postoperatively.[8] Metabolism and microscopic anatomic architecture in the cerebral cortex and the cerebral white matter may also change along with these postoperative improvements in cognitive function, but the relationship among these factors remains unclear.

Recently, diffusion tensor magnetic resonance imaging (DTI) has been used to quantitatively evaluate alterations in the white matter in patients with various conditions, including mild traumatic brain injury, brain tumors, idiopathic normal pressure hydrocephalus, and Alzheimer's disease.[9-12] Among the parameters derived from DTI, diffusion anisotropy is most commonly quantified by using fractional anisotropy (FA) values.[13] FA values correlate with cognitive function in patients with cerebral small vessel disease, chronic traumatic brain injury, and Alzheimer disease.[14-17]

There are various methods of analyzing FA data, such as region-of-interest analysis, tract-specific analysis, and voxelwise statistical analysis. Although region-of-interest analysis and tract-specific analysis are simple methods that can be used to directly derive values for structures that are anatomically the same among individuals, these methods are limited in that they are sensitive only to changes in those few parts of the brain that they can accurately measure.[11] In contrast, voxelwise statistical analysis does not require prespecification or prelocalization of regions of interest, and it can be used to automatically perform statistical analysis of the whole brain by voxelwise comparisons between 2 groups.[11] Tract-based spatial statistics (TBSS) is a recently developed voxelwise statistical method that has a specific registration algorithm for FA maps and can thereby minimize misregistration.[18] Further, whole-brain analysis using TBSS may be useful for the characterization of white matter changes that occur in patients undergoing CEA, because the hemodynamics in the whole cerebral hemisphere ipsilateral to surgery often changes postoperatively in such patients.[8]

The purpose of the present study was to use TBSS to determine whether postoperative changes of FA values in the cerebral white matter derived from DTI are associated with cognitive improvement after uncomplicated CEA.

## PATIENTS AND METHODS

### Patient Selection

Patients were prospectively enrolled in this study if they were intended to undergo CEA and if they satisfied the following inclusion criteria: age ≤75 years; symptomatic or asymptomatic ipsilateral cervical internal carotid artery (ICA) stenosis (≥70%) on angiography study via magnetic resonance (MR), computed tomography, or arterial catheterization according to the method of the North American Symptomatic Carotid Endarterectomy Trial[1,19]; preoperative useful residual function (modified Rankin disability scale 0 or 1); no infarction in the cerebral cortical area perfused by one or more branches of the middle cerebral artery as confirmed by MR imaging including T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences that was performed 2 weeks before surgery; and provision of written informed consent. Patients with the following postoperative criteria were excluded from the study: presence of episodes of ipsilateral carotid territory ischemic symptoms that had occurred ≤2 months before presentation to our department, new neurological deficits lasting for 2 weeks after surgery, additional ischemic lesions on MR imaging including T2-weighted and FLAIR sequences performed 2 weeks after surgery in comparison with preoperative MR imaging, and cerebral hyperperfusion determined on intraoperative transcranial regional cerebral oxygen saturation monitoring as described below. A 1.5-T whole body imaging system (Signa MR/I; General Electric, Milwaukee, Wisconsin) was used for the evaluation of ischemic lesions before and after surgery. Whether the anterior cerebral artery (ACA) contralateral to surgery was perfused by the ipsilateral ICA (the A1 of the ipsilateral ACA was not hypoplastic and the contralateral A1 was hypoplastic) was also confirmed on postoperative MR angiography by using this 1.5-T imaging system.

This protocol was reviewed and approved by the institutional ethics committee, and written informed consent was obtained from all patients or their next of kin.

### Preoperative and Intraoperative Management

All patients received antiplatelet therapy until the morning of the day on which CEA was performed. Surgery was conducted under general anesthesia. No intraluminal shunt or patch graft was used in these procedures. A bolus of heparin (5000 international units) was given before ICA clamping. A near-infrared spectroscope, TOS 96 (Tostec, Tokyo, Japan) with a dual-channel system was used to continuously measure regional cerebral oxygen saturation throughout surgery, and the presence or absence of the development of cerebral hyperperfusion was determined intraoperatively as previously described.[20]

### FA Measurements by DTI and TBSS Analysis

DTI was performed by using a 3.0-T superconductive MR imager with a gradient slew rate of 150 mT $m^{-1} \cdot ms^{-1}$ (Signa EXCITE HD; GE Healthcare) and an 8-channel head coil within 7 days before and 1 month after surgery. The following pulse sequences were used for DTI covering the entire brain: axial single-shot, spin-echo, echo-planar imaging; repetition time, 10 000 ms; echo time, 66 ms; 6 motion-probing gradient directions (b value 1000 $s/m^2$); matrix size, 128 × 128; field of view, 24 cm; slice thickness, 4.0 mm with 1.5-mm interslice gaps (voxel size, 1.9 × 1.9 × 4.0 mm); number of slices, 24; number of excitations, 3; parallel imaging reduction factor, 2; and acquisition time, 3 minutes 40 seconds.

TBSS analyses for DTI were performed by using FSL 5.0 (FMRIB Software Tools, Oxford, http://www.fmrib.ox.ac.uk/fsl).[18] After skull stripping and correction for spatial misregistration due to eddy currents and head motions, FA values were calculated for whole brain volumes. The FA maps of patients who underwent CEA on the left side were flipped horizontally so that the right and left cerebral hemispheres were defined as the cerebral hemisphere ipsilateral to surgery and the contralateral cerebral hemisphere, respectively. The FA maps were then aligned to the FMRIB58-FA standard space by using a nonlinear registration algorithm. With the use of TBSS implemented in FSL, mean FA images of each subject were created and were then thinned and thresholded at FA >0.20 to generate a mean FA skeleton that represents the centers of major white matter tracts common to all subjects. The FA map of each subject was projected onto the FA skeleton to obtain skeleton FA maps, which were fed into voxelwise analyses to compare the preoperative and postoperative FA values. Further, right hemispheric (ipsilateral to surgery) and left hemispheric (contralateral to surgery) white matter regions-of-interest (ROIs) were defined as an intersection

between the FA skeleton maps and cerebral white matter labels based on the Harvard-Oxford Subcortical Structural Atlas implemented in FSL, as illustrated in Figure 1. Mean FA values in the ipsilateral and contralateral white matter ROIs were then calculated before and after surgery in each subject. Differences between the 2 mean FA values (postoperative values minus preoperative values) were also calculated and defined as the Δ mean FA in each subject.

To determine the location of voxels with postoperative increase in FA value in each cerebral white matter in a subgroup, a voxelwise group comparison of the FA values before and after surgery was performed by using the FSL function "randomize" with 5000 permutations. Then, statistical maps of $P < .05$ for postoperative increase in FA value were obtained by using a paired $t$ test with a correction for multiple comparison by the family-wise error rate.[21]

Ten healthy volunteers (8 men, 2 women; mean age, 33 years; range, 21-55 years) without any history of hypertension, diabetes mellitus, atrial fibrillation, pulmonary disease, or presence of organic brain lesions, including leukoaraiosis or asymptomatic lacunar infarction on MR imaging served as controls. These participants underwent 2 separate DTI studies in the same manner as described above. The interval between the 2 studies ranged from 1 month to 2 months.

