL (Fig. 7e and g). The statistical analysis indicated that the overextended migration of the Dcx+ cells caused by memantine administration was partially rescued by the lentivirus-mediated expression of DISC1 (Fig. 7h). These findings suggest that DISC1 is involved in the neuronal migration regulated by NMDA-R signaling.

Discussion

NMDA-R signaling plays important roles not only in excitatory neuronal transmission but also in neurogenesis (Nacher and McEwen 2006; Heng *et al.* 2007). Cumulative studies have demonstrated that treatment with an NMDA-R antagonist enhances the proliferation of progenitor cells and increases the number of newly generated neurons in the adult hippocampus (Cameron *et al.* 1995; Nacher *et al.* 2001; Maekawa *et al.* 2009; Namba *et al.* 2009). In addition, the integration and the survival of newly generated neurons are also regulated by NMDA-R activity (Tashiro *et al.* 2006). However, the involvement of NMDA-R signaling in neuronal migration, which is an important step in adult hippocampal neurogenesis, remains unclear. In this study, we showed that the inhibition of NMDA-R activity using specific antagonists resulted in the aberrant positioning of Dcx-positive immature neurons in the adult hippocampus. A BrdU-labeling experiment suggested that this aberrant positioning was caused by the overextended migration of newly generated neurons. These findings suggest that NMDA-R signaling is involved in neuronal migration in addition to its role in the proliferation of progenitor cells and the integration and survival of newly generated neurons in the adult hippocampus.

DISC1 has been largely studied as a key regulator of adult neurogenesis. The expression of *DISC1* in the adult brain is restricted to two types of neurons: hippocampal dentate granule neurons and interneurons of the olfactory bulb (Austin *et al.* 2004). These neurons are continuously produced throughout life. A recent study demonstrated that the down-regulation of *DISC1* using the retrovirus-mediated expression of DISC1-small hairpin RNA in the adult hippocampus led to accelerated neuronal integration, resulting in aberrant morphological development and the mis-positioning of newly generated granule neurons (Duan *et al.*

