striatum is considered insufficient, at least in primates [35,43]. Although L-dopa may function as a neurotransmitter or modify behaviour through DA-independent mechanisms, central inhibition of AADC with 3-hydroxybenzylhydrazine (NSD-1015) has been shown to result in the abolition of the L-dopa motor effect [44], corroborating the classical concept that L-dopa is pharmacologically inert and its effects are mediated by DA and metabolites. Thus, most of the L-dopa produced in the striatum after gene transfer must be converted to DA *in situ*. Along with decreases in the levels of BH_4, TH and DA observed in PD, the activity of GCH in the striatum is also decreased in this disease [45]. As a low level of endogenous BH_4 does not yield sufficient TH activity, GCH is thought to regulate TH activity by regulating BH_4 biosynthesis, thus indirectly controlling DA production in TH-containing DA neurons [45-47]. In dominantly inherited dopa-responsive dystonia, a mutation in the gene encoding GCH results in a decrease in the level of BH_4, a further decrease in TH activity and a decrease in DA production [48]. Although BH_4 can cross the blood–brain barrier, uptake of exogenous BH_4 from the blood is low [49] and the primary source of BH_4 in the brain is via intracellular biosynthesis. Thus, GCH gene transfer into striatal cells may offer a more efficient method of supplying BH_4 than its exogenous administration.

3.2 Dopamine production in animal models

The availability of well-characterised rodent and non-human primate models makes PD a suitable candidate for gene therapy experiments. These models use neurotoxins that selectively elicit DA neuronal death in the SNc and deplete nigrostrital DA. A rat model of PD is generated by injecting 6-hydroxydopamine (6-OHDA) into the striatum or nigrostriatal pathway [50]. Systemic or intracarotid administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) into monkeys replicates all the cardinal signs of PD, including tremor, rigidity, bradykinesia and postural instability. The primate MPTP model is useful in evaluating motor functions and in assaying transduction efficiency in the larger striatum.

The limited packaging capacity of rAAV vectors (< 5 kb) makes it impossible to use a single vector to express all three enzymes. However, one cell could be simultaneously transduced with multiple rAAV vectors encoding different enzymes. Although the addition of AADC via genetically modified fibroblasts to a system expressing TH and GCH has been reported to result in reduced production of L-dopa, due to feedback inhibition of DA on TH [51,52], direct gene transfer of AADC and TH into the striatum using rAAV vectors has been found to be beneficial in 6-OHDA-lesioned rats [10,36]. Local production of striatal DA was higher in cells expressing GCH, TH and AADC than in cells expressing TH and AADC, and these results have been confirmed in a primate model [38]. rAAV vectors have been shown to efficiently introduce genes encoding DA-synthesising enzymes into the striatum of the MPTP model, resulting in the restoration of motor functions with robust transgene expression and elevated DA synthesis in the treated putamen.

So far, dyskinesia has not been observed in preclinical studies of rAAV vector-mediated gene delivery of DA-synthesising enzymes. Moreover, rAAV vector-mediated delivery of TH and GCH was found to reverse peak-dose dyskinesia in a rat model of PD [53]. Continuous DA production in the striatum may account for the reduced likelihood of dyskinesia. It has been clinically shown that, compared with drugs that have a long duration of effect, short-acting pulsatile DA agonists are more likely to induce dyskinesia in PD patients [5]. Compared with conventional oral therapies, continuous intraduodenal infusion of L-dopa/carbidopa has a greater effect on motor performance improvement, but without increasing dyskinesia [54].

Gene transfer of AADC alone, in combination with oral administration of L-dopa, could be a shortcut to start clinical trials in PD patients [35,39]. Although these patients would still need to take L-dopa to control their PD symptoms, DA production could be regulated by altering the dose of L-dopa. It could be argued that the major reason for failure of L-dopa therapy is that adverse effects overwhelm the therapeutic response (shrinking of the therapeutic window). Many of the late-stage complications of PD result from the very high doses of L-dopa required to induce a therapeutic response. Experimental and clinical experience using inhibitors of catechol-*O*-methyl-transferase (COMT) indicates that a reduction in the dose of L-dopa is important in controlling the dyskinesia associated with prolonged DA half-life [55]. Restoring decarboxylating capacity by gene transfer could potentially allow lower doses of L-dopa and reduce the long-term adverse effects associated with escalating L-dopa therapy [10,35,39,41,56].

4. Protection of the nigrostriatal pathway

An alternative approach to the treatment of PD is to protect the nigrostriatal pathway from progressive degeneration by providing genes encoding growth factors [57], antioxidant molecules or antiapoptotic substances [58]. The slow progressive nature of degeneration in PD makes this approach attractive for arresting or even reversing parkinsonian symptoms. One potential candidate for this strategy is glial cell line-derived neurotrophic factor (GDNF) [57,59], a small glycoprotein that provides strong tropic support for DA neurons [60]. Application of the GDNF protein, however, is limited by its short half-life and its poor ability to cross the blood–brain barrier. An attempt to treat PD patients by direct injection of GDNF protein into the ventricles was unsuccessful [61]. A Phase I safety trial of continuous delivery of GDNF into the postero-dorsal putamen of five PD patients showed alleviation of off-medication motor symptoms and dyskinesia [62], and positron emission tomography (PET) detected increased DA storage, both in the putamen and the substantia nigra, suggesting retrograde transport of GDNF. Delivery of the gene encoding GDNF by viral vectors would be advantageous for PD patients, in that a single injection

would provide sustained GDNF production in the brain. This method does not require implantation of an infusion device, which is likely to increase patient morbidity as well as long-term risks of infection. Many studies have demonstrated that delivery of the *GDNF* gene via rAAV [63-67], adenoviral [68-73] or lentiviral [74,75] vectors protects nigral DA neurons in rodent and primate models of PD. Intrastriatal administration of rAAV vector expressing GDNF prevented the degeneration of DA neurons and promoted behavioural recovery, even 4 – 5 weeks after the onset of progressive degeneration [66,67]. This delayed rescue is important for clinical application, as a substantial number of DA neurons are lost prior to the appearance of symptoms characteristic of PD.

Striatal GDNF appears to be important for the occurrence of functional reinnervation. However, the expression of GDNF in nigral DA neurons may be detrimental, because intense local sprouting may prevent regeneration of the lesioned axons toward the striatum [64,76,77]. Expression of low levels of GDNF in the striatum is sufficient to provide protective effects without affecting DA synthesis [78]. Although it remains to be verified whether neurotoxin-induced animal models faithfully reflect the human disease, neuroprotective gene therapy with neurotrophic factors holds promise as a novel treatment of PD. Furthermore, neuroimaging techniques [79] and genetic analysis of some familial cases [1,3] have provided an opportunity for detecting at-risk individuals prior to the appearance of the symptoms characteristic of PD, thus enabling the application of earlier gene therapy with GDNF.

5. Suppression of the overactive subthalamic nucleus

Hyperactivity of the subthalamic nucleus (STN) is considered a major functional abnormality of PD [80]. Based on a model of basal ganglia circuitry, depletion of DA in the striatum increases the activity of neurons expressing the D2 receptor. These neurons send their inhibitory projections to the external segment of the globus pallidus (GPe), which leads to the over-inhibition of GPe neurons. Reduced inhibitory input from GPe in the STN results in overactivation of the nucleus, and excessive drive of the STN to the internal segment of the globus pallidus (GPi) and to the substantia nigra pars reticulata (SNr) exerts inhibitory effects on the thalamocortical projection and brainstem nucleus. During the past decade, deep brain stimulation of the STN, which may modify STN output, has become a routine treatment for advanced PD patients, providing improvements in motor function, although the fundamental mechanism of STN stimulation remains to be defined. The success of STN stimulation in PD patients, together with the symptomatic relief mimicked by infusion of the $GABA_A$ agonist into the STN [81] or directly into the SNr [82] in a primate model of PD, suggests another gene therapy approach: rAAV vector-mediated gene transfer of glutamate decarboxylase (GAD), an enzyme involved in the biosynthesis of the inhibitory neurotransmitter γ-aminobutylic acid (GABA), into the STN [83,84]. The underlining physiological changes in PD include, in addition to increases in the firing rate of the STN, the tendency of pallidal neurons to fire in more irregular patterns, as well as abnormal oscillatory synchronisation in the basal ganglia [85]. A Phase I clinical trial is underway at present to determine the extent to which *GAD* gene transfer into the STN remedies these abnormalities.

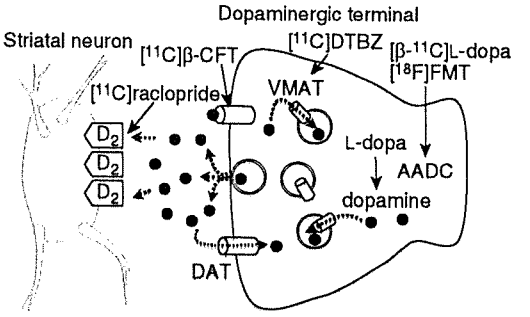

Figure 2. Schematic diagram of a DA synapse, demonstrating specific ligands for PET. [β-^{11}C]L-dopa and [^{18}F]FMT are AADC substrates. Transporter markers include high-affinity ligands for the neuronal DAT, including the cocaine analogue [^{11}C]β-CFT, as well as ligands that bind the VMAT2, such as [^{11}C]DTBZ. Ligands for the D_2 receptor, such as [^{11}C]raclopride, compete with DA and reflect the synaptic DA level. Black circles represent DA.
[β-^{11}C]L-dopa: [β-^{11}C]L-3,4-dihydroxyphenylalanine;
[^{11}C]β-CFT: [^{11}C]2β-carbomethoxy-3β-(4-fluorophenyl)tropane;
AADC: Aromatic-L-amino acid decarboxylase; DA: Dopamine;
DAT: DA transporter; [^{11}C]DTBZ: [^{11}C]dihydrotetrabenazine;
[^{18}F]FMT: 6-[^{18}F]-*m*-tyrosine; PET: Positron emission tomography;
VMAT: Vesicular monoamine transporter.