## Neuropsychological Evaluation

For each patient, a battery of neuropsychological tests was administered, consisting of the Wechsler Adult Intelligence Scale Revised (WAIS-R),[22] the Wechsler Memory Scale,[23] and Rey-Osterreith Complex Figure test (Rey test).[24] WAIS-R generates a verbal and performance intelligence quotient (IQ). The Rey test evaluates copy and recall of a complex figure. Thus, 5 scores (WAIS-R verbal IQ, WAIS-R performance IQ, Wechsler Memory Scale, Rey copy, and Rey recall) were used to evaluate cognitive function.

The neuropsychological tests were performed within 7 days before surgery and were repeated 1 month after surgery. All examinations were administered by a trained neuropsychologist who was blinded to the patient's clinical information.

Postoperative cognition was categorized as improved or not improved for each patient based on the definition described previously.[7] In brief, 40 healthy volunteers served as controls and underwent the same neuropsychological tests on 2 separate occasions (intertest interval, 1-2 months).[7] Differences in each neuropsychological test score between the 2 tests (the second test score—the first test score) were calculated. For each neuropsychological test score of patients undergoing CEA, a significant increment was defined as a postoperative test score > preoperative score plus the value of the mean + 2 standard deviations (2SD) of the difference between the 2 test scores in the controls. A patient was defined as having postoperative cognitive improvement when there was a significant increment in postoperative neuropsychological scores on one or more of the 5 tests (WAIS-R verbal IQ, WAIS-R performance IQ, Wechsler Memory Scale, Rey copy, and Rey recall).[7]

## Statistical Analysis

Data are expressed as the mean ± SD. Changes between mean FA values of white matter ROIs in each cerebral hemisphere in the first and second studies were evaluated with the use of the Wilcoxon signed rank test. The relationship between each characteristic and Δ mean FA in each cerebral hemisphere was evaluated by using the Mann-Whitney $U$ test. The relationship between each characteristic and postoperative cognitive improvement was evaluated with univariate analysis by the use of the Mann-Whitney $U$ test or the $\chi^2$ test. A multivariate statistical analysis of factors related to postoperatively improved cognition was also performed with the use of a logistic regression model, and odds ratios with 95% confidence intervals were calculated. Variables with $P < .2$ in the univariate analyses were selected for analysis in the final model. For all statistical analyses, significance was set at the $P < .05$ level.

# RESULTS

Over a period of 31 months, a total of 119 patients underwent CEA. Of these, 91 patients satisfied the preoperative inclusion criteria. However, 1 patient experienced a new major neurological deficit that lasted for 2 weeks after surgery, and another patient developed additional asymptomatic ischemic lesions on MR imaging including T2-weighted and FLAIR sequences performed 1 month after surgery in comparison with preoperative MR imaging. Nine additional patients were determined to have cerebral hyperperfusion on intraoperative monitoring of the regional cerebral oxygen saturation with the use of the near-infrared spectroscope. These 11 patients did not undergo DTI study and neuropsychological testing after surgery and were

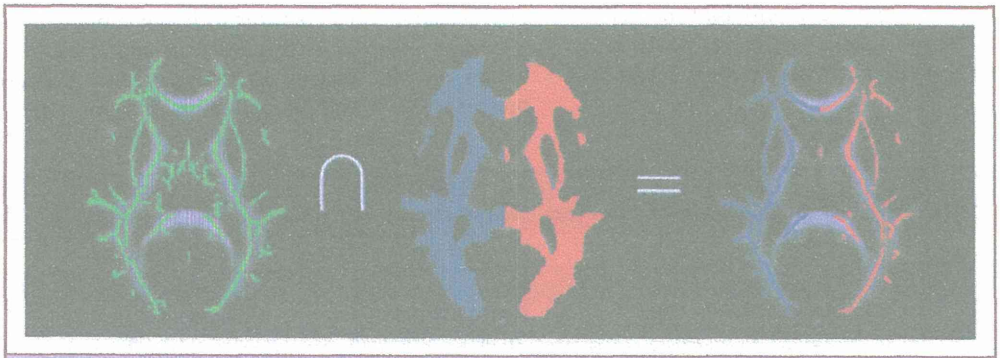

**FIGURE 1.** *Skeleton-based region-of-interest (ROI) analysis procedure. The template for white matter labels in the right (blue) and left (red) cerebral hemispheres (Middle) is registered to the skeleton of the mean fractional anisotropy (FA) map in each subject (green) (Left), resulting in defining ROIs of white matter FA in each cerebral hemisphere (Right).*

excluded from the present study. Thus, the remaining 80 patients were analyzed. None of these patients experienced further ischemic symptoms during the period between initial evaluation and surgical intervention.

The mean age of the 80 patients (73 men, 7 women) was 68 ± 6 years (range, 47-75 years). Concomitant disease states and symptoms were recorded, including 71 patients with hypertension, 32 patients with diabetes mellitus, and 41 patients with dyslipidemia. Fifty patients had ipsilateral symptomatic ICA stenosis, and the remaining 30 patients had asymptomatic ICA stenosis. Preoperative MR imaging demonstrated infarction in the hemisphere ipsilateral to the ICA stenosis in 44 patients and no infarction in 36 patients. Overall average degree of ICA stenosis was 87.2 ± 8.7% with a range of 70% to 99% according to the method of the North American Symptomatic Carotid Endarterectomy Trial.[19] The contralateral ICA was occluded in 3 patients, and 6 additional patients had 60% to 99% stenosis. The ACA contralateral to surgery was perfused by the ipsilateral ICA on MR angiography in 27 patients. Mean duration of ICA clamping was 37 minutes (range, 26-49 minutes). The interval of the neuropsychological testing and measurement of FA was ≤4 days in all patients.

The mean ± SD of mean FA values of white matter ROIs in each cerebral hemisphere in the first and second studies among 80 patients and 10 controls is summarized in Table 1. When analyzed as a group, mean FA values in the 20 cerebral hemispheres in the control participants did not differ between the first study and the second study. In contrast, mean FA values in the cerebral hemispheres ipsilateral and contralateral to surgery in the patients undergoing CEA were significantly greater after surgery than before surgery.

The Δ mean FA in patients ranged from −0.0200 to 0.0392 (0.0037 ± 0.0087) in the cerebral hemisphere ipsilateral to surgery and from −0.0187 to 0.0223 (0.0027 ± 0.0069) in the contralateral cerebral hemisphere. The relationship between each characteristic and Δ mean FA in each cerebral hemisphere is summarized in Table 2. Although variables were not related to Δ mean FA in the ipsilateral cerebral hemisphere, Δ mean FA in the contralateral cerebral hemisphere was significantly greater in patients with bilateral lesions or in patients in whom the contralateral ACA was perfused by the ipsilateral ICA than in patients without those respective features.