© 2011 The Authors

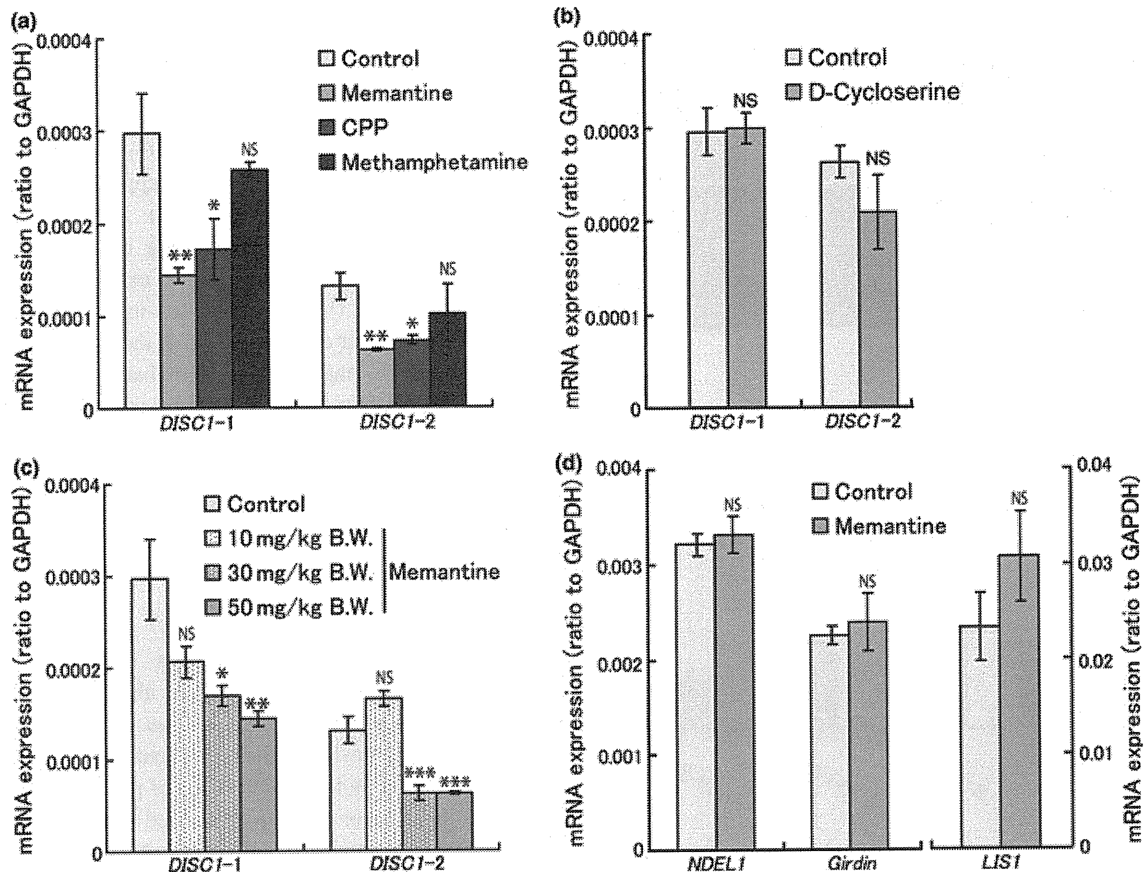

Fig. 6 Administration of NMDA-R antagonists reduces the *DISC1* mRNA expression but does not affect *NDEL1*, *Girdin*, and *LIS1* mRNA expressions in the adult dentate gyrus. (a) Quantitative analysis of the *DISC1* mRNA expression in control or memantine-, CPP-, or methamphetamine-injected mice. (b) Quantitative analysis of the *DISC1* mRNA expression in control or D-cycloserine-injected mice. (c) Dose-dependent effect of memantine on *DISC1* mRNA expression. (d) Quantitative analysis of *NDEL1*, *Girdin*, and *LIS1* mRNA expressions in control or memantine-injected mice. The ratio of mRNA expression was evaluated using GAPDH as an internal control and real-time PCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS, not significant.

2007). Since the overextended migration in memantine-treated mice was very similar to the mispositioning caused by the down-regulation of *DISC1*, we examined the expression of *DISC1* in memantine-treated mice and found a reduction in *DISC1* expression. This finding suggests that DISC1 is critical for the positioning of newly generated neurons and that its expression is regulated by NMDA-R signaling in the adult hippocampus.

The involvement of NMDA-R signaling in neurogenesis during embryonic development has also been studied. Recent studies have demonstrated that NMDA-R antagonists, including D(-)-2-amino-5-phosphonopentanoic acid (APV), MK-801, Cerestat, and memantine, increase the proliferation of neuronal stem/progenitor cells (Hirasawa *et al.* 2003; Jin *et al.* 2006; Uchino *et al.* 2010). Furthermore, treatment with an NMDA-R antagonist caused the abnormal migration of pyramidal neuron in the embryonic neocortex (Behar *et al.* 1999; Hirasawa *et al.* 2003; Uchino *et al.* 2010). Our recent study showed that the inhibition of NMDA-R prevented changes from a multipolar to bipolar morphology in migrating immature neurons and delayed neurite extension in the direction of the leading process and axon-like processes (Uchino *et al.* 2010). Interestingly, Kamiya *et al.* (2005) recently reported that small hairpin RNA-mediated suppression of DISC1 or the expression of its dominant-negative mutation in E14.5 embryos caused retarded migration and morphological defects, including neurite outgrowth in the developing neocortex. DISC1 is broadly expressed in various brain regions, including the ventricular zone during embryonic development (Austin *et al.* 2004). We further observed that prenatal treatment with an NMDA-R antagonist reduced the expression of *DISC1* in the developing neocortex (Figure S3). These results indicate that the expression of DISC1, which is associated with neuronal migration and morphological maturation including neurite outgrowth, is regulated by NMDA-R signaling not only in the adult hippocampus, but also during neocortical development.