6. *In vivo* monitoring of transgenes by PET

PET is a valuable technique for imaging altered DA function in PD [86-88]. The effects of therapeutic gene delivery can be assessed using specific positron-labelled ligands developed for evaluating each process of DA turnover [89] (Figure 2). In addition, the level and duration of transgene expression can be directly monitored *in vivo* when the tracer is a substrate for the transgene product [90]. The first tracer used to visualise and assess the integrity of DA presynaptic systems was 6-[^{18}F]-fluoro-L-dopa (FDOPA), a fluoro-analogue of L-dopa. FDOPA is taken up into the DA terminals, decarboxylated by AADC, trapped and stored in synaptic vesicles. FDOPA uptake is highly correlated with viable DA cells in MPTP-lesioned monkeys [91] and in post-mortem human PD brains [92]. One shortcoming that complicates the use of this agent is that metabolites of FDOPA, such as 3-*O*-methyl–FDOPA, formed by the action of the ubiquitous enzyme COMT, enter the brain and diminish image contrast. An alternative tracer is [β-^{11}C]L-dopa, which undergoes less 3-*O*-methylation [93,94] and has a shorter half-life (20 min for ^{11}C). The use of this tracer has made it possible to obtain sequential imaging data not only

during the early steps of DA metabolism (e.g., decarboxylation by AADC), but also during DA receptor binding and DA transporter availability in the same subject on the same day. With an ~ 2-h interval between PET scans, each can be performed without any interference by radioactivity from the previous scan. In addition, PET scans can be performed using the non-catecholic tracer 6-[^{18}F]-*m*-tyrosine (FMT), which is also a good substrate for AADC and is not metabolised by COMT [87,90,95]. As FMT uptake has about twice the sensitivity of FDOPA uptake, FMT and [β-^{11}C]L-dopa are the ligands of choice for assessing the distribution and activity of AADC delivered by rAAV vectors.

To evaluate the functional effects of gene therapy in PD, DA release following pharmacological challenges can be measured indirectly *in vivo*, as reflected by reductions of DA receptor availability to selective antagonists such as [^{11}C]raclopride [96-98]. A 1% change in striatal [^{11}C]raclopride binding corresponds to at least an 8% change in synaptic DA levels [99]. PET measurements with [^{15}O]H_2O can also monitor alterations in regional cerebral blood flow, both during resting conditions and in response to stimuli. In PD, activation was found to be reduced in the premotor and prefrontal cortices during performance of a paced motor task [100].

7. Regulation of transgene expression

Most of the existing rAAV vectors rely on strong viral promoters that exhibit constitutive expression of transgenes. Excess production of therapeutic proteins, however, can often cause adverse effects. Vector constructs that allow the regulation of gene expression are necessary to maintain functional concentrations within therapeutic windows. However, so far there are no clinically available regulatable systems based on rAAV vectors, although several systems have been developed for preclinical studies. The most popular systems incorporate inducible promoters, which can respond to drugs and/or hormones. Inducing agents employed in these systems include RU486 (mefipristone) [101], rapamycin [102] and tetracycline [103]. Another system by which transgene expression can be shut down is viral vector-mediated delivery of bacteriophage Cre recombinase [104]. In this system, the therapeutic gene is flanked by *lox*P sequences, allowing the Cre recombinase to excise the transgene. RNA technology, including RNA interference [105,106] and ribozymes [107,108], has become more popular, and these systems can be used to reduce transgene expression. In addition, cell type-specific promoters may be useful for targeting specific brain regions, although rAAV vectors based on AAV2 preferentially transduce neurons.

8. Expert opinion and conclusion

rAAV vectors have been found to expand the potential of gene therapy, allowing the treatment of a wide range of neurological diseases, including PD [25,109]. Two Phase I clinical trials testing rAAV vectors for PD have been initiated in the US. These trials involve the delivery of the *GAD* gene into the STN and the delivery of the *AADC* gene into the putamen. Vectors used in these protocols do not contain a regulatory component for controlling gene expression. However, stimulation or coagulation of STN potentially reverses the unanticipated effects of GAD expression, and DA cannot be synthesised in the absence of L-dopa administration after transfer of the *AADC* gene. Incorporation of a regulatory component is necessary to increase safety in future clinical trials that are intended to allow local DA synthesis without L-dopa administration. However, DA receptor antagonists, such as haloperidol, could be used in cases of DA overproduction.

Fetal cell transplantation has been applied clinically to patients with advanced PD, with the intention of replacing the degenerated DA neurons. If the primary mechanism underlying recovery in these cell therapies is restoration of dopaminergic neurotransmission, a more straightforward approach would be direct delivery of genes encoding DA-synthesising enzymes into the striatum. However, replacement of DA in the striatum may have no effect on non-motor problems in PD, including affective and autonomic disturbances associated with pathologies not involving dopaminergic pathway degeneration. Broad regions of the brain beyond the motor parts of the basal ganglia are involved in the manifestation of L-dopa-induced dyskinesia [110]. This should be considered when delivery of the *TH* and *GCH* genes is intended to reduce dyskinesia. If reconstruction of the neural network, including the nigrostriatal pathway, is necessary to ameliorate the more complex symptoms of PD, such as dementia, a combination of gene therapy and cell transplantation would be the next strategy for overcoming this complex task. Ongoing clinical trials should provide information showing the extent to which rAAV-mediated gene therapy alleviates motor symptoms in advanced PD patients who responded to L-dopa therapy at early stages.

Recently developed neuroimaging techniques and genetic analysis in some familial cases of PD may provide information enabling the identification of at-risk individuals before characteristic symptoms appear. Earlier gene therapy with neuroprotective molecules could be applied to these at-risk individuals, as well as to patients known to have PD. The elucidation of the mechanism by which genetic mutations lead to the loss of DA neurons in familial forms of PD, as well as the detection of factors that increase the risk for PD, will provide new targets for gene therapy [111].

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Cerebrospinal Fluid Neprilysin is Reduced in Prodromal Alzheimer's Disease

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Amyloid β peptide (Aβ) has been implicated in Alzheimer's disease (AD) as an initiator of the pathological cascades. Several lines of compelling evidence have supported major roles of Aβ-degrading enzyme neprilysin in the pathogenesis of sporadic AD. Here, we have shown a substantial reduction of cerebrospinal fluid (CSF) neprilysin activity (CSF-NEP) in patients with AD-converted mild cognitive impairment and early AD as compared with age-matched control subjects. The altered CSF-NEP likely reflects changes in neuronal neprilysin, since transfer of neprilysin from brain tissue into CSF was demonstrated by injecting neprilysin-carrying viral vector into the brains of neprilysin-deficient mice. Interestingly, CSF-NEP showed an elevation with the progression of AD. Along with a close association of CSF-NEP with CSF tau proteins, this finding suggests that presynaptically located neprilysin can be released into CSF as a consequence of synaptic disruption. The impact of neuronal damages on CSF-NEP was further demonstrated by a prominent increase of CSF-NEP in rats exhibiting kainate-induced neurodegeneration. Our results unequivocally indicate significance of CSF-NEP as a biochemical indicator to pursue a pathological process that involves decreased neprilysin activity and Aβ-induced synaptic toxicity, and the support the potential benefits of neprilysin up-regulation in ameliorating neuropathology in prodromal and early AD.