Based on the neuropsychological assessments performed before and after surgery, 11 (14%) patients were defined as having postoperatively improved cognition. Results of the univariate analysis of factors related to postoperative cognitive improvement are summarized in Table 3. Patients with postoperative cognitive improvement were significantly younger than those without; the incidence of the bilateral lesions was significantly greater in patients with postoperative cognitive improvement than in those without; the Δ mean FA value in the cerebral hemisphere ipsilateral and contralateral to surgery was significantly greater in patients with postoperative cognitive improvement than in those without. Other variables were not significantly associated with postoperative cognitive improvement. After eliminating variables that were closely related to others, the following items with values of $P < .2$ in univariate analyses were adopted as confounders in the logistic regression model for multivariate analysis: age, bilateral lesions, contralateral ACA perfused by the ipsilateral ICA, and Δ mean FA value in the cerebral hemisphere ipsilateral and contralateral to surgery. This analysis revealed that only Δ mean FA value in the cerebral hemisphere ipsilateral to surgery was significantly associated with postoperative cognitive improvement (95% confidence interval, 2.632-9.877; $P = .008$).

The voxelwise group analysis revealed that white matter FA values in a subgroup of 11 patients with postoperative cognitive improvement were significantly increased after surgery not in the whole ipsilateral cerebral hemisphere, in the contralateral ACA territory, and in the watershed zone between the contralateral ACAs and middle cerebral arteries (Figure 2).

## DISCUSSION

The present study using TBSS analysis demonstrated that postoperative increase in cerebral white matter FA on DTI is associated with cognitive improvement after uncomplicated CEA.

Cerebral ischemic events caused by cerebral hemispheric hypoperfusion during ICA clamping or intraoperative emboli from the surgical site may result in cognitive impairment after CEA.[7] Because we intended to determine the association between FA value changes in the cerebral white matter and cognitive improvement only in patients with uncomplicated CEA, patients with new major neurological deficits and/or additional ischemic lesions on MR imaging after surgery were excluded from the present study. Postoperative cerebral hyperperfusion, even when

**TABLE 1. Comparison of Mean FA Values of White Matter ROIs in Each Cerebral Hemisphere in the First and Second Studies[a,b]**

| | | First Study | Second Study | *P* Value |
|---|---|---|---|---|
| Patients (n = 80) | Ipsilateral hemisphere (n = 80) | 0.4191 ± 0.0293 | 0.4228 ± 0.0291 | <.001 |
| | Contralateral hemisphere (n = 80) | 0.4278 ± 0.0253 | 0.4304 ± 0.0259 | .001 |
| Controls (n = 10) | Bilateral hemispheres (n = 20) | 0.4549 ± 0.0142424 | 0.4552 ± 0.0131 | .467 |

[a]FA, fractional anisotropy; ROIs, regions-of-interest.
[b]Values are expressed as mean ± standard deviation.

**TABLE 2. Relationship Between Characteristics and Δ Mean FA in Each Hemisphere[a]**

| Variables | Δ Mean FA in Ipsilateral Hemisphere | | Δ Mean FA in Contralateral Hemisphere | | |
|---|---|---|---|---|---|
| | Mean ± SD | *P* Value | Mean ± SD | *P* Value | |
| Age, y | ≥68 (n = 52) | 0.0032 ± 0.0069 | .595 | 0.0024 ± 0.0058 | .057 |
| | <68 (n = 28) | 0.0039 ± 0.0113 | | 0.0041 ± 0.0077 | |
| Sex | Male (n = 73) | 0.0026 ± 0.0091 | .083 | 0.0029 ± 0.0067 | .403 |
| | Female (n = 7) | 0.0066 ± 0.0044 | | 0.0014 ± 0.0055 | |
| Hypertension | Yes (n = 71) | 0.0035 ± 0.0085 | .301 | 0.0034 ± 0.0068 | .192 |
| | No (n = 9) | 0.0015 ± 0.0072 | | 0.0013 ± 0.0074 | |
| Diabetes mellitus | Yes (n = 32) | 0.0028 ± 0.0084 | .294 | 0.0018 ± 0.0080 | .381 |
| | No (n = 48) | 0.0043 ± 0.0091 | | 0.0029 ± 0.0073 | |
| Dyslipidemia | Yes (n = 41) | 0.0026 ± 0.0101 | .166 | 0.0027 ± 0.0078 | .791 |
| | No (n = 39) | 0.0041 ± 0.0073 | | 0.0033 ± 0.0045 | |
| Symptomatic lesion | Yes (n = 50) | 0.0032 ± 0.0094 | .187 | 0.0015 ± 0.0071 | .287 |
| | No (n = 30) | 0.0052 ± 0.0069 | | 0.0035 ± 0.0073 | |
| Infarction on preoperative MRI | Yes (n = 44) | 0.0026 ± 0.0103 | .087 | 0.0024 ± 0.0083 | .702 |
| | No (n = 36) | 0.0051 ± 0.0069 | | 0.0030 ± 0.0061 | |
| Degree of ICA stenosis, % | ≥87 (n = 50) | 0.0039 ± 0.0082 | .639 | 0.0031 ± 0.0057 | .473 |
| | <87 (n = 30) | 0.0031 ± 0.0093 | | 0.0024 ± 0.0082 | |
| Bilateral lesions | Yes (n = 9) | 0.0079 ± 0.0119 | .112 | 0.0094 ± 0.0089 | .011 |
| | No (n = 71) | 0.0031 ± 0.0082 | | 0.0015 ± 0.0055 | |
| Contralateral ACA perfused by ipsilateral ICA | Yes (n = 27) | 0.0073 ± 0.0059 | .076 | 0.0064 ± 0.0055 | .027 |
| | No (n = 53) | 0.0018 ± 0.0093 | | 0.0010 ± 0.0068 | |
| Duration of ICA clamping, min | ≥37 (n = 42) | 0.0049 ± 0.0091 | .714 | 0.0033 ± 0.0062 | .472 |
| | <37 (n = 38) | 0.0034 ± 0.0087 | | 0.0024 ± 0.0076 | |

[a]FA, fractional anisotropy; SD, standard deviation; ICA, internal carotid artery; ACA, anterior cerebral artery; MRI, magnetic resonance imaging.

asymptomatic, is another possible mechanism of cognitive impairment after CEA.[7,25] Intraoperative monitoring of the regional cerebral oxygen saturation using near-infrared spectroscopy is a reliable method of identifying patients with hyperperfusion after CEA.[20,26] Thus, patients characterized as having cerebral hyperperfusion by that mode of monitoring were also excluded from the present study.

