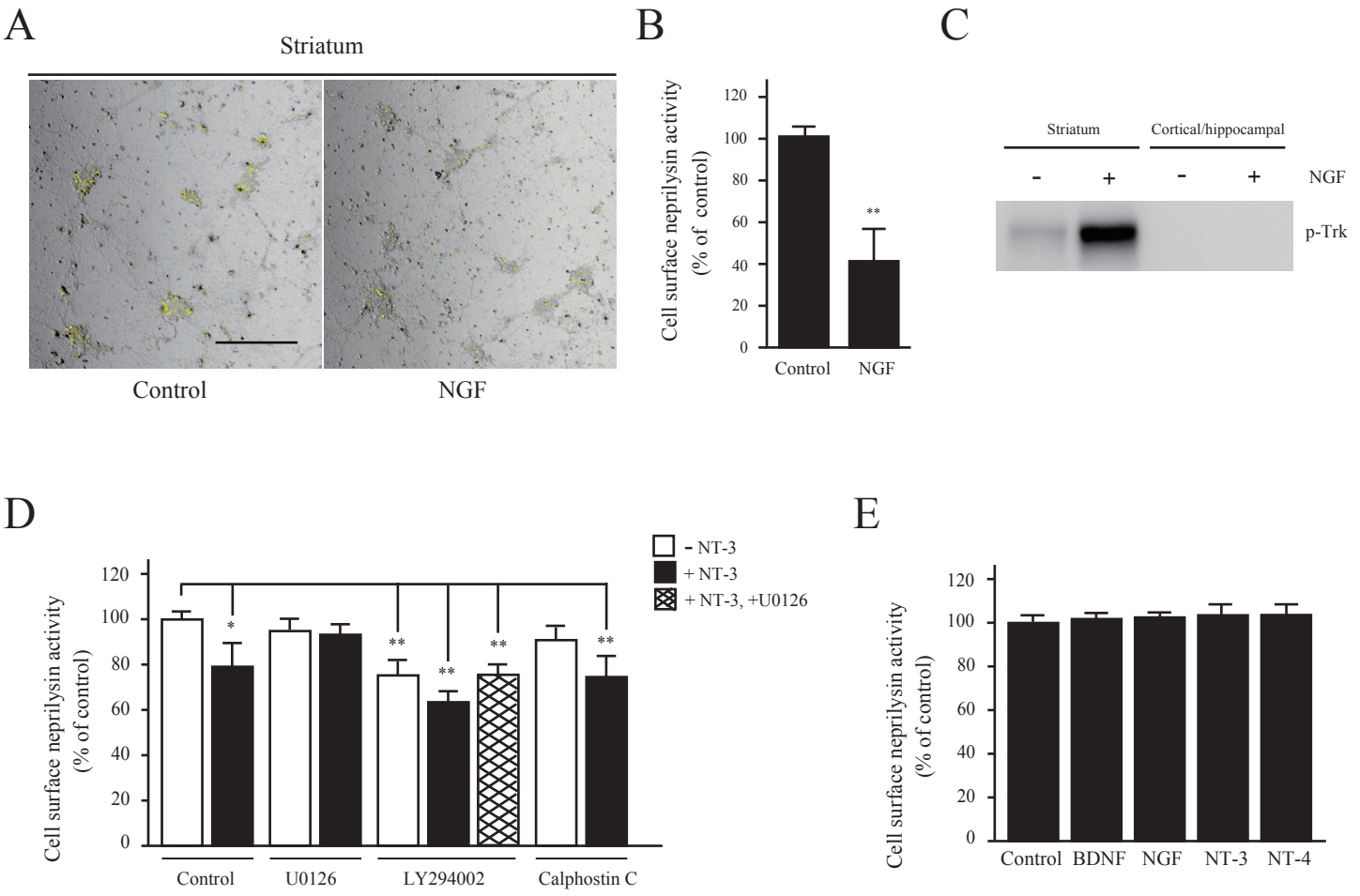
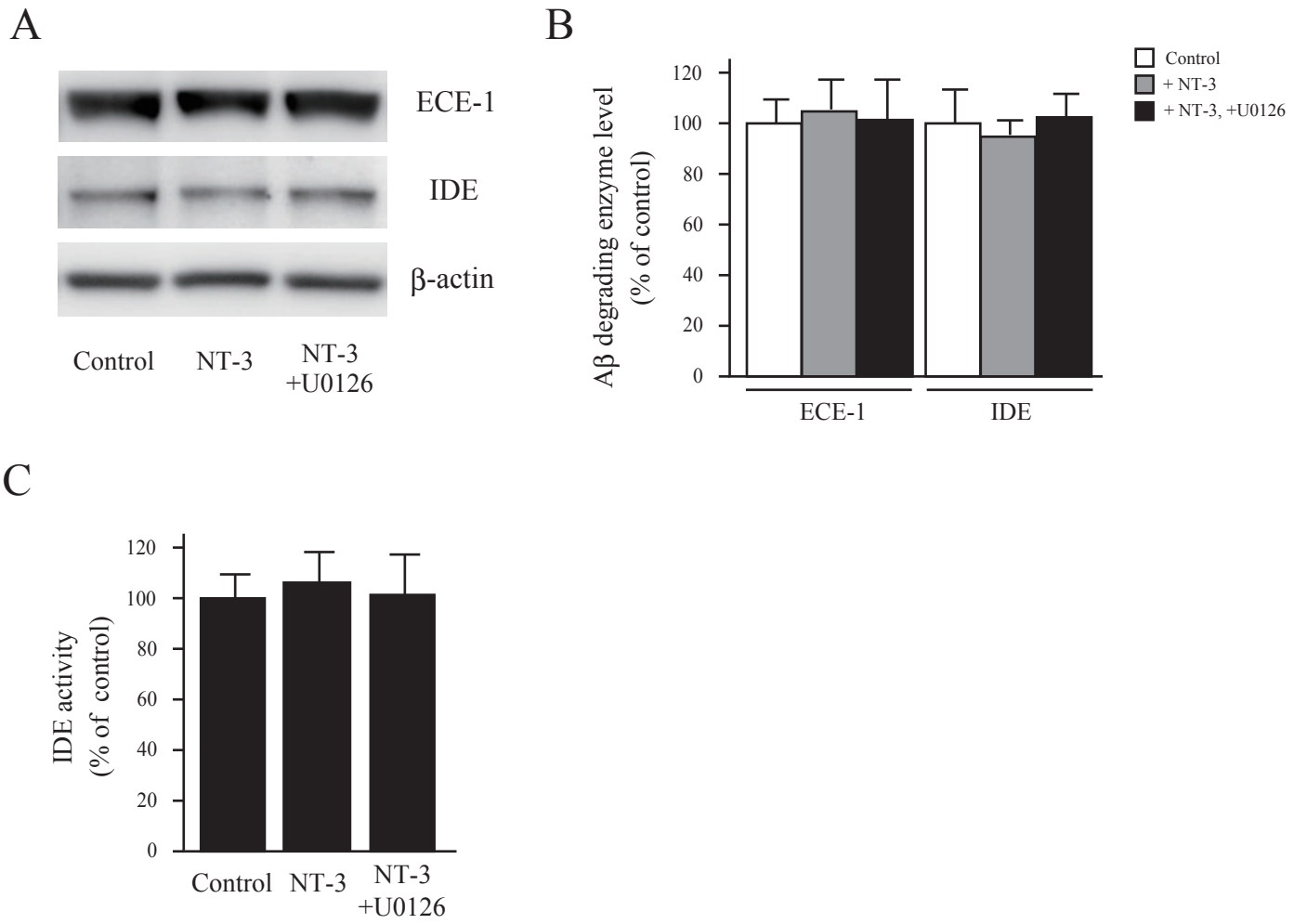
Figure S1.