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Numerous investigations have supported the contention that senile plaques and neurofibrillary lesions, composed primarily of amyloid β peptide (Aβ) and tau proteins, respectively, are not only descriptive characteristics of histopathology in brains with Alzheimer's disease (AD), but also mechanistically related to the pathogenesis of AD. That all of the genetic mutations causally linked to familial AD induce overproduction of either total Aβ or relatively amyloidogenic Aβ42[1] further provides supportive evidence for the role of Aβ accumulation as an initiator of the pathological cascade toward the onset of AD.[2]

The diagnosis of AD is definite based on magnitudes of these hallmark lesions after an autopsy, whereas exploitation of AD-specific biochemical markers reflecting central pathogenic processes, such as degeneration of neurites and synapses and alterations of Aβ and tau, for antemortem diagnosis is still ongoing. Since 1995, two categories of cerebrospinal fluid (CSF) markers, CSF-tau and CSF-Aβ42, have emerged and have proved to be useful indicators to assist clinical diagnosis of AD in living patients.[3–5] Furthermore, several recent studies have demonstrated usefulness of CSF-tau in differentiation of prodromal AD from AD-unrelated cognitive decline among patients with mild cognitive impairment (MCI).[6,7] In contrast, altered processing of amyloid precursor protein and Aβ has not been detected by biochemical markers such as CSF-Aβ42 in MCI patients,[6] although it is conceived to be upstream of tau abnormalities in the cascade of AD pathogenesis. Because therapeutic approaches, including existing drugs[8] and emerging treatments modifying Aβ pathology,[9,10] presumably have the greatest potential of being effective in the predementia phase of AD, accurate prediction of conversion to AD in patients with MCI by means of biological indices representing abnormal metabolism of amyloid precursor protein/Aβ is particularly crucial. This gives us a rationale of analyzing accessible body fluid in search for altered levels of

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Table 1. Clinical Characteristics of Study Subjects (mean ± SE)

Characteristic	Control	sMCI	pMCI	AD
No. of patients	27	5	33	32
Male/female ratio	7/19	5/0	10/13	9/23
Age (yr)	70.0 ± 1.5	74.4 ± 6.1	74.5 ± 1.0	73.1 ± 1.3
Years of education	10.8 ± 0.4	9.2 ± 0.1	11.7 ± 0.5	9.8 ± 0.9
MMSE score at baseline	28.6 ± 0.3	26.8 ± 0.8	25.3 ± 0.3	16.8 ± 1.0
Delayed recall score on WMS-R	95.0 ± 5.5	66.0 ± 4.3	58.5 ± 2.0	—
Years of follow-up	1.9 ± 0.1	1.9 ± 0.2	2.0 ± 0.4	1.3 ± 0.1
Changes in MMSE score by the end of follow-up	0.3 ± 0.1	−0.7 ± 0.3	−3.3 ± 0.4	−1.7 ± 0.6
Annual changes in MMSE score	0.14 ± 0.07	−0.39 ± 0.14	−1.74 ± 0.23	−1.48 ± 0.52

SE = standard error; sMCI = stable mild cognitive impairment; pMCI = progressive MCI; AD = Alzheimer's disease; MMSE = Mini-Mental State Examination; WMS-R = Wechsler Memory Scale–Revised.

molecules in close association with pathogenic Aβ accumulation in the brain.

One notable feature of Aβ metabolism is that it is a normal physiological process occurring in diverse cell types. Because there has been no overt evidence for an increased production of Aβ in sporadic AD, the molecular mechanism of Aβ degradation is of growing interest. The neutral endopeptidase neprilysin (EC 3.4.24.11) is one of the enzymes implicated in physiological Aβ catabolism.[11,12] In neurons, it is localized primarily to the presynaptic terminals, with its ectodomain facing extracellular matrix,[11,13] and thus is capable of degrading extracellular Aβ released from nerve ends. Recent genetic approaches using neprilysin-deficient mice have demonstrated the ability of neprilysin to cleave endogenous Aβ.[12,14] Moreover, a decline of neprilysin levels has also been found in the brains of patients with early-stage sporadic AD,[15] suggesting critical roles played by reduced neprilysin activity in the incipient process of Aβ accumulation.

The purpose of the study reported here was to assess applicability of monitoring neprilysin activities in CSF (CSF-NEP) and plasma (plasma-NEP) of patients with MCI and AD for prediction of clinical course and for gaining insights into molecular events early in Aβ pathogenesis. The results showed a significant decrease of CSF-NEP, which developed to AD, in patients with MCI and in patients with mild AD, indicating usefulness of CSF-NEP assay as an informative clinical adjunct.

Subjects and Methods

Subjects

We studied 96 patients (mean age ± standard error, 72.5 ± 0.8 years) who underwent evaluations for memory disturbance at the Tohoku University Hospital Outpatient Clinic on Dementia. Clinical assessments by geriatricians and neuropsychological examinations, including Mini-Mental State Examination (MMSE) and Wechsler Memory Scale Revised were performed for all patients, as described in detail previously.[16] Our established criteria[16] based on the current consensus[17] were used for diagnosis of amnestic MCI, and a diagnosis of AD was made in accordance with the National Institute of Neurological and Communication Disorders-Alzheimer's Disease and Related Disorders Association criteria.[18] Consequently, 38 patients fulfilled the diagnostic criteria for amnestic MCI, 32 patients were diagnosed as having AD, and 26 patients were found to be cognitively normal at baseline investigation.

During the 2-year follow-up period, 33 of the patients with amnestic MCI progressed to AD and were thus classified into progressive MCI (pMCI). Five patients with amnestic MCI who showed unchanged or improved cognitive functions remained, and they were categorized as having stable MCI (sMCI). Twenty-eight patients with pMCI and all of the patients who were diagnosed as having AD at baseline were treated with a 5mg daily dose of donepezil hydrochloride.

At baseline examination, plasma and CSF samples were collected from each patient. CSF-tau was determined using a sandwich enzyme-linked immunosorbent assay designed for measurement of total tau (INNOTEST hTau antigen; Innogenetics, Gent, Belgium), as described elsewhere.[4] CSF-Aβ42 was also quantified with a specifically constructed sandwich enzyme-linked immunosorbent assay system.[6] The sample collection was performed after written informed consent was obtained from each participant or a family member. Demographic profiles of the patients examined in this study are summarized in Table 1.

Plasma and Cerebrospinal Fluid Neprilysin Activity Assay

Before high-throughput analysis, neprilysin in CSF was identified by immunoblotting for a subset of CSF samples with antibody against human neprilysin (goat polyclonal; 1:400 dilution; Genzyme-TECHNE, Minneapolis, MN). Lysates of murine primary cortical neurons overexpressing human neprilysin[19] were used as control samples. Subsequently, CSF-NEP in all patients was fluorometrically assayed as thiorphan-inhibitable peptidase activity, based on the previously described protocol.[14] Briefly, a 20μl CSF sample was used for enzymatic cleavage of succinyl-Ala-Ala-Phe-AMC, with or without thiorphan, a specific inhibitor of neprilysin. Similarly, plasma-NEP was biochemically quantified by using 5μl plasma samples.

The application of fluorometric assay to CSF samples was

validated by examining correlation between intensity of immunoblotting signal and measured CSF-NEP. In addition, we assessed the specificity of CSF-NEP assay by immunodepleting CSF samples with antibody against human neprilysin (Genzyme-TECHNE; goat polyclonal) and protein G agarose slurry (Oncogene/Calbiochem, Cambridge, MA). Antibody against $GABA_A$ receptor α_1 subunit (Santa Cruz, Santa Cruz Biotechnology, CA; goat polyclonal) was also used as a control antibody for immunodepletion.

Biochemical Characterization of Cerebrospinal Fluid Neprilysin

Cortical brain samples from autopsy-confirmed AD cases were homogenized with 4 volumes of 50mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.6) containing 150mM sodium chloride (NaCl) and protease inhibitor cocktail and centrifuged at 200,000× *g* for 20 minutes at 4°C. The resultant pellet was rehomogenized with 3 volumes of the above-mentioned buffer plus 1% Triton X-100 (Sigma Labs, St. Louis, MO) and centrifuged at 200,000× *g* for 20 minutes at 4°C. The supernatant was then used for immunoblot analysis. CSF samples from the patients were processed with sodium dodecyl sulfate polyacrylamide gel electrophoresis Clean-Up Kit (Amersham Biosciences, Piscataway, NJ). Lysates of murine primary cortical neurons overexpressing human neprilysin[19] and recombinant protein corresponding to the extracellular domain of human neprilysin (Genzyme-TECHNE) were also used as control samples. In addition, aliquots of the protein samples were chemically deglycosylated by using trifluoromethanesulfonic acid.[20] The nondeglycosylated and deglycosylated protein samples (~10μg) were applied to immunoblotting with antibody against human neprilysin (Genzyme-TECHNE).

Animal Experiments

To prove the transport of neuronal neprilysin from the brain into CSF, we assayed CSF-NEP in neprilysin-deficient mice after intrahippocampal injection of recombinant adeno-associated viral vector expressing human neprilysin (rAAV-NEP), which was prepared as described elsewhere.[14] Twelve neprilysin-deficient mice (generously provided by Dr C. Gerard, Harvard Medical School), aged 18 to 20 months, were injected with 0.6μl rAAV-NEP preparations (~1.3×10^{10} genome copies) into the bilateral dentate gyri of the hippocampus (stereotactic coordinates: anteroposterior, 2.4mm; mediolateral, 2.0mm; and dorsoventral, 2.0mm). At 10 weeks after injection, the mice were anesthetized with pentobarbital, and CSF was isolated from the cisterna magna compartment under a dissecting microscope, based on the protocol by DeMattos and colleagues.[21] CSF samples (20–30μl from each mouse) were combined into three pooled volumes. After the collection of CSF, blood sampling was performed by cardiac puncture. The mice were then transcardially perfused with phosphate-buffered saline (PBS), and the hippocampi were dissected, homogenized with 9 volumes of 50mM Tris-hydrochloride buffer (pH 7.6) containing 150mM NaCl and EDTA-free protease inhibitor cocktail, and centrifuged at 200,000× *g* for 20 minutes at 4°C. The resultant pellet was rehomogenized with 2 volumes of above-mentioned buffer plus 1% Triton X-100 and centrifuged at 200,000× *g* for 20 minutes at 4°C. The supernatant was used as solubilized membrane fraction for biochemical analyses. For comparison, CSF, plasma, and hippocampal samples were also obtained from 12 wild-type and 12 neprilysin-deficient mice, aged 20 to 22 months, that were untreated. Plasma- and CSF-NEP were measured as described earlier, and a similar experimental procedure was applied to assay for hippocampal neprilysin activity by using 10μg protein in the membrane fraction. In the plasma and hippocampal analyses, 3 of 12 mice were randomly chosen in each study group, and samples from the selected mice were used for the assays. In addition, we also performed immunoblotting of neprilysin in pooled CSF samples using antimouse neprilysin antibody (Genzyme-TECHNE; goat polyclonal). Recombinant mouse neprilysin (Genzyme-TECHNE) was used as a control.