In the present study, mean FA values in the cerebral white matter in the controls did not differ between the first study and the second study, indicating that the TBSS analysis has good reproducibility for repeat measurement of FA values. This finding also supports the notion that white matter FA values in the cerebral hemispheres ipsilateral and contralateral to surgery usually increase

**TABLE 3. Comparison of Characteristics Between Patients Who Experienced Postoperative Improvement in Cognition and Those That Did Not[a]**

| | Group | | |
|---|---|---|---|
| | Improved Cognition (n = 11) | No Improvement in Cognition (n = 69) | *P* Value |
| Age, y, mean ± SD | 65.5 ± 5.2 | 68.5 ± 6.4 | .032 |
| Male sex | 82% (9/11) | 93% (64/69) | .245 |
| Hypertension | 91% (10/11) | 88% (61/69) | >.999 |
| Diabetes mellitus | 36% (4/11) | 41% (28/69) | >.999 |
| Dyslipidemia | 64% (7/11) | 49% (34/69) | .520 |
| Symptomatic lesion | 55% (6/11) | 64% (44/69) | .739 |
| Infarction on preoperative MRI | 55% (6/11) | 55% (38/69) | >.999 |
| Degree of ICA stenosis, %, mean ± SD | 86.8 ± 10.1 | 87.2 ± 8.5 | .971 |
| Contralateral ACA perfused by ipsilateral ICA | 55% (6/11) | 30% (21/69) | .169 |
| Bilateral lesions | 36% (4/11) | 7% (5/69) | .018 |
| Duration of ICA clamping, min, mean ± SD | 38.6 ± 5.1 | 37.0 ± 5.7 | .405 |
| Δ mean FA in ipsilateral hemisphere, mean ± SD | 0.0153 ± 0.0091 | 0.0019 ± 0.0072 | <.001 |
| Δ mean FA in contralateral hemisphere, mean ± SD | 0.0082 ± 0.0068 | 0.0018 ± 0.0066 | .024 |

[a]FA, fractional anisotropy; SD, standard deviation; ICA, internal carotid artery; ACA, anterior cerebral artery; MRI, magnetic resonance imaging.

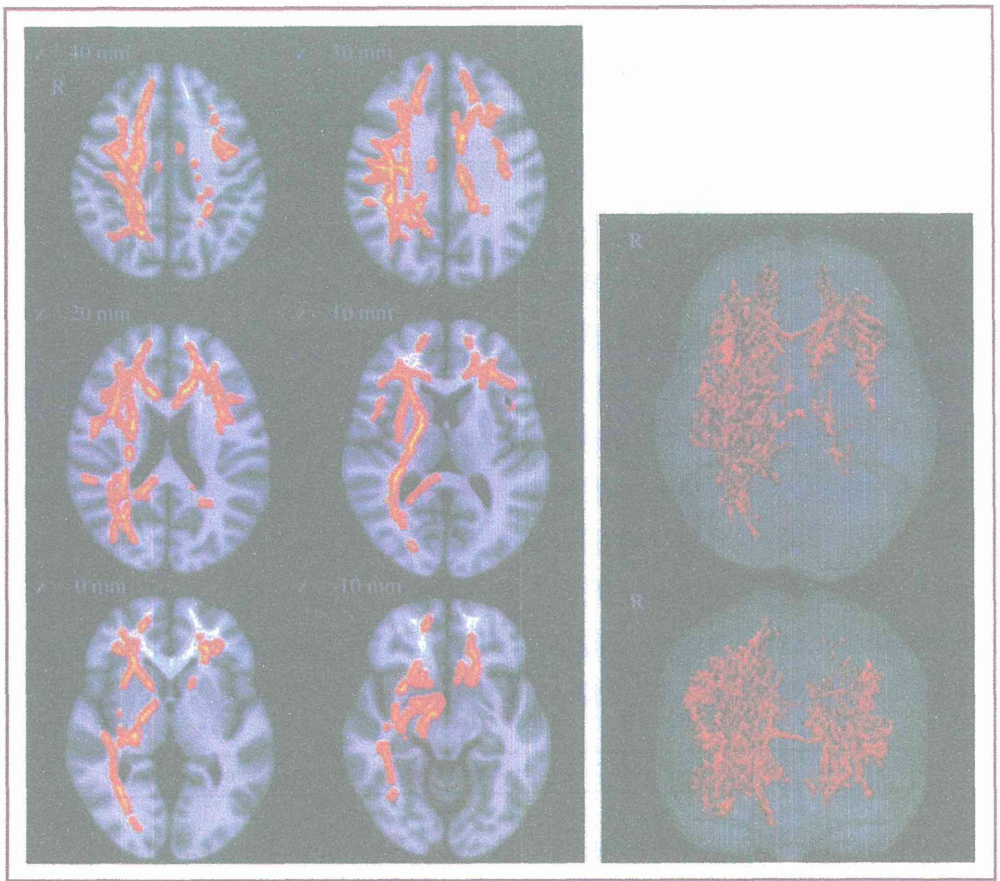

**FIGURE 2.** *Results of voxelwise group analysis for postoperative increase in FA in a subgroup of patients with postoperative cognitive improvement. Voxels with significant postoperative increase in cerebral white matter FA are expressed as red-yellow dots on axial slice images (Left) and axial and coronal 3-dimensional images (Right). Z value in each axial slice image indicates the height from the anterior commissure-posterior commissure line.*

after uncomplicated CEA and that the degree of postoperative increase in white matter FA values in the ipsilateral cerebral hemisphere is significantly associated with postoperative cognitive improvement.

Several studies suggested that diffusion anisotropy is mainly influenced by the magnitude of myelination in animal models[27,28] or remyelination within acute lesions in normal-appearing white matter of patients with multiple sclerosis.[29] A recent study using animal models also demonstrated that remyelination is strongly correlated with the recovery of cognitive dysfunction following chronic cerebral ischemia, suggesting that treatment leading to remyelination could ameliorate the cognitive dysfunction associated with chronic cerebral ischemia.[30] Another study using magnetic resonance spectroscopy showed that choline-containing compounds in the cerebral hemisphere ipsilateral to surgery is increased after CEA in patients with postoperatively improved cognition.[31] This postoperative increase in choline may imply recovery of abnormally reduced myelin membrane turnover (ie, remyelination), resulting in cognitive improvement after CEA.[31] In combination with observations from the present study, these previous findings supported the notion that normalization of cerebral hemodynamics after CEA may lead to remyelination in the cerebral white matter, resulting in postoperative improvements in cognition. However, the correlation between the postoperative increase in brain perfusion and the postoperative increase in FA has not been demonstrated.

In the above-mentioned study that compared postoperative changes in choline (measured by using magnetic resonance spectroscopy) with changes in cognitive function after CEA, magnetic resonance spectroscopy was performed within 7 days before surgery and between 2 and 4 weeks after surgery. Thus, we supposed that the remyelination may occur within 1 month after surgery, and, therefore, DTI was performed at 1 month after surgery in the present study.