© 2011 The Authors

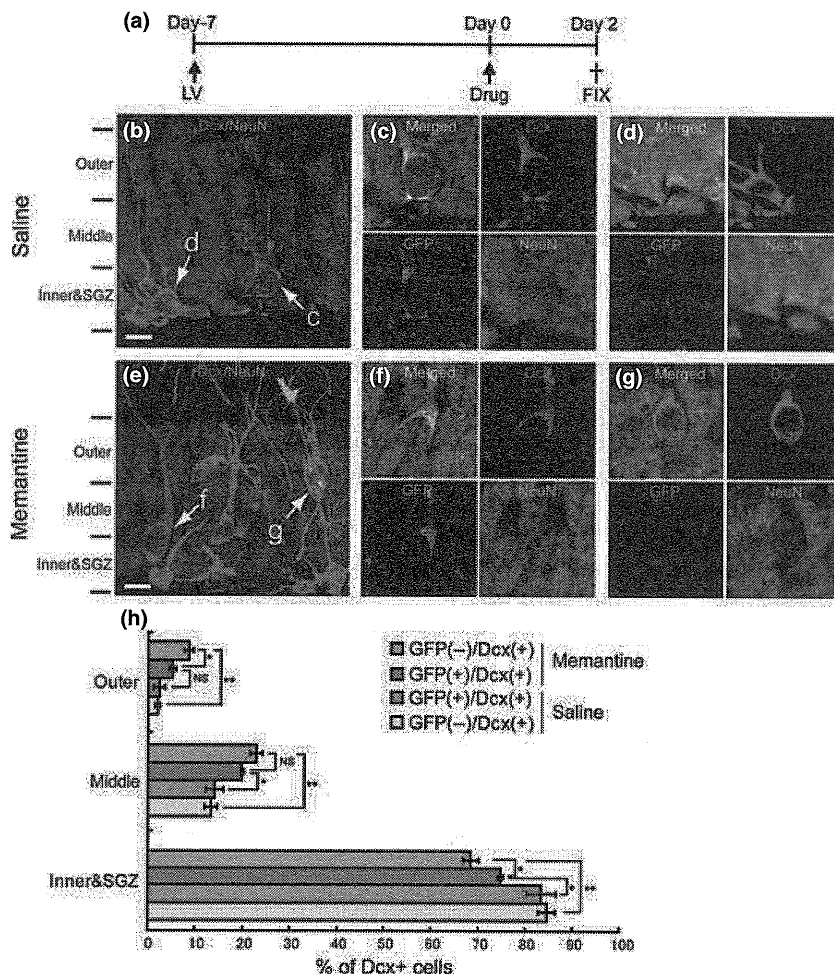

Fig. 7 The aberrant positioning of Dcx+ cell caused by memantine is partially rescued by lentiviral-mediated DISC1 expression. (a) Schematic illustration of the experimental design. (b–g) Representative immunostaining images of the Dcx+ cells (red), the GFP+ cells (green), and the NeuN+ cells (blue) in the control (b–d) or memantine-injected mice (e–g). Scale bars = 10 μm. (h) Quantitative analysis of the location of the infected or non-infected Dcx+ cells in the GCL. *$p < 0.05$, **$p < 0.01$. NS, not significant.

The present results provide the new notion that DISC1 acts downstream of NMDA-R signaling. However, the aberrant positioning caused by NMDA-R antagonist was partially, but not completely, rescued by the lentiviral-mediated expression of *DISC1*. Considering that recent studies have shown that NMDA-R antagonist treatment caused changes in expression and activity of various molecules (O'Donnell *et al.* 2003; Paulson *et al.* 2003; Namba *et al.* 2010; Uchino *et al.* 2010), this result raises the possibility that other molecules associated with NMDA-R signaling may be involved in neuronal migration. One intriguing molecule is CaMKII, which is a major downstream mediator of NMDA-R signaling. Interestingly, in the adult DG of mice heterozygous for CaMKII deficiency, an increased number of immature neurons and their aberrant positioning were observed (Yamasaki *et al.* 2008). In this study, we examined the expression of CaMKII and its autophosphorylation in memantine-treated mice, which revealed no prominent changes in both the expression and autophosphorylation of CaMKII compared with control mice (Figure S4). However, the data do not necessarily exclude the contribution of CaMKII signaling in transducing the effects of memantine administration, since it is known that the modification of NMDA-R signaling leads to the changes of CaMKII interaction with other proteins without the alteration of its expression levels (Dhavan *et al.* 2002). Further studies are needed to determine how NMDA-R signaling regulates neuronal migration and DISC1 expression.

In summary, we found that the inhibition of NMDA-R signaling causes the overextended migration of newly generated neurons in the adult hippocampus, indicating that NMDA-R regulates the migration of newly generated neurons in addition to the proliferation of progenitor cells, and the survival and integration of newly generated neurons. Taken together with the role of NMDA-R in excitatory neuronal transmission, these findings suggest that NMDA-R plays an important role in the formation and maintenance of hippocampal neuronal network followed by neurogenesis.

Acknowledgements

We thank Dr Kotaro Hattori for technical help and useful discussion. This work was supported by The Ministry of Health, Labour and

© 2011 The Authors