Supplemental Fig. 1. NGF reduces cell surface neprilysin activity in primary striatal neurons. Primary striatal neurons derived from wild-type mouse embryos were isolated as described previously (47), after which 2.0×10^5 cells were plated on glass coverslips, which were cultured at one coverslip per well in 24-well plates. After 14 days in vitro, the primary neurons were assayed. A,B. Neprilysin activity staining was performed with primary striatal neurons infected by Semliki-forest virus expressing human neprilysin (SFV-hNEP) and stimulated by NGF (100 ng/ml) for 24 hours. Scale bar represents 100 μm. Quantification of the fluorescence signal was performed and the signal from NGF-treated cells was compared to that of non-stimulated control cells and presented as an average ± S.D (n = 4). **P < 0.01 compared with control. C. Primary striatal neurons were incubated with NGF for 30 min and Trk phosphorylation was evaluated by western blot analysis. D. After 24 hours addition of inhibitors for the signaling transduction pathways involving MAP kinase/ERK kinase (MEK1/2), phosphatidylinositol-3 kinase (PI3K) and protein kinase C (PKC) to primary cortical/hippocampal neurons revealed that the MEK1/2 inhibitor U0126 (1 μM) could abolish the NT-3-induced decrease in neprilysin activity. The PKC inhibitor Calphostin C (200 nM) had no effect, whereas the PI3K inhibitor LY294002 (50 μM) alone induced a decrease in cell surface neprilysin activity, possibly through a Trk-independent pathway. Data represent the mean ± S.D (n = 5, P < 0.05). *P < 0.05, **P < 0.01. E. After 30 min stimulation with BDNF, NGF, NT-3 or NT-4 (all at 100 ng/ml), cell surface neprilysin activity was measured using primary cortical/hippocampal neurons.