In the present study, the presence of bilateral ICA stenoocclusive diseases or the presence of a contralateral ACA perfused

by the ipsilateral ICA was related to the degree of postoperative increase in white matter FA in the cerebral hemisphere contralateral to surgery. Further, the degree of postoperative increase in the white matter FA was greater in patients with postoperatively improved cognition than in those without, although this relationship was not statistically significant on multivariate analysis. Further, according to the voxelwise group analysis, white matter FA values in patients with postoperative cognitive improvement were significantly increased after surgery even in the contralateral ACA territory and the watershed zone between the contralateral ACA and middle cerebral artery. These data suggested that improvement in cerebral hemodynamics in the contralateral cerebral hemisphere after CEA leads to remyelination in the white matter in that cerebral hemisphere, which may, in part, contribute to postoperative cognitive improvement.

## CONCLUSION

The present study using TBSS analysis demonstrated that postoperative increase in cerebral white matter FA on DTI is associated with cognitive improvement after uncomplicated CEA. Normalization of cerebral hemodynamics after CEA may lead to remyelination in the cerebral white matter, resulting in postoperative improvements in cognition.

### Disclosures

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## REFERENCES

1. Chaturvedi S, Bruno A, Feasby T, et al. Carotid endarterectomy: an evidence-based review: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. *Neurology.* 2005;65(6):794-801.
2. Aharon-Peretz J, Tomer R, Gabrieli I, Aharonov D, Nitecki S, Hoffman A. Cognitive performance following endarterectomy in asymptomatic severe carotid stenosis. *Eur J Neurol.* 2003;10(5):525-528.
3. Iddon JL, Sahakian BJ, Kirkpatrick PJ. Uncomplicated carotid endarterectomy is not associated with neuropsychological impairment. *Pharmacol Biochem Behav.* 1997;56(4):781-787.
4. Irvine CD, Gardner FV, Davies AH, Lamont PM. Cognitive testing in patients undergoing carotid endarterectomy. *Eur J Vasc Endovasc Surg.* 1998;15(3):195-204.
5. Lunn S, Crawley F, Harrison MJ, Brown MM, Newman SP. Impact of carotid endarterectomy upon cognitive functioning. A systematic review of the literature. *Cerebrovasc Dis.* 1999;9(2):74-81.
6. Rao R. The role of carotid stenosis in vascular cognitive impairment. *Eur Neurol.* 2001;46(2):63-69.
7. Yoshida K, Ogasawara K, Kobayashi M, et al. Improvement and impairment in cognitive function after carotid endarterectomy: comparison of objective and subjective assessments. *Neurol Med Chir (Tokyo).* 2012;52(3):154-160.
8. Chida K, Ogasawara K, Aso K, et al. Postcarotid endarterectomy improvement in cognition is associated with resolution of crossed cerebellar hypoperfusion and increase in 123I-iomazenil uptake in the cerebral cortex: a SPECT study. *Cerebrovasc Dis.* 2010;29(4):343-351.
9. Chu Z, Wilde EA, Hunter JV, et al. Voxel-based analysis of diffusion tensor imaging in mild traumatic brain injury in adolescents. *AJNR Am J Neuroradiol.* 2010;31(2):340-346.
10. Giussani C, Poliakov A, Ferri RT, et al. DTI fiber tracking to differentiate demyelinating diseases from diffuse brain stem glioma. *Neuroimage.* 2010;52(1):217-223.
11. Hattori T, Ito K, Aoki S, et al. White matter alteration in idiopathic normal pressure hydrocephalus: tract-based spatial statistics study. *AJNR Am J Neuroradiol.* 2012;33(1):97-103.
12. Liu Y, Spulber G, Lehtimäki KK, et al. Diffusion tensor imaging and tract-based spatial statistics in Alzheimer's disease and mid cognitive impairment. *Neurobiol Aging.* 2011;32(9):1558-1571.
13. Masutani Y, Aoki S, Abe O, et al. MR diffusion tensor imaging: recent advance and new techniques for diffusion tensor visualization. *Eur J Radiol.* 2003;46(1):53-66.
14. Schiavone F, Charlton RA, Barrick TR, Morris RG, Markus HS. Imaging age-related cognitive decline: a comparison of diffusion tensor and magnetization transfer MRI. *J Magn Reson Imag.* 2009;29(1):23-30.
15. O'Sullivan M, Morris RG, Huckstep B, Jones DK, Williams SC, Markus HS. Diffusion tensor MRI correlates with executive dysfunction in patients with ischaemic leukoaraiosis. *J Neurol Neurosurg Psychiatry.* 2004;75(3):441-447.
16. Kraus MF, Susmaras T, Caughlin BP, Walker CJ, Sweeney JA, Little DM. White matter integrity and cognition in chronic traumatic brain injury: a diffusion tensor imaging study. *Brain.* 2007;130(pt 10):2508-2519.
17. Stebbins GT, Murphy CM. Diffusion tensor imaging in Alzheimer's disease and mild cognitive impairment. *Behav Neurol.* 2009;21(1):39-49.
18. Smith SM, Jenkinson M, Johansen-Berg H, et al. Tract-based spatial statistics: voxelwise analysis of multi-subject diffusion data. *Neuroimage.* 2006;31(4):1487-1505.
19. North American Symptomatic Carotid Endarterectomy Trial Collaborators. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. *N Engl J Med.* 1991;325(7):445-453.
20. Ogasawara K, Konno H, Yukawa H, Endo H, Inoue T, Ogawa A. Transcranial regional cerebral oxygen saturation monitoring during carotid endarterectomy as a predictor of postoperative hyperperfusion. *Neurosurgery.* 2003;53(2):309-314.
21. Smith SM, Nichols TE. Threshold-free cluster enhancement: addressing problems of smoothing, threshold dependence and localization in cluster inference. *Neuroimage.* 2009;44(1):83-98.
22. Shinagawa F, Kobayashi S, Fujita K. *Japanese Wechsler Adult Intelligence Scale-Revised.* Tokyo, Japan: Nihon Bunka Kagakusha; 1990.
23. Koyama M. *Clinical Psychology of Brain Damage.* Tokyo, Japan: Gakuen Sha; 1985:48-54.
24. Lezak MD. *Neuropsychological Assessment.* 3rd ed. New York, NY: Oxford University Press; 1995.
25. Ogasawara K, Yamadate K, Kobayashi M, et al. Postoperative cerebral hyperperfusion associated with impaired cognitive function in patients undergoing carotid endarterectomy. *J Neurosurg.* 2005;102(1):38-44.
26. Ogasawara Y, Ogasawara K, Suzuki T, et al. Preoperative 123I-iomazenil SPECT imaging predicts cerebral hyperperfusion following endarterectomy for unilateral cervical internal carotid artery stenosis. *Am J Nucl Med Mol Imaging.* 2012;2(1):77-87.
27. Gulani V, Webb AG, Duncan ID, Lauterbur PC. Apparent diffusion tensor measurements in myelin-deficient rat spinal cords. *Magn Reson Med.* 2001;45(2):191-195.
28. Nair G, Tanahashi Y, Low HP, Billings-Gagliardi S, Schwartz WJ, Duong TQ. Myelination and long diffusion times alter diffusion-tensor-imaging contrast in myelin-deficient shiverer mice. *Neuroimage.* 2005;28(1):165-174.
29. Fox RJ, Cronin T, Lin J, et al. Measuring myelin repair and axonal loss with diffusion tensor imaging. *AJNR Am J Neuroradiol.* 2011;32(1):85-91.
30. Chida Y, Kokubo Y, Sato S, et al. The alterations of oligodendrocyte, myelin in corpus callosum, and cognitive dysfunction following chronic cerebral ischemia in rats. *Brain Res.* 2011;1414(9):22-31.
31. Saito H, Ogasawara K, Nishimoto H, et al. Postoperative changes in cerebral metabolites associated with cognitive improvement and impairment after carotid endarterectomy: a 3T Proton MR Spectroscopy Study. *AJNR Am J Neuroradiol.* 2013;34(5):976-982. doi: 10.3174/ajnr.A3344.