Because neprilysin in degenerating neurons can be released into CSF due to disruption of membrane structures to which neprilysin is anchored, CSF-NEP is likely to be affected by not only the level of brain neprilysin, but also the magnitude of neurodegeneration. Hence, we examined CSF-NEP in rats after low- and high-dose administrations of kainic acid (KA), an inducer of excitotoxic insults in the CNS neurons. At 7 weeks old, nine female Sprague–Dawley rats were divided into three groups. Rats in the control group were intraperitoneally injected with PBS, and the low- and high-dose KA groups underwent intraperitoneal administration of 10 and 50mg/kg KA dissolved in PBS, respectively. CSF and blood samples were collected in the control rats and rats treated with low-dose KA at 48 hours after injection as in the mouse experiment. All rats in the high-dose KA group exhibited a lethal status epilepticus within 5 hours of injection, and CSF and blood were sampled immediately after death of the animals. After CSF and blood collections, rats in all groups were transcardially perfused with PBS, and the brains were removed. The right hemisphere was fixed overnight with 4% paraformaldehyde in phosphate buffer (pH 7.4). Protein level and enzymatic activity of neprilysin in the CSF, plasma, and hippocampal samples were quantified by immunoblotting and fluorometric assay, respectively, as described earlier. We also analyzed levels of tau in CSF by immunoblotting with anti-tau antibody (Tau-1; mouse monoclonal; Chemicon, Temecula, CA). Before immunoblotting, albumin and IgG were removed from CSF preparations using a ProteoExtract Albumin/IgG Removal Kit (Calbiochem, San Diego, CA), followed by dephosphorylation of the samples using alkaline phosphatase (Sigma). For histochemical and immunohistochemical analyses, representative 10μm frozen sections of the right hemisphere were generated. Extents of excitotoxic insults and synaptic loss were investigated by immunofluorescence staining with antibodies against calpain-cleaved α-spectrin (rabbit polyclonal)[22] and vesicular GABA transporter (117G4; rabbit polyclonal; Synaptic Systems GmbH, Goettingen, Germany), respectively. Amount of neprilysin was also examined by using antineprilysin antibody (56C6; mouse monoclonal; Novocastra Laboratories, Newcastle, United Kingdom). Immunostaining signals were amplified with a TSA-Direct kit (NEN Life Science Products, Boston, MA).

Statistical Analyses

For group comparisons of clinical and biochemical variables, one-way analysis of variance was done, followed by Bonfer-

roni multiple comparison test. Correlations between two variables were tested by the t statistic.

Results

Reduction of Cerebrospinal Fluid Neprilysin Activity in Early Stages of Alzheimer's Disease Pathogenesis

In 11 CSF samples, a close correlation between intensity of neprilysin immunoblotting signal and measured CSF-NEP was observed (Figs 1A, B). Moreover, CSF-NEP was substantially reduced by immunodepleting the samples with antineprilysin antibody (see Fig 1C). These findings justify the application of CSF-NEP assay to high-throughput analysis of CSF samples with sufficient specificity.

Quantified CSF-NEP in the control, pMCI, and AD groups is shown in Figure 1D. CSF-NEP was signifi-

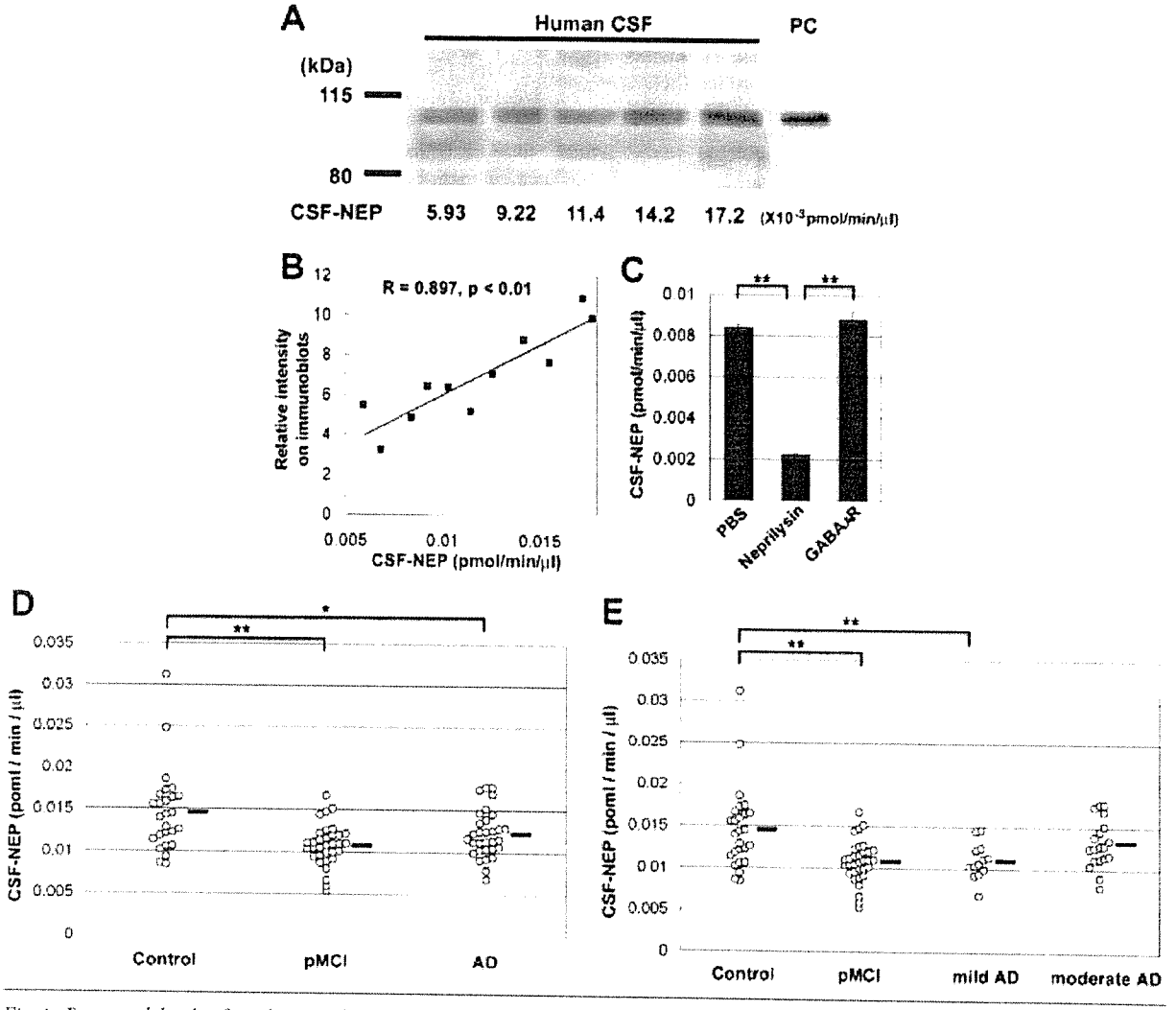

Fig 1. Decreased levels of cerebrospinal fluid neprilysin activity (CSF-NEP) in patients with incipient and mild Alzheimer's disease (AD). (A) Representative immunoblotting of neprilysin in human CSF samples demonstrates that apparent molecular mass of CSF neprilysin is nearly the same as that of the human neprilysin from primary culture (PC) of cortical neurons. The same volume of CSF preparation was loaded in each lane. CSF-NEP determined by fluorometric assay of enzymatic peptidolysis using the same sample is shown at the bottom. (B) CSF-NEP showed a close correlation with intensity of neprilysin immunoblotting signal in 11 human CSF samples. (C) CSF-NEP was reduced substantially by immunodepletion with antineprilysin antibody (neprilysin) relative to those in control subjects treated with either phosphate-buffered saline or antibody against anti-$GABA_A$ receptor α_1 subunit ($GABA_AR$). All assays were performed in triplicate. Bars *represent standard error. (D) A significant reduction of CSF-NEP was observed in patients with progressive mild cognitive impairment and AD compared with control subjects. Each* circle *represents the value obtained for a single individual, and* horizontal lines *represents the mean value in each group. (E) Patients with mild AD (Mini-Mental State Examination [MMSE] score, >17) showed a significant decline of CSF-NEP, whereas levels of CSF-NEP in patients with moderate AD (MMSE score, 9–17) were similar to those in control subjects.* $^*p < 0.05$; $^{**}p < 0.01$.