47. Brami-Cherrier, K., Valjent, E., Garcia, M., Pages, C., Hipskind, R. A., and Caboche, J. (2002) J. Neurosci. 22, 8911-8921

Figure S2.



Supplemental Fig. 2. No effect of NT-3 on the expression levels of other Aβ-degrading enzyme, ECE-1 and IDE or the activity of IDE. A,B. Expression levels of endogenous ECE-1 or IDE in primary cortical/hippocampal neurons after 24 hours incubation of NT-3 (100 ng/ml) was investigated by western blot using anti-ECE-1 (Abgent) or anti-IDE (Proteintech) antibodies respectively. The intensities were quantified by densitometric analysis and data represent the mean ± S.D (n=3). C. Enzymatic activity of IDE was evaluated by Insulysin/IDE immunocapture activity assay kit (Calbiochem) according to the manufacturer' s instructions. IDE activity assay was performed with primary cortical/hippocampal neurons stimulated by NT-3 (100 ng/ml) for 24 hours. Data represent the mean ± S.D (n=3). ECE-1 activity could not be examined because an assay system of mouse ECE-1 activity has not yet been established.

Figure S3.

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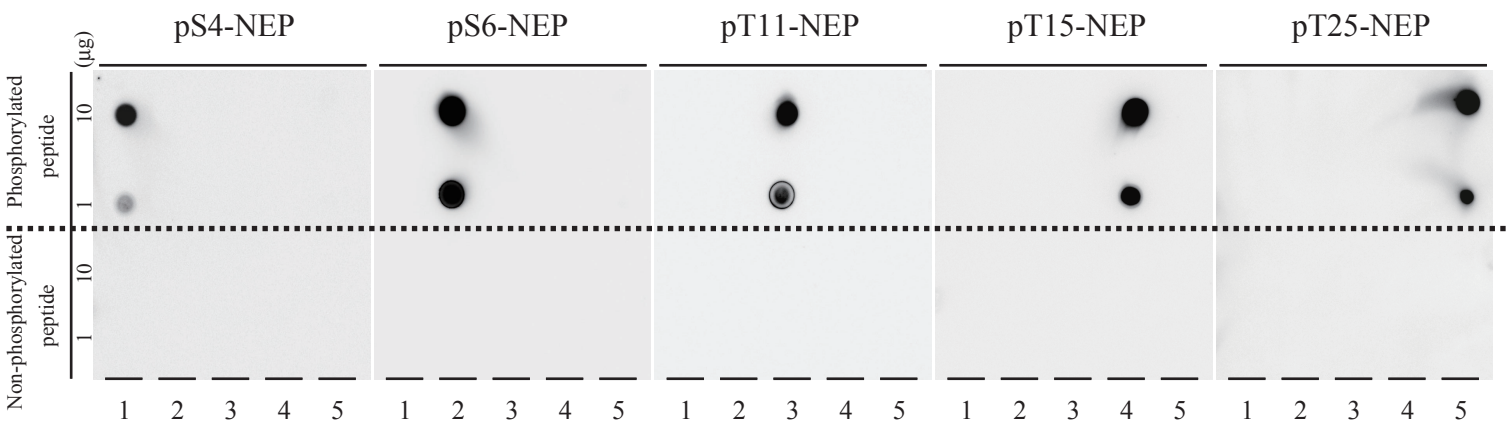

Supplemental Fig. 3. Specificities of antibodies generated against phosphorylated serine and threonine residues in the neprilysin intracellular domain. A. Specificities of the phospho-human-neprilysin antibodies were confirmed by dot blot analysis. Phosphorylated peptides, with peptide sequences corresponding to the antigens used for immunization [(1) GKpSESQMC, (2) GKSEpSQC, (3) QMDIpTDINTC, (4) TDINpTPKPKC and (5) KQRWpTPLEC] and non-phosphorylated peptides [(1) GKSESQMC, (2) GKSESQC, (3) QMDITDINTC, (4) TDINTPKPKC and (5) KQRWTPLEC] were spotted on membranes as described previously (21). The blotted membranes were incubated with the generated phospho-human-neprilysin antibodies, pS4-NEP, pS6-NEP, pT11-NEP, pT15-NEP and pT25-NEP, as indicated. The antibodies did not show any cross-reactivity against the non-phosphorylated peptides.