## COMMENTS

The article entitled "Postoperative Increase in Cerebral White Matter Fractional Anisotropy on Diffusion Tensor Magnetic Resonance Imaging Is Associated With Cognitive Improvement After

Uncomplicated Carotid Endarterectomy: Tract-Based Spatial Statistics Analysis" demonstrates an important issue related to cognitive performance, ie, cognitive domains encompass multiple brain regions all of which participate in cognitive performance. The authors have demonstrated that improved cognitive function in white matter connectivity by using new techniques: fractional anisotropy (FA) in the cerebral white matter derived from diffusion tensor magnetic resonance imaging, and a new method of analyzing FA data, tract-based spatial statistics (TBSS). It would be interesting to see if cognitive dysfunction also had its correlates in changes in FA. The present study is one of the first articles to demonstrate the relation of changes of anatomic architecture in the cerebral white matter and improvements in cognitive function after CEA.

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This is a rigorous and extremely well done study that characterizes the previously reported phenomenon of improved cognitive function following carotid endarterectomy and assesses whether this improvement relates to cerebral white matter changes after surgery through use of Fractional anisotropy (FA) magnetic resonance imaging measurements. Their results demonstrate again that in a small but significant number of patients (14% in this series) carotid endarterectomy appears to improve cognition - presumably by addressing a deficit in cerebral hemodynamics. Potential confounds such as inclusion of patients with recent symptomatic stroke (a population where cognitive improvement over time would be expected) were appropriately excluded. They also convincingly showed that the change in FA following surgery correlated with postoperative cognitive improvement.

Because FA improvements have been linked in preclinical and clinical studies to remyelination, the authors conclude that post-endarterectomy improvements in cerebral hemodynamics lead to white matter remyelination and ultimately enhanced cognitive function. This may very well be the case, but it is important to note that cerebral hemodynamics was not assessed in this study. We do not know whether the patients that showed improved cognition following surgery were in fact those that had impaired cerebral hemodynamics pre-operatively. Additional studies will be required to make this causal link. Overall, this is a high quality study that provides support for the notion that cognitive dysfunction should be included in future clinical trials examining indications and benefits of carotid endarterectomy. It also argues that FA might be useful as a surrogate marker for this clinical endpoint.

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# $\beta_2$-Adrenergic and $M_2$-muscarinic receptors decrease basal t-tubular L-type $Ca^{2+}$ channel activity and suppress ventricular contractility in heart failure

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ABSTRACT

L-type $Ca^{2+}$ channels (LTCC) play a crucial role in cardiac excitation–contraction coupling. We previously found that in failing ventricular myocytes of mice chronically treated with isoproterenol, basal t-tubular (TT) LTCC activity was halved by activation of protein phosphatase (PP)2A whereas basal surface sarcolemmal (SS) LTCC activity was doubled by inhibition of PP1. Interestingly, chronic treatment of these mice with pertussis toxin almost completely normalized TT and SS LTCC densities and cardiac contractility. In the present study, we therefore sought to identify the $G_{i/o}$ protein-coupled receptors in cardiac myocytes (i.e. $\beta_2$-adrenergic, $M_2$-muscarinic and $A_1$-adenosine receptors) that are responsible for these abnormalities in heart failure by chronically administrating mice a selective antagonist of each receptor (ICI118,551, atropine and 8-cyclopentyl-1,3-dipropilxanthine (DPCPX), respectively) with isoproterenol. Compared with mice treated with isoproterenol alone, mice treated with isoproterenol plus ICI118,551 or atropine, but not DPCPX showed significantly lower lung weight/tibial length, higher fractional shortening, lower left ventricular end-diastolic pressure and higher $dP/dt_{max}$ and $dP/dt_{min}$. In addition, ventricular myocytes of mice treated with isoproterenol plus ICI118,551 or atropine, but not DPCPX exhibited significantly higher TT and lower SS LTCC current densities than those of mice treated with isoproterenol alone due to normalization of the PP activities. These results indicate that $\beta_2$-adrenergic, $M_2$-muscarinic, but not $A_1$-adenosine receptors contribute to reduced ventricular contractility at least partially by decreasing basal TT LTCC activity in heart failure. Therefore, antagonists of $\beta_2$-adrenergic and/or $M_2$-muscarinic receptors can be good adjuncts to $\beta_1$-adrenergic receptor antagonists in the treatment of heart failure.



## 1. Introduction

Despite recently advanced medical therapies, heart failure is one of the leading risks of death in developed nations (Go et al., 2013). Thus, development of medical treatment based on further understanding of the pathophysiology of heart failure is required to improve the quality of life of patients with heart failure. Maladaptive remodeling of ventricular excitation–contraction (EC) coupling caused by such as chronic β-adrenergic receptor stimulation, and activation of the renin-angiotensin-aldosterone system plays a predominant role in an impaired cardiac function in heart failure (Mann, 2008; Osadchii, 2007; Rockman et al., 2002). The underlying primary defect is a decrease in sarcoplasmic reticulum (SR) $Ca^{2+}$ content due to decreased activity and/or expression of SR $Ca^{2+}$-ATPase 2, increased expression of sarcolemmal $Na^+/Ca^{2+}$ exchangers and diastolic $Ca^{2+}$ leak from the SR via ryanodine receptors (Bers, 2008).

L-type $Ca^{2+}$ channel (LTCC) currents play a crucial role in cardiac EC coupling (McDonald et al., 1994). In most cardiac hypertrophy and failure, whole-cell LTCC current density is reported not to change markedly (Bers, 2008; Mukherjee and Spinale, 1998). However, we previously found for the first time that basal t-tubular (TT) LTCC activity was halved by activation of protein phosphatase (PP) 2A whereas basal surface sarcolemmal (SS) LTCC activity was doubled by inhibition of PP1 in failing ventricular myocytes of mice chronically treated with isoproterenol (Horiuchi-Hirose et al., 2011;

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Kashihara et al., 2012). Because $G_{i/o}$ proteins are known to activate PP2A (Herzig et al., 1995; Ke et al., 2004; Liu and Hofmann, 2003) and suppress PP1 in cardiac myocytes (Zhu et al., 2008), we examined the effect on isoproterenol-treated mice of pertussis toxin, a selective inhibitor of the coupling between G protein-coupled receptors and $G_{i/o}$ proteins (Kashihara et al., 2012). Pertussis toxin significantly increased basal TT LTCC activity, decreased basal SS LTCC activity and increased cardiac contractility independently of protein kinase A in isoproterenol-treated mice.