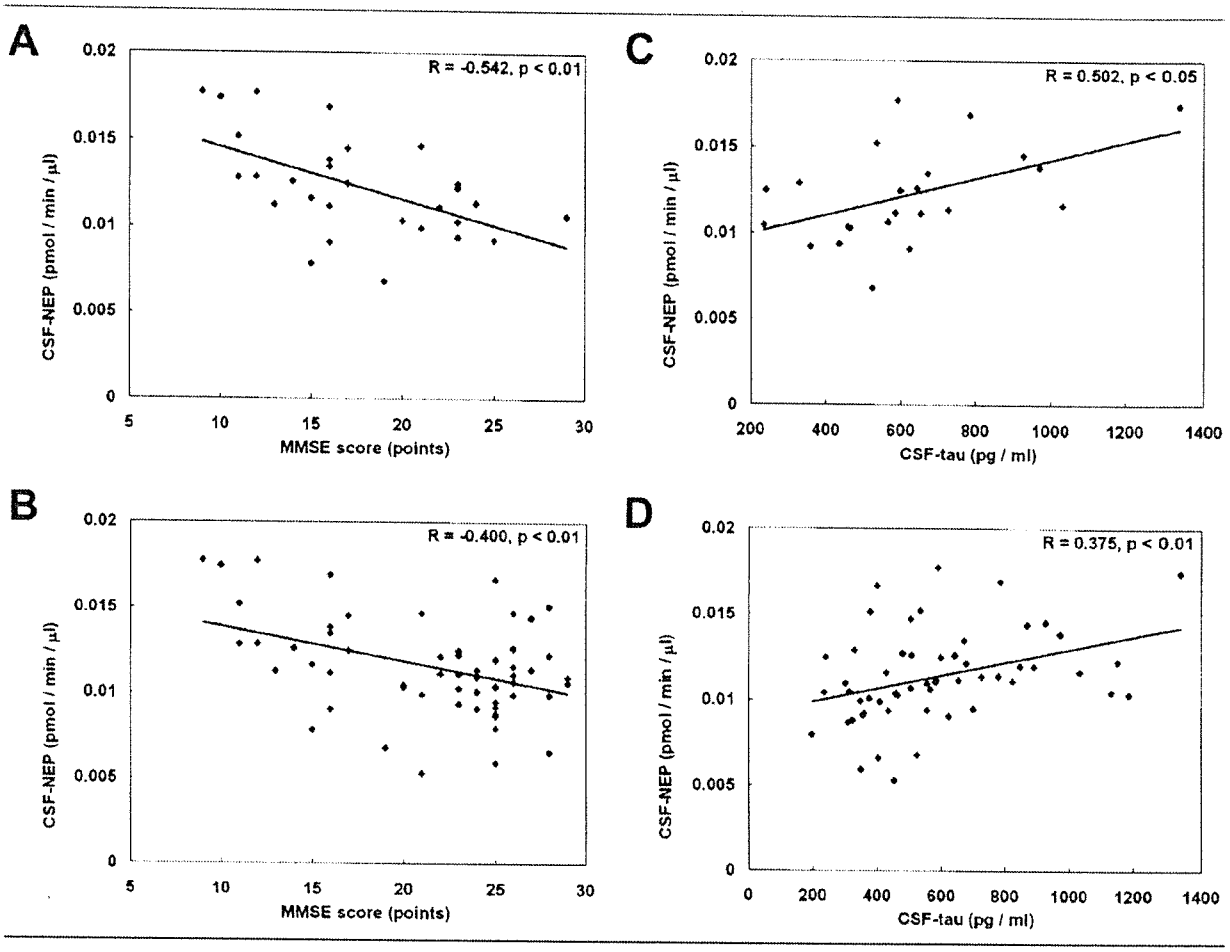

Fig 2. Association of cerebrospinal fluid neprilysin activity (CSF-NEP) (CSF-NEP) with disease severity assessed by Mini-Mental State Examination (MMSE) score and CSF-tau level in patients with progressive mild cognitive impairment (pMCI) and Alzheimer's disease (AD). (A) CSF-NEP was increased with disease progression in AD patients and was significantly correlated with MMSE score. (B) A significant correlation between CSF-NEP and MMSE score was also observed when patients with pMCI were added to the study group. (C) CSF-NEP showed a significant and positive correlation with CSF-tau in AD patients. (D) Correlation between CSF-NEP and CSF-tau levels remained significant when patients with pMCI were also included in the analysis. Solid lines *represent the linear regression of the data.*

cantly decreased in pMCI patients (0.0108 ± 0.0004pmol/min/µl; mean ± SE) compared with subjects in the control group (0.0146 ± 0.0008pmol/min/µl). There also was a significant decrease of CSF-NEP in AD patients (0.0121 ± 0.0005pmol/min/µl) compared with the control group. When the AD patients were subdivided into mild (MMSE scores, >17 points) and moderate AD groups (MMSE scores, 9–17 points), patients with mild AD showed a significant reduction of CSF-NEP (0.0107 ± 0.0006pmol/min/µl) relative to the control subjects (see Fig 1E). By contrast, CSF-NEP in patients with moderate AD (0.0132 ± 0.0007pmol/min/µl) was similar to the control level (see Fig 1E). Alteration of CSF-NEP during progression of AD was more intensively analyzed by plotting CSF-NEP data in AD patients against their MMSE scores. Notably, levels of CSF-NEP in AD patients showed a significant inverse correlation with MMSE scores (Fig 2A). The correlation remained significant after data from pMCI patients were added to the plot (see Fig 2B). Thus, it is conceivable that occurrence of a prominent CSF-NEP reduction is confined to early stages of AD pathogenesis, and thereafter CSF-NEP is likely to be reversed to greater levels with advance of the disease.

AD patients with high levels of CSF-NEP showed higher levels of CSF-tau, thus the correlation between the two variables was significant (see Fig 2C). There also was a significant correlation between CSF-NEP and CSF-tau when patients with pMCI were combined with AD patients for analysis (see Fig 2D). Because CSF-tau is supposed to increase as a function of cytoskeletal disruption and abnormal tau accumulation in neurons, these findings support an increased diffusion of neuronal

neprilysin to the extracellular matrix and CSF as a consequence of injuries of neuronal membranes.

CSF-NEP was unrelated to CSF-Aβ42 in AD patients ($R = -0.112$, $p > 0.05$; data not shown), whereas correlation between these two variables became significant when patients with AD and pMCI were included for analysis ($R = -0.262$, $p < 0.05$; data not shown).

CSF-NEP was not correlated with age and sex in any of the studied groups. In addition to the aforementioned simple correlation analyses, multiple regression with stepwise selection option was used as an exploratory tool for identification of primary factors that determine levels of CSF-NEP in patients with pMCI and AD. As listed in Table 2, MMSE score and CSF-tau were selected as independent variables. CSF-tau showed the greatest partial correlation to CSF-NEP, and MMSE score also had a tendency to be correlated to CSF-NEP. Other variables, including age, sex, and CSF-Aβ42 were eliminated by the stepwise selection; thus, it is likely that the marked influence of the disease severity on both CSF-NEP and CSF-Aβ42 produced an apparent correlation between these two CSF measures in the simple correlation analysis.

CSF-NEP in sMCI subjects (0.0147 ± 0.001pmol/min/μl) was similar to that in control subjects ($p > 0.05$) and was significantly greater than that in pMCI subjects ($p < 0.05$), implying applicability of CSF-NEP measurement to differentiation between pMCI and sMCI at baseline examination.

Neprilysin in CSF was further characterized by immunoblotting (Fig 3). Deglycosylated neprilysin in the brain and CSF showed the same molecular mass, which was larger than the apparent molecular weight of extracellular domain of neprilysin and corresponded to the predicted size of the full-length form. Therefore, secretion of neprilysin from neurons to the extracellular matrix likely occurs without shedding of the full-length enzyme.

Plasma-NEP did not differ among the control (1.669 ± 0.306pmol/min/μl), pMCI (1.777 ± 0.429pmol/min/μl), and AD (1.944 ± 0.395pmol/min/μl) groups. Patients with mild (2.036 ± 0.725pmol/min/μl) and moderate AD (1.816 ± 0.494pmol/min/μl) exhibited similar levels of plasma-NEP. Moreover, there was no significant correlation between levels of CSF-NEP and plasma-NEP in any of the examined groups ($R = 0.103$, $p > 0.05$; data not shown). Hence, the CSF-NEP changes in patients with pMCI and early AD observed in this study were unrelated to the status of plasma neprilysin, but they conceivably reflected an altered transfer of neprilysin from the brain to CSF in these patients. This notion was further tested by the following experiments using rAAV-treated, neprilysin-deficient mice and KA-treated rats.

Table 2. Multiple Regression Analysis for Patients with pMCI and AD

Independent Variable	Partial Correlation Coefficient	p
MMSE	−0.237	0.096
CSF-tau	0.292	0.042

$R^2 = 0.152$, $F = 3.93$, $p = 0.027$.
Age, sex, and CSF-Aβ42 were eliminated from independent variables by the stepwise selection.
pMCI = progressive mild cognitive impairment; AD = Alzheimer's disease; MMSE = Mini-Mental State Examination; CSF = cerebrospinal fluid.