Figure S4.

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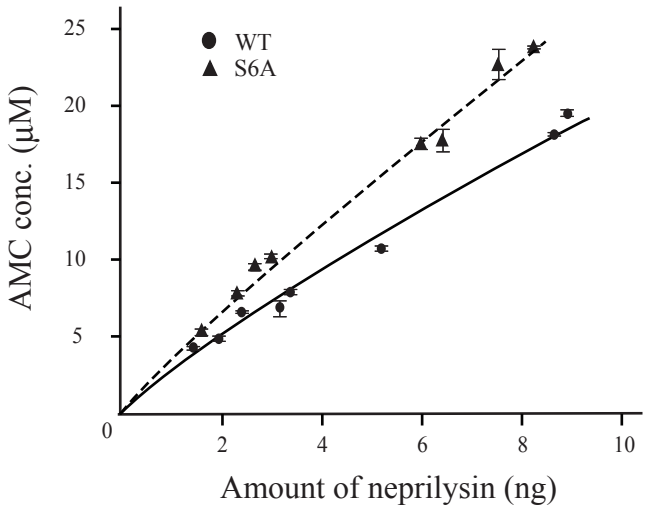
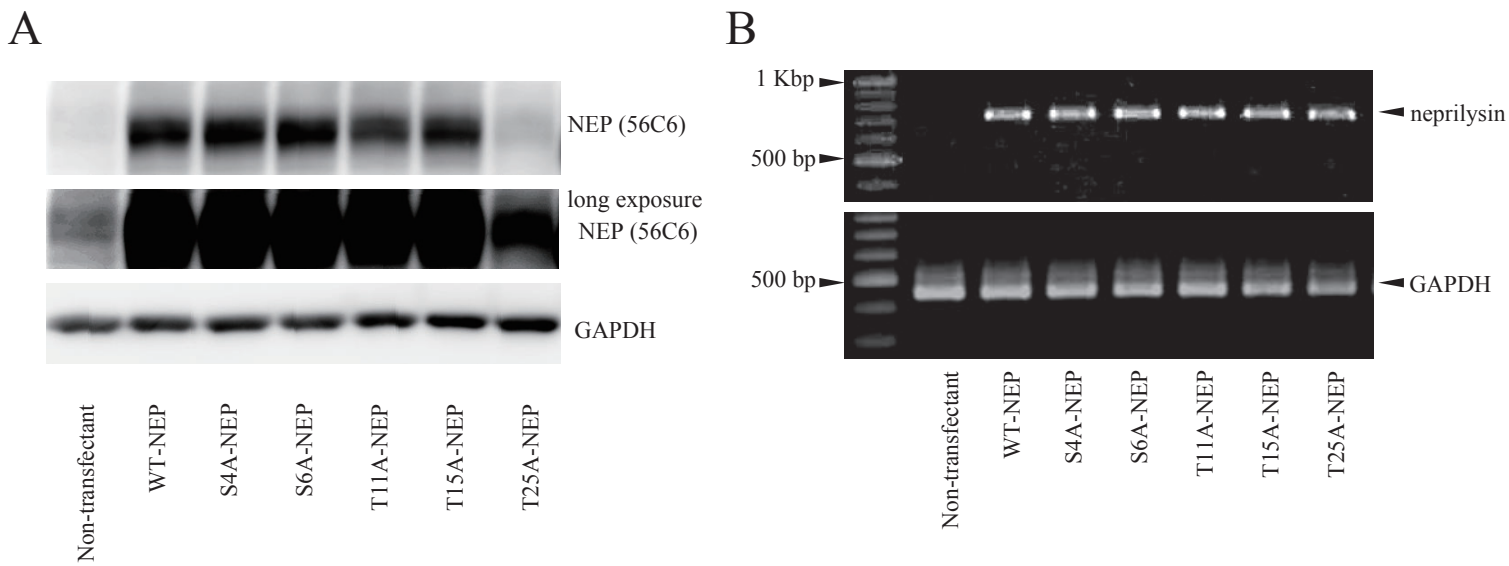


Supplemental Fig. 4. Correlation between neprilysin expression and cell surface activity. A. AMC production was measured using SH-SY5Y cells expressing WT-NEP or S6A-NEP. To ensure accurate determination of expression levels of the transfectants, quantitative western blot, using recombinant neprilysin as a standard, was performed. For each mutant transfectant, eight levels of expression, using four different DNA:lipofectin reagent ratios were established. The initial linear range was used as a measure of cell surface neprilysin activity for each mutant. Various expression levels of S6A-NEP resulted in significantly higher cell surface neprilysin activity compared to that in cells expressing WT-NEP.