In this study, we sought to identify the G protein-coupled receptors that lead to abnormal LTCC activity and cardiac dysfunction in heart failure. In the heart, $M_2$-muscarinic and $A_1$-adenosine receptors are the principal G protein-coupled receptors coupled with $G_{i/o}$ proteins. Moreover, the phosphorylation of $\beta_2$-adrenergic receptors by protein kinase A or G protein-coupled receptor kinase, which are expected to occur in heart failure, switches their coupling from $G_s$ to $G_i$ (Daaka et al., 1997; Liu et al., 2009); therefore, we examined whether any of these three types of receptors are involved in the abnormalities in isoproterenol-treated mice with a selective antagonist for each receptor.

In the present study, we found that chronic activation of $\beta_2$-adrenergic and $M_2$-muscarinic, but not $A_1$-adenosine receptors decreases cardiac contractile function at least partially by decreasing basal TT LTCC activity in heart failure. Therefore, antagonists of $\beta_2$-adrenergic and $M_2$-muscarinic receptors can be good adjuncts to $\beta_1$-adrenergic receptor antagonists in the treatment of heart failure.

A part of the preliminary results of this study has been published elsewhere in the form of an abstract (Kashihara et al., 2013).

## 2. Materials and methods

### 2.1. Chemicals

Chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated. Isoproterenol (Sigma-Aldrich, Inc., St. Louis, MO, USA) and atropine were dissolved in saline. ICI118,551 (Sigma-Aldrich) and 8-cyclopentyl-1,3-dipropilxanthine (DPCPX) (Sigma-Aldrich) were dissolved in 100% dimethyl sulfoxide (DMSO) at 50 and 6.6 mM, respectively. They were then diluted 3.3 and 20 times, respectively, with saline before administration to mice. Okadaic acid (Calbiochem, La Jolla, CA, USA) and fostriecin were dissolved at 1.0 mM in 100% DMSO. Final 0.1% DMSO did not significantly affect LTCC currents in isolated myocytes.

### 2.2. Animal model

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). All experiments were carried out in accordance with the Guidelines for Animal Experimentation of Shinshu University and approved by the Committee for Animal Experimentation. Male C57BL/6 adult mice (8–10 weeks) were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). Cardiac hypertrophy and failure were induced in the mice by subcutaneous injection of 6 mg/kg/day isoproterenol once daily from Day 0 to 21 as previously described (Horiuchi-Hirose et al., 2011). Age-matched normal control mice received the same volume of saline only. To examine the effect of the inhibition of $\beta_2$-adrenergic, $M_2$-muscarinic and $A_1$-adenosine receptors on isoproterenol-induced cardiac hypertrophy and failure, mice were treated with 0.5 mg/kg/day ICI118,551, 3 mg/kg/day atropine or 1 mg/kg/day DPCPX along with isoproterenol. ICI118,551 and atropine were applied to mice through osmotic minipumps (model 1004; Alzet, Cupertino, CA, USA) whereas DPCPX was applied daily intraperitoneally because it is too hydrophobic to be used with osmotic minipumps. The dose of ICI118,551 (0.5 mg/kg/day) was the maximum applicable to mice with the pump. The blood concentration of ICI118,551 in mice applied with this dose of the agent was $0.54 \pm 0.07$ nM whereas the $pA_2$ value of this agent for $\beta_1$ and $\beta_2$-adrenergic receptors is 7.17 and 9.26, respectively. The dose of atropine was determined according to previous reports (Jumrussirikul et al., 1998; Uechi et al., 1998). In pilot studies, mice injected with 1, 3 or 10 mg/kg/day of atropine along with 6 mg/kg/day isoproterenol exhibited the fractional shortening of $32.7 \pm 1.9\%$, $36.4 \pm 1.0\%$, $35.0 \pm 1.4\%$, respectively. Thus, we used the maximum effective dose of atropine (i.e. 3 mg/kg/day). The dose of DPCPX was determined according to a previous report (Koeppen et al., 2009). The half maximum inhibitory dose of intravenously applied N6-cyclopentyladenosine, a selective $A_1$ adenosine receptor agonist in causing negative chronotropic effect was 7.09 and 226 μg/kg in mice injected with saline or 1 mg/kg/day DPCPX, respectively. Pumps were implanted under pentobarbital sodium (30 mg/kg) anesthesia administered intraperitoneally on Day −1. These antagonists were applied to mice from Day −1 to 21, whereas isoproterenol was applied from Day 0 to 21.

All animals were used for experiments on Day 22. Animals were sacrificed by sodium pentobarbital (30 mg/kg) anesthesia administrated intraperitoneally. The hearts and lungs were excised from the animals, rinsed in ice-cold modified Tyrode solution containing (in mmol/l): NaCl, 136.5; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.53; HEPES (Dojindo Laboratories, Kumamoto, Japan), 5.5; and glucose, 5.5 (pH=7.4 with NaOH) and weighed. Because the body weight of all groups of mice treated with drugs was significantly higher than that of saline-treated mice (Horiuchi-Hirose et al., 2011), the heart weight (HW; in mg) and lung weight (LW; in mg) were normalized to the tibial length (TL; in mm), which was not significantly different among the five groups of mice (i.e. HW/TL and LW/TL).

### 2.3. Gross and histological evaluation of hearts

The morphology of the heart was evaluated as previously described (Horiuchi-Hirose et al., 2011). Briefly, the hearts were fixed in 20% phosphate-buffered formalin at room temperature for 24 h and cut transversely to obtain ventricular cross sections. The tissues were then embedded in paraffin and cut into 4-μm thick slices. Histological sections were stained with hematoxylin–eosin and examined with a light microscope (BX-51; Olympus Corp., Tokyo, Japan). Wall thickness and the chamber diameter of the left ventricle were measured with an objective micrometer as the scale on digital microscopic images taken from histological sections.

### 2.4. Measurement of heart rate (HR) and mean systemic blood pressure (MBP)

HR and MBP in conscious, warmed and restrained mice were measured by the tail-cuff method with a programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) once a week between Day −1 and 22. Mice were restrained for 3 min with a temperature-controlled warming holder (37 °C) before these measurements. Each estimation was the average of three records taken at 1 min intervals. These measurements were performed 20 h after the isoproterenol injection.

### 2.5. Ultrasound cardiography

Cardiac function was assessed with ultrasound cardiography (GE Yokogawa Medical System K.K., Tokyo, Japan) in anesthetized

mice as previously described (Horiuchi-Hirose et al., 2011). Briefly, hearts were viewed at the level of the papillary muscles in the short axis. On M-mode tracings, the average of three consecutive beats was used to measure the following parameters: left ventricular (LV) end-diastolic diameter (EDD); LV end-systolic diameter (ESD); and fractional shortening (FS), calculated as (EDD−ESD)/EDD × 100%.