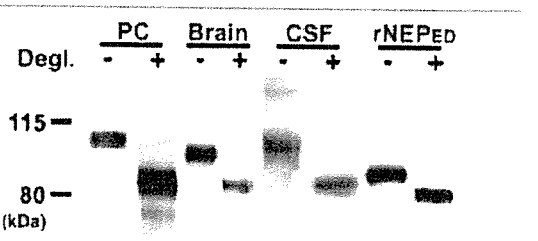

Fig 3. Biochemical characteristics of neprilysin in the brain and cerebrospinal fluid (CSF) from the human subjects. Non-deglycosylated neprilysin in the CSF sample from an AD patient migrated at approximately 95kDa, which was nearly the same as the apparent molecular mass of the human neprilysin from primary culture (PC) of cortical neurons, whereas neprilysin in the membrane-associated fraction extracted from the brain migrated slightly faster than the CSF neprilysin. After deglycosylation, the apparent molecular weight of neprilysin in the PC, brain, and CSF became approximately 85kDa, which corresponds to the predicted size of full-length, unmodified neprilysin. The recombinant extracellular domain of human neprilysin ($rNEP_{ED}$) exhibited smaller apparent molecular mass than other samples.

Physiological Transfer of Neprilysin from the Brain to Cerebrospinal Fluid Demonstrated in Mice

Neprilysin activity was assayed in the hippocampus, CSF, and plasma of neprilysin-deficient mice injected with rAAV-NEP, as well as untreated wild-type and neprilysin-deficient mice. Hippocampal neprilysin activity was nearly undetectable in the untreated neprilysin-deficient mice, whereas it prominently increased at 10 weeks after injection with rAAV-NEP (Fig 4A). There remained unnegligible signals in CSF-NEP assay for untreated neprilysin-deficient mice, which may be produced by degradation of the substrate by thiorphan semisensitive endopeptidases (see Fig 4B). In rAAV-NEP–treated, neprilysin-deficient mice, CSF-NEP showed a significant increase to approximately 70% of CSF-NEP in wild-type mice (see Fig 4B). By contrast, plasma-NEP in neprilysin-deficient mice treated with rAAV-NEP stayed at an undetectable level, similar to

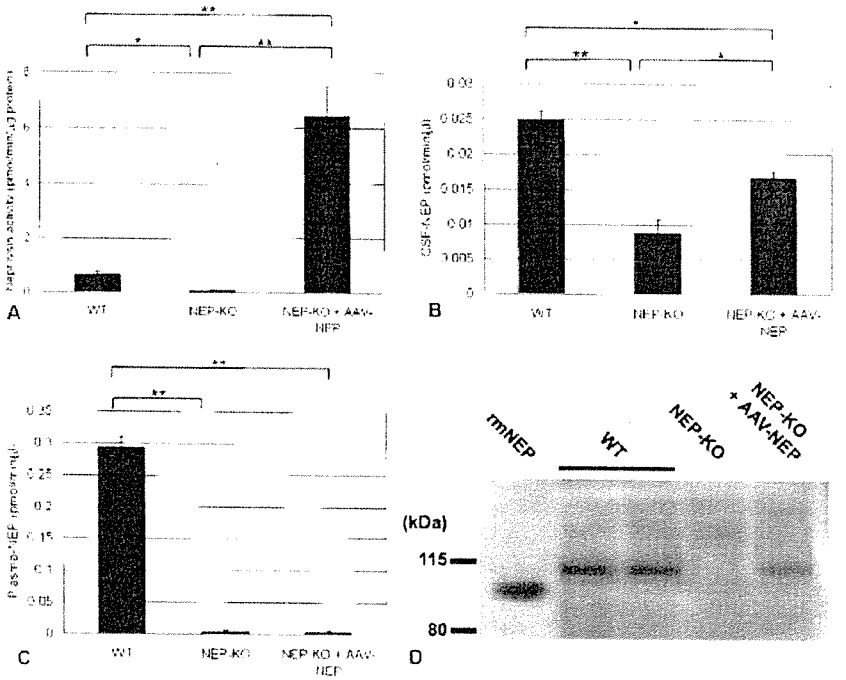

Fig 4. Physiological transfer of neprilysin from the brain into CSF demonstrated by neprilysin-deficient mice injected with recombinant adeno-associated viral vector expressing human neprilysin (rAAV-NEP). (A) Hippocampal neprilysin activity was nearly undetectable in untreated neprilysin-deficient mice (NEP-KO). By contrast, NEP-KO mice at 10 weeks after intrahippocampal rAAV-NEP injection (NEP-KO + AAV-NEP) showed a pronouncedly high level of CSF-NEP, which was approximately 10-fold greater than the endogenous neprilysin activity in wild-type (WT) mice. (B) CSF samples of NEP-KO mice did not produce overt signals compared with those of WT mice. After administration of rAAV-NEP, CSF-NEP in NEP-KO mice was increased to 70% of the endogenous level in WT mice. (C) Unlike CSF-NEP, plasma-NEP did not display an apparent increase after treatment with rAAV-NEP. (D) Immunoblotting of neprilysin in mouse CSF showed consistency with CSF-NEP assay. Each lane was loaded with either CSF sample pooled from three mice or recombinant murine neprilysin (rmNEP). Bars *represent standard error.* $^{*}p < 0.05$; $^{**}p < 0.01$.

untreated neprilysin-deficient mice (see Fig 4C). The transfer of neprilysin from the brains of rAAV-NEP–injected, neprilysin-deficient mice into CSF was also clearly demonstrated by immunoblotting of neprilysin in CSF samples (see Fig 4D). The predominance of the association between neprilysin activities in the brain and CSF over the plasma-CSF correlation suggests strong impacts of brain neprilysin activity on CSF-NEP through transfer of neuronal neprilysin into CSF in physiological conditions.

Pathological Transfer of Neprilysin from the Brain to Cerebrospinal Fluid in Kainic Acid–Treated Rats

In rats injected with KA, there was a KA-induced increase of CSF-NEP in a dose-dependent fashion; low-dose, KA-treated rats showed a slight and insignificant increase of CSF-NEP, and a pronounced increase in CSF-NEP (68-fold greater than the control level) was observed in rats treated with high-dose KA (Fig 5A). Unlike CSF-NEP, plasma-NEP did not significantly differ among the three groups (see Fig 5B). Immunoblotting of neprilysin also indicated a remarkable increase of neprilysin in CSF samples from rats treated with high-dose KA (see Figs 5C [top panel], D [left panel]). In addition to neprilysin, levels of tau in CSF were prominently increased in rats injected with high-dose KA (see Figs 5C [bottom panel], D [right panel]). These data support an aberrantly increased transfer of both neprilysin and tau from damaged brain to CSF, providing a molecular basis for the close association between CSF-NEP and CSF-tau in patients with pMCI and AD (see Figs 2C, D).

Immunoblotting of neprilysin showed that neprilysin in membrane-associated protein fraction from the hippocampi was significantly reduced in rats injected with high-dose KA (Figs 6A, B [left panel]). Neprilysin in Tris/NaCl-soluble fraction had a tendency to increase in KA-treated rats in a dose-dependent fashion, although the increase was not statistically significant (see Figs 6A, B [right panel]). Immunohistochemistry for fragmented α-spectrin indicated extensive activation of calpains in the entire hippocampus except the dentate gyrus after administration of KA at a high dose (see Figs 6C, D). A marked reduction of neprilysin immu-

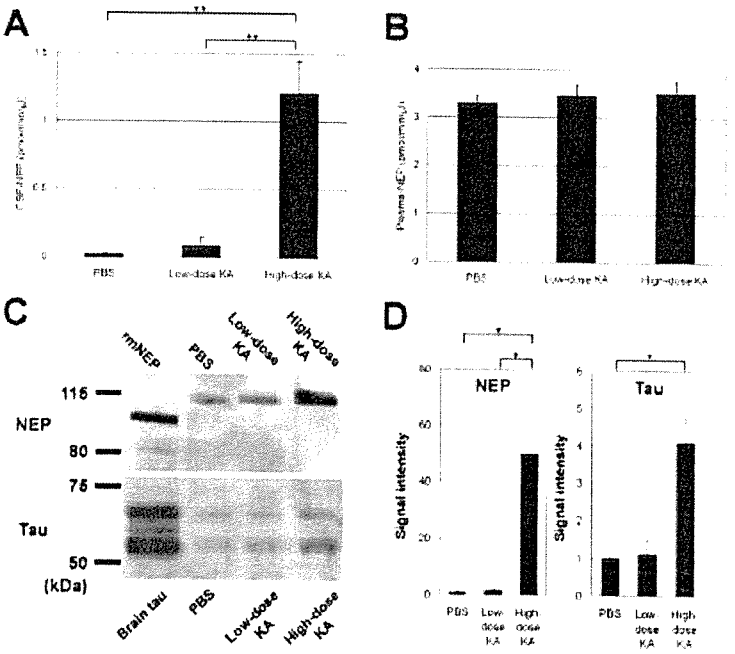

Fig 5. Increased transfer of neprilysin from the brain into cerebrospinal fluid (CSF) in a pathological condition as demonstrated by kainic acid (KA) challenge for rats. (A) Cerebrospinal fluid neprilysin activity (CSF-NEP) was increased after KA administration in a dose-dependent fashion. (B) Plasma-NEP did not significantly differ between rats injected with phosphate-buffered saline and KA. (C) Immunoblotting of neprilysin (top) also indicates a prominent increase of neprilysin in CSF from rats treated with high-dose KA. The CSF sample from a rat injected with high-dose KA was diluted fivefold, and equal volume was loaded in each lane. In addition to neprilysin, levels of tau proteins in CSF (bottom) were significantly increased in rats treated with high-dose KA. Dephosphorylated soluble fraction from rat brain tissue was applied as a control in tau immunoblotting. (D) Intensitometric data constituted from immunoblotting signals demonstrate significant increase in levels of NEP and tau in CSF from rats treated with high-dose KA. Bars *represent standard error.* $^{*}p < 0.05$; $^{**}p < 0.01$.

noreactivity (see Figs 6E, F) accompanying loss of presynaptic signals in interneurons (see Figs 6G, H) was found in the hippocampal CA1 region of the rats treated with high-dose KA compared with the control rats (merged images are shown in Figs 6I, J). These results suggest that increased CSF-NEP in KA-treated rats can be caused by pathological transfer of neprilysin from surface of injured presynaptic membrane to CSF.