Figure S5.



Supplemental Fig. 5. Expression of endogenous neprilysin and neprilysin mutants in SH-SY5Y cells. NEP mRNA and protein levels in SH-SY5Y cells overexpressing WT-, S4A-, S6A-, T11A-, T15A- and T25A-NEP mutants were analyzed by western blotting and RT-PCR. A. Equal amounts of whole cell lysate were subjected to SDS-PAGE followed by western blot, and probing with anti-neprilysin and anti-GAPDH antibodies. In addition, to visualize the expression of endogenous human neprilysin in SH-SY5Y cells, a long exposure of the blot is also included. B. Total RNA was isolated from SH-SY5Y cells using the EASY Prep RNA kit according to the manufacturer' s protocol (Takara). The RNA was subjected to reverse transcription using an RNA PCR kit (AMV) Ver.3.0 (Takara) according to the manufacturer' s protocol. Human neprilysin and GAPDH cDNA products were amplified with primer sets for neprilysin or GAPDH as follows: hNEP 5' -GGCTCCACTGGAGATCAGCCTCTCG-3' and 5' -GGTTTTCATCGATGGGCAATC-3' and GAPDH 5' -ACCACAGTCCATGCCATCAC-3' and 5' -TCCACCACCCTGTTGCTG-3' . The expected product sizes were 721 bp and 451 bp, respectively. Note that even though T25A-NEP mRNA is present, the T25A-NEP protein level is significantly lower than that of the other transfectants.

Figure S6.

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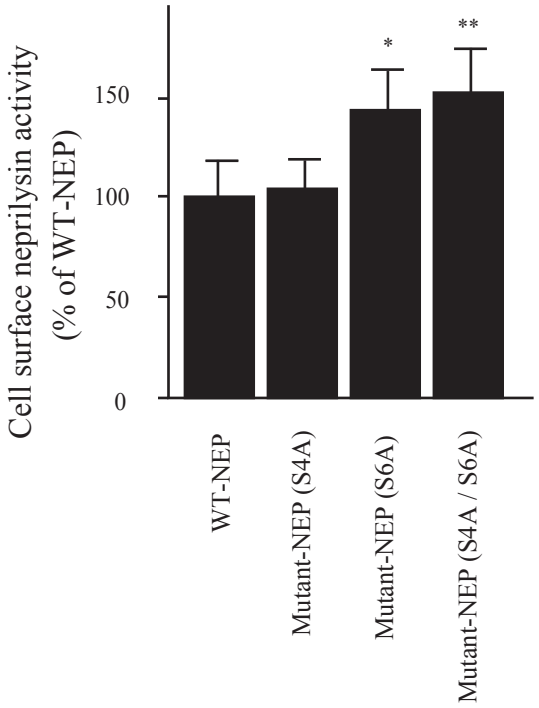

Supplemental Fig. 6. Cell surface neprilysin activity of the double mutant S4A /S6A- NEP-ICD. In addition to the observed increase in phosphorylation at S6 in NEP-ICD upon 24 hour NT-3 stimulation, the phosphorylation of S4 was enhanced in a MEK-dependent manner (Fig 5B,C). Furthermore, PP1a inhibition with tautomycetin increased phosphorylation of S4 (Fig.6A). These data together suggested that phosphorylation of S4 could, together with S6, cooperatively regulate cell surface neprilysin activity. In order to clarify this possibility, we prepared a double mutant S4A/S6A -NEP to examine whether the role of S4 had been underestimated in our analysis of neprilysin activity. S4/S6-NEP was transformed into SH-SY5Y cells and cell surface neprilysin activity was measured as described in materials and methods. The result showed that cell surface neprilysin activity of S4A/S6A-NEP was similar to S6A-NEP, reinforcing that only S6 of neprilysin regulates cell surface neprilysin activity via phosphorylation and dephosphorylation. Data represent the mean ± S.D (n = 3). *P < 0.05, **P < 0.01, compared to WT-NEP.