### 2.6. Cardiac catheterization

Under 2,2,2-tribromoethanol (250 mg/kg) anesthesia administrated intraperitoneally, a micro-tipped pressure catheter transducer (model 420 LP; Samba Sensor, Gothenburg, Sweden) was inserted into the right carotid artery and advanced into the LV cavity. After stabilization for 5 min, the signal was continuously recorded with a Samba 201 system (Samba Sensor) coupled with a PowerLab system (Chart4; ADInstruments, Dunedin, New Zealand), stored and displayed on a computer. LV preload was assessed by end-diastolic pressure (LVEDP; in mmHg); contractility, by $dP/dt_{max}$ (in mmHg/s); and relaxation, by $dP/dt_{min}$ (in mmHg/s).

### 2.7. Isolation of cardiac myocytes

Ventricular myocytes were enzymatically isolated as previously described (Horiuchi-Hirose et al., 2011). Briefly, the heart mounted on a Langendorff apparatus was digested with nominally $Ca^{2+}$-free Tyrode solution containing 0.80 mg/ml collagenase, 0.06 mg/ml protease (Sigma-Aldrich, Inc.), 1.20 mg/ml hyaluronidase (Sigma-Aldrich, Inc.), 0.03 mg/ml DNase I (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and 0.50 mg/ml bovine serum albumin at 37 °C for 2 min. Isolated ventricular myocytes were suspended in $Ca^{2+}$-free Tyrode solution containing 1 mg/ml bovine serum albumin at room temperature, and the $Ca^{2+}$ concentration was gradually increased to 1.8 mmol/l.

### 2.8. Detubulation

Detubulation of isolated ventricular myocytes was carried out with osmotic shock as previously described (Horiuchi-Hirose et al., 2011; Kawai et al., 1999). Briefly, myocytes were treated with modified Tyrode solution containing formamide (1.5 mol/l) for 15 min and returned to the modified Tyrode solution. A membrane-staining dye, di-8-ANEPPS clearly stained t-tubules in non-detubulated but barely in detubulated myocytes (Horiuchi-Hirose et al., 2011), suggesting that detubulation detached the t-tubular invaginations from the surface sarcolemma because hydrophobic di-8-ANEPPS could stain the t-tubules that were occluded yet connected to the sarcolemma. In different experiments, detubulation significantly reduced cell membrane capacitance (Table 1), indicating that intra-t-tubular space was electrically disconnected from extracellular space. Taken together, it is likely that t-tubular LTCC currents were not measured in the whole-cell configuration in detubulated myocytes.

### 2.9. Electrophysiology

The current of isolated ventricular myocytes was studied in the whole-cell configuration of the patch-clamp technique at 35–37 °C with a patch-clamp amplifier (EPC8; HEKA Instruments Inc., Bellmore, NY, USA) as previously described (Horiuchi-Hirose et al., 2011). Series resistance was always kept < 7 MΩ and was routinely compensated using an amplifier by ~75%. This resulted in a voltage drop in series resistance of < 3.5 mV in the presence of 2 nA membrane currents. LTCC currents were measured with pipette solution containing (in mmol/l): D-glutamate, 90; N-methyl-D(-)-glucamine, 10; $MgCl_2$, 5; tetraethylammonium chloride, 20; EGTA, 10; HEPES, 20; and ATP 3 (pH=7.3 with CsOH). The bathing solution contained (in mmol/l): N-methyl-D(-)-glucamine, 150; CsCl, 5.4; $CaCl_2$, 2; $MgCl_2$, 1.2; 4-aminopyridine, 2; HEPES, 5; and glucose, 5.5 (pH=7.4 with HCl). The membrane potential was stepped from −90 mV to −50 mV for 200 ms and then for 500 ms to potentials between −110 and +70 mV with a 10 mV increment every 5 s. LTCC currents were isolated as the current inhibited by $Cd^{2+}$ (100 μmol/l) plus nifedipine (10 μmol/l) (Yamada et al., 2008). The peak amplitude of LTCC currents evoked by the 500 ms test pulse was plotted against the membrane potential.

**Table 1**
The peak LTCC current amplitude at 0 mV and the membrane capacitance of non-detubulated (ND) and detubulated (D) myocytes.

| | $I_{Ca}$ at 0 mV (nA) | Membrane capacitance (pF) |
|---|---|---|
| CONT | | |
| ND | 1.38 ± 0.06 | 168.8 ± 6.6 |
| D | 0.53 ± 0.03[a] | 117.1 ± 4.3[a] |
| ISO | | |
| ND | 1.60 ± 0.11 | 229.7 ± 9.5 |
| D | 1.11 ± 0.06[a] | 159.4 ± 8.2[a] |
| ISO+ICI | | |
| ND | 1.91 ± 0.12 | 226.3 ± 7.4 |
| D | 0.76 ± 0.07[a] | 150.0 ± 6.8[a] |
| ISO+ATR | | |
| ND | 1.78 ± 0.06 | 227.3 ± 6.8 |
| D | 0.78 ± 0.06[a] | 156.7 ± 5.7[a] |
| ISO+DPCPX | | |
| ND | 1.80 ± 0.12 | 239.0 ± 5.4 |
| D | 1.16 ± 0.08[a] | 166.1 ± 3.5[a] |

Values are the mean ± S.E.M. $N$=6–8 for each group. CONT, control, ISO, isoproterenol; ICI, ICI118,551; ATR, atropine.

[a] $P < 0.05$ vs. ND.

To assess SS and TT LTCC current densities, the peak LTCC current amplitude at 0 mV and the membrane capacitance were measured in non-detubulated and detubulated myocytes (Table 1). SS and TT LTCC current densities were calculated from these values as previously described (Horiuchi-Hirose et al., 2011). Briefly, the LTCC current density in SS and TT ($D_{SS}$ and $D_{TT}$, respectively) was calculated with the following equations:

$$D_{SS} = (I_D - \alpha I_N)/(C_D - \alpha C_N) \quad (1)$$

$$D_{TT} = (I_N - I_D)/(C_N - C_D), \quad (2)$$

where $I_N$ and $I_D$ are the peak LTCC current amplitude at 0 mV of non-detubulated and detubulated myocytes, respectively; $C_N$ and $C_D$, the membrane capacitance of non-detubulated and detubulated myocytes, respectively; and $\alpha$, fraction of TT membranes remaining in detubulated myocytes. $\alpha$ was estimated as 0.17 from binary image analysis of detubulated myocytes stained with di-8-ANEPPS. Because non-detubulated and detubulated myocytes were different groups of myocytes, the above calculation was carried out with the mean membrane capacitance and current amplitude of each group.

The liquid junction potential of the pipette solution in relation to the modified Tyrode solution was −10 mV and taken into consideration throughout this study.

### 2.10. Statistical analysis

Data are shown as the mean ± S.E.M. Statistical significance was evaluated with Student's unpaired $t$-test. For multiple comparisons of data, analysis of variance with Bonferroni's test was used. $P < 0.05$ was considered significant.