Discussion

The principal outcome of this study was to demonstrate that CSF-NEP levels in patients with AD pathologies represent well both down-regulation of brain neprilysin early in the course of the aging-MCI-AD continuum and emanation of neprilysin from damaged neurons with exacerbation of the disease from early to intermediate stages. Importantly, decline of presynaptic neprilysin is putatively one of the earliest cytopathological events in AD pathogenesis[23,24] and is likely to intensify the local concentration of Aβ in the vicinity of synaptic structures. As favored by circumstantial evidence,[25] accumulation of Aβ may disrupt the integrity of synapses, conceivably causing further decrement of presynaptically localized neprilysin. In light of our findings, we conclude that CSF-NEP is potentially an informative biochemical marker to monitor this vicious cycle of synaptic pathogenesis, which can accelerate an imbalance between neprilysin activity and Aβ level in living patients with cognitive deficiency.

A literature of clinical studies has emerged indicating that CSF-tau assay permits prediction of AD-converted MCI and differentiation of prodromal AD from AD-unrelated MCI,[6,7] whereas persistent increase of CSF-tau at a nearly stable level regardless of disease stage[26] may hinder a chance to use CSF-tau as an antemortem index of neuropathological severity of AD. Unlike CSF-tau, CSF-Aβ42 is known to decline as the disease advances[6]; therefore, it may be useful to estimate magnitude of AD pathology in living patients. However, measurement of CSF-Aβ42 does not allow detection of abnormal Aβ metabolism in prodromal AD because of a great overlap among normal, sMCI, and pMCI subjects.[6,27] Based on the data obtained in this study, CSF-NEP assay is capable of distinguishing pMCI patients from normal subjects with a sensitivity of 76% and a specificity of 74% when a cutoff threshold is

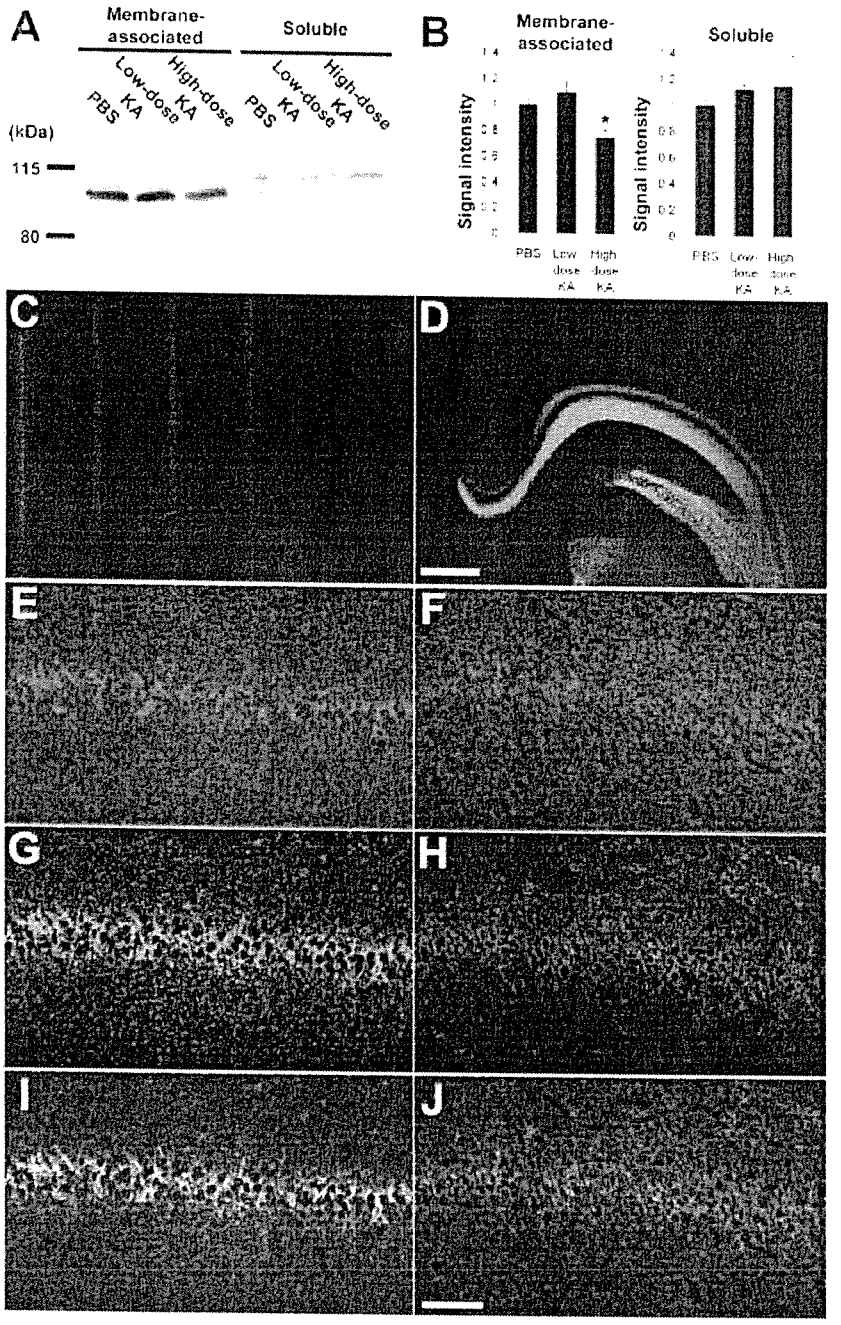

Fig 6. Reduction of neprilysin in disrupted presynaptic terminals observed in the hippocampal formations of rats treated with high-dose kainic acid (KA). (A) Representative immunoblotting indicates reduced level of membrane-associated neprilysin in high-dose KA group. Note that soluble neprilysin exhibits a larger molecular mass than membrane-associated neprilysin, presumably because of a higher magnitude of glycosylation. (B) Significant decrease of membrane-associated neprilysin in rats treated with high-dose KA was demonstrated by intensitometry of neprilysin immunoblotting signals. Bars *represent standard error. (C–J) Immunostaining of hippocampal sections from rats treated with phosphate-buffered saline (left) and high-dose KA (right). Scale bars = 250μm (C, D); 100μm (E–J).* $^{*}p < 0.05$.

assigned at 0.012 pmol/min/μl. These values may not appear particularly impressive as compared with CSF-tau, but they clearly support feasibility of using CSF-NEP as a valuable clinical adjunct to prediction of conversion from MCI to AD in view of Aβ pathogenesis. Moreover, alteration of CSF-NEP as a function of dis-

ease severity enables evaluation of neuropathological progression as patients are longitudinally examined.

It should be noted that a reduction of CSF-NEP in pMCI is indicative of a decreased level of neuronal neprilysin and a consequent diminishment of physiological transfer of neprilysin from the brain to CSF. An increased level of CSF-NEP in neprilysin-deficient mice after intrahippocampal administration of rAAV-NEP has provided unequivocal evidence for such a transfer in nonpathological conditions. The molecular mechanisms by which neprilysin is released from healthy neurons to extracellular matrix remain to be elucidated. Our immunoblotting data indicate release of neprilysin from neurons without enzymatic shedding, unlike other membrane-bound metalloproteases.[28,29] Further biochemical assessments including mass spectrometric analysis of immunocaptured samples are required to identify membrane-unbound species of neprilysin that are transferable to CSF.

As mentioned earlier, the significant association of CSF-NEP with both MMSE score and CSF-tau suggests aberrant release of neprilysin from degenerating neurons. Because there was a lack of a significant correlation between CSF-tau and MMSE score in accordance with previous findings,[26] we postulate that diffusion of neprilysin from the central nervous system to CSF in neurodegeneration has primarily two distinct origins: neurons in the middle of active neuritic and synaptic disruptions, and neurons at the end stage of the degenerative process. Based on a marked and transient increase in CSF-tau levels after acute brain injuries,[30,31] CSF-tau levels supposedly reflect the number of neurons undergoing active degenerative processes. Diffusion of neprilysin from neurons to extracellular medium can first unfold on this acute and active neuropathology, as this experiment demonstrates using KA-treated rats. Neurons at the terminal stage of degenerative changes are unlikely to release a substantial quantity of tau, because tau in such neurons is depleted in the axonal compartment or is stuck to fibrillary aggregates in the somatodendritic compartment, or both.[32] However, advanced stages of cellular injury are presumed to still allow neurons to remain productive of neprilysin, and disruption or instability of membrane structures may promote emanation of neprilysin from these cells. The number of these terminally damaged neurons increases as the disease progresses, leading to an increase of CSF-NEP in tight association with disease severity.

Our data also suggest potential benefits of neprilysin up-regulation in treating patients with pMCI and mild AD and usefulness of CSF-NEP for biochemically evaluating efficacy of the treatment in these patients. In fact, suppression of Aβ levels and amyloid plaque formation in amyloid precursor protein transgenic mice by genetically up-regulating neprilysin has been demonstrated by several independent groups.[14,33,34] Possibility of pharmacological modulation of regulatory mechanisms for neprilysin activity has also been raised by various lines of supportive evidence.[23,35] CSF-NEP could have a predictive value for identifying who would be a responder to neprilysin activation among patients with pMCI and early-stage AD.

In conclusion, this study has provided strong clinical and experimental indication that compromised neprilysin activity, Aβ-triggered neuronal injury, and a conjunction of these two changes in the brain can be monitored by CSF-NEP assay from predementia phase of AD. Quantification of CSF-NEP may also play a role in the diagnostic work-up of MCI to identify patients in transition from MCI to AD and patients afflicted by a depletion of brain neprilysin. This will be of particular importance when drugs with potential up-regulatory effects on neprilysin activity reach the clinical trial stage.

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ARTICLE

Specific and Efficient Transduction of *Cochlear* Inner Hair Cells with Recombinant Adeno-associated Virus Type 3 Vector

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Recombinant adeno-associated virus (AAV) vectors are of interest for cochlear gene therapy because of their ability to mediate the efficient transfer and long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues with minimal vector-related cytotoxicity. In the present study, seven AAV serotypes (AAV1–5, 7, 8) were used to construct vectors. The expression of EGFP by the chicken β-actin promoter associated with the cytomegalovirus immediate-early enhancer in cochlear cells showed that each of these serotypes successfully targets distinct cochlear cell types. In contrast to the other serotypes, the AAV3 vector specifically transduced cochlear inner hair cells with high efficiency *in vivo*, while the AAV1, 2, 5, 7, and 8 vectors also transduced these and other cell types, including spiral ganglion and spiral ligament cells. There was no loss of cochlear function with respect to evoked auditory brain-stem responses over the range of frequencies tested after the injection of AAV vectors. These findings are of value for further molecular studies of cochlear inner hair cells and for gene replacement strategies to correct recessive genetic hearing loss due to monogenic mutations in these cells.

Key Words: adeno-associated virus, serotype, gene transfer, cochlea, hair cells

INTRODUCTION

The total number of hair cells in the cochlea is finite. They are not renewed and there is very little (if any) redundancy in this population. The irreversible loss of cochlear hair cells is presumed to be a fundamental cause of permanent sensorineural hearing loss. Gene transfer into hair cells presents numerous opportunities for protecting these cells. There is considerable interest in the development of viral vectors to deliver genes to the cochlea to counteract hearing impairment, and recent studies have focused on vectors based on adenovirus [1–3], herpes simplex virus [4–6], lentivirus [7], and adeno-associated virus (AAV) [8,9]. The patterns of vector-encoded transgene expression have been found to differ significantly among vectors. Cochlear hair cells can be efficiently transduced with adenovirus vectors [10–12]. However, these vectors were found to provoke a strong immune response that could damage recipient cells and compromise cochlear function [10,13,14]; they are also incapable of mediating prolonged transgene expression [15,16]. Although AAV vectors might overcome these problems, the transduction of hair cells by AAV2-derived vectors is controversial [8,10,17]. To our knowledge, other AAV serotypes have not yet been tested as cochlear gene transfer vectors *in vitro* or *in vivo*. AAV vectors are of interest in the context of gene therapy because they mediate efficient transfer and long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues with minimal vector-related cytotoxicity.

In this study, we assessed the utility of seven AAV serotypes as vectors with the chicken β-actin promoter associated with a cytomegalovirus immediate-early

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enhancer (CAG)-driven enhanced green fluorescent protein (EGFP) gene [18] in the murine cochlea. Vectors were introduced by microinjection through the round window membrane [19]. As a result, we determined that the specific and efficient gene transduction of inner hair cells could be achieved by using AAV type 3 vectors.

RESULTS

Expression Profile of EGFP in the Cochlea

Several cell types line the cochlear duct and support the hair cells (Fig. 1A). We carefully made a small opening in the tympanic bulla and injected vectors derived from the AAV1–4, 7, and 8 pseudotypes into the cochlea of two strains of mice (C57BL/6J and ICR) through the round window membrane (Fig. 1B). The mode of EGFP expression in various murine cochlear hair cells had a close similarity and was essentially equal for both strains. We determined the distribution of AAV vector-mediated EGFP expression throughout the cochlea for all serotypes tested (Table 1). A principal finding is that the inner hair cells in the organ of Corti showed clear evidence of EGFP expression with all of the AAV serotype-derived vectors except for the AAV4 vector (Fig. 2). This result indicates that most of the vectors (AAV1–5, 7, and 8) could efficiently transduce cochlear inner hair cells *in vivo* when slowly infused into the scala tympani. The AAV3-based vector was the most efficient and specific of the serotypes in transducing cochlear inner hair cells (Fig. 3). Transduction with 5×10^{10} genome copies (gc)/cochlea of the AAV3 vector resulted in robust transgene expression in the inner hair cells. The spiral ganglion cells showed significantly higher levels of fluorescence per unit area with the AAV5-based vector (Fig. 2n), and the spiral ligament cells were transduced prominently with the AAV1 and AAV7 vectors (Figs. 2d and 2r). Histological sections of cochleae injected with the AAV4 vector identified EGFP-positive cells predominantly in connective tissue within the mesothelial cells beneath the organ of Corti and in mesenchymal cells lining the perilymphatic fluid spaces (Figs. 2j and 2l). Furthermore, we detected intense expression with the AAV5- and AAV8-based vectors in the inner sulcus cells and in Claudius' cells (Figs. 2p and 2x). We did not detect notable levels of gene expression in the outer hair cells, supporting pillar cells, or stria vascularis cells for any serotype.

Long-term Expression of EGFP

We examined cochlear expression of the EGFP transgene in animals sacrificed at 1–12 weeks. Expression persisted in cochlear tissues for up to 3 months after infusion, while the extent of expression peaked at 2 weeks.

Transgene Activity

We determined the percentage of inner hair cells transduced with the AAV3 vector. The mid- to high-frequency regions of the cochlea were efficiently transduced, as shown in Fig. 3. Almost all of the inner hair cells in the basal and middle cochlear regions were transduced with the AAV3 vector (Fig. 4). Transgene expression was not detected in the hair cells of the apical turn of the cochlea. The predominant expression in the middle and basal cochlear turns is reasonable, as the virus

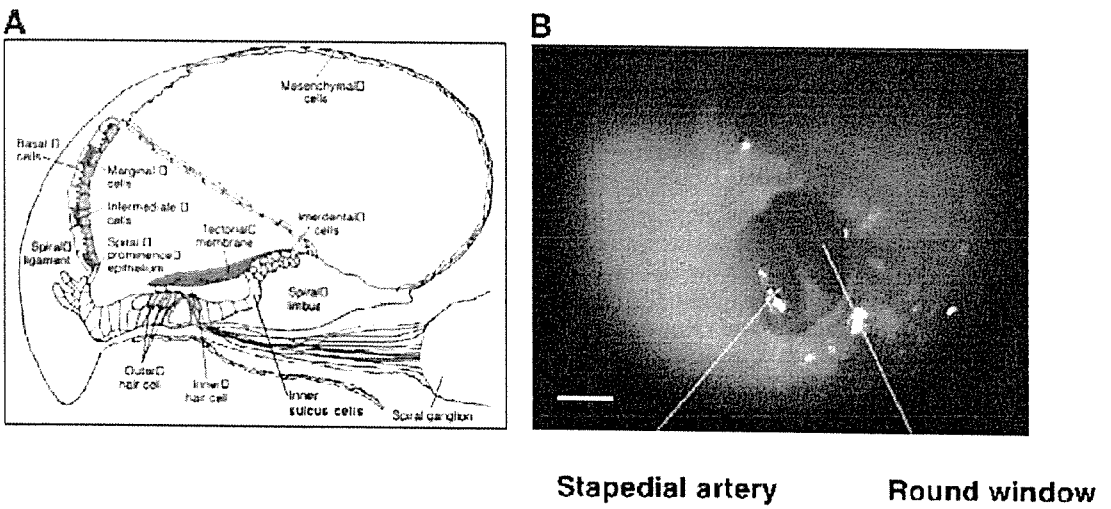

FIG. 1. (A) Schematic diagram of a cross section of the cochlea, demonstrating the scala vestibuli, scala tympani, and scala media or cochlear duct. The organ of Corti rests on the basilar membrane, with the hair cell cilia embedded in the gelatinous tectorial membrane. The outer margin of the cochlear duct contains the stria vascularis. Reproduced, by permission of the publisher, from [44]. (B) Direct visualization of the round window membrane in the right ear. The upper side of the picture is the back of the mouse and the right side is the head of the animal. The stapedial artery, a branch of the internal carotid artery, transverses an open bony semicanal within the round window niche. Bar denotes 500 μm, 15× original magnification.