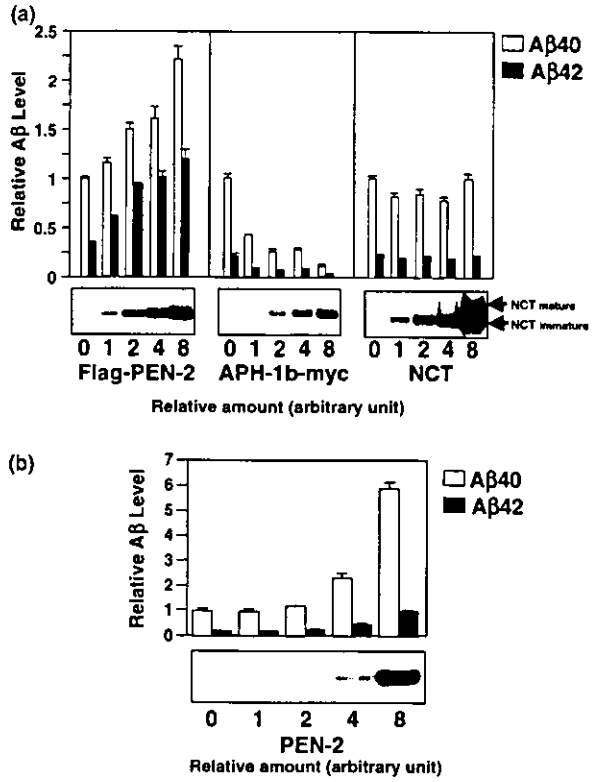

Fig. 2 Effect of various levels of PEN-2, APH-1b or nicastrin (NCT) on amyloid β-protein (Aβ) generation. (a) After PEN-2, APH-1b or NCT was retrovirally expressed in fibroblasts (2×10^5) expressing β-amyloid precursor protein (APP)695 (presenilin, endogenous levels), Aβ40 and Aβ42 secreted from cells during a 48-h culture were quantified by ELISA. The Aβ levels were normalized to soluble APP and then expressed as relative to basal Aβ40 levels obtained from mock-transfected cells as described in (a). Numbers used for arbitrary units denote the relative concentration of the virus solution containing pMX carrying the indicated cDNA used for the infection (the concentration of the original virus solution is defined as 8 arbitrary units). Values are means ± SD of two independent dishes ($n = 2$). The RIPA-solubilized lysates (10 μg) were immunoblotted with the anti-FLAG antibody (for PEN-2), anti-myc antibody (for APH-1b) or anti-NCT antibody. FLAG-PEN-2, N-terminally FLAG-tagged PEN-2; APH-1b-myc, C-terminally myc-tagged APH-1b. Data are representative of four independent experiments. (b) PEN-2 without the FLAG tag was also retrovirally expressed and secreted Aβ was quantified as described in (a). The RIPA-solubilized lysates (10 μg) were immunoblotted with the anti-PEN-2 antibody.

PS1 NTF and CTF. Figure 3(c) demonstrates that no drastic increase in γ-secretase activity was observed with various expression levels of NTF or CTF. In this experiment, it appears that γ-secretase activity is saturable at the minimum expression levels of NTF and CTF. Therefore, we next determined the effect of the expression of PS cofactors on Aβ generation mediated by the coexpression of PS1 NTF and CTF at the expression level at which Aβ increase is saturated.

Effect of expression of presenilin cofactors on amyloid β-protein generation mediated by the coexpression of N- and C-terminal fragments in presenilin-null cells

As shown in Figs 4(a) and (b), only PEN-2 expression significantly increased Aβ generation mediated by the coexpression of PS1 NTF/CTF, while the expression of NCT or APH-1 alone did not cause a significant increase in Aβ generation. Interestingly, the levels of PS1 NTF and CTF were greatly increased by the coexpression of NCT and APH-1b, accompanied by the stimulation of NCT maturation as observed in cells expressing the PS1 holoprotein with all of the cofactors (Fig. 4b). Therefore, it appears that PS1 NTF and CTF are stabilized by the binding of both APH-1 and NCT to PS1 NTF and CTF. Moreover, it was noted that the expression of PEN-2 together with the expression of APH-1b and NCT further stimulated NCT maturation. However, it was noted that the increase in Aβ generation by PEN-2 expression was not associated with the stimulation of NCT maturation or the increase in the levels of PS1 NTF and CTF. It was also noted that the expression of APH-1b clearly inhibited the PEN-2-mediated increase in Aβ generation.

We next investigated whether the inhibitory effect of APH-1b on the PEN-2-mediated increase in Aβ generation results from the inhibition of the binding of PEN-2 to PS due to APH-1b expression. For this purpose, we investigated whether the level of PEN-2 bound to PS1 changes with an increase in the expression level of APH-1b (Fig. 4c). APH-1b did not inhibit the binding of PEN-2 to PS1 (Fig. 4c). In contrast, an increase in the level of APH-1b bound to PS1 caused an increase in the level of PEN-2 bound to PS1 (Fig. 4c, lower panel), although Aβ generation decreased with an increase in the expression level of APH-1b (Fig. 4c, upper panel). We also observed that APH-1b did not inhibit the binding of NCT to PS1 (Fig. 4c, lower panel). This result indicates that the inhibition of the PEN-2-mediated increase in Aβ generation by APH-1b is not due to the inhibition of the binding of PEN-2 to PS1.

PEN-2 does not enhance presenilin 1 heterodimer formation

The PS NTF/CTF heterodimers are considered to constitute the active site in the γ-secretase complex (Esler *et al.* 2000; Li *et al.* 2000b). Therefore, PEN-2 could stimulate PS1 NTF/CTF heterodimer formation in PS-null cells coexpressing PS1 NTF and CTF because PEN-2 increases Aβ generation. To test this possibility, we first determined whether PS1 NTF and CTF coexpressed in PS-null cells form the PS1 NTF/CTF heterodimer. The anti-PS1 NTF antibody coimmunoprecipitated PS1 CTF and the anti-PS1 CTF antibody also coimmunoprecipitated PS1 NTF (Fig. 5a). This result clearly indicates that PS1 NTF and CTF form the PS1 NTF/CTF heterodimer complex in PS-null cells. However, PEN-2 did not stimulate the formation of the heterodimer (Fig. 5a). We also examined the effect of PEN-2 expression on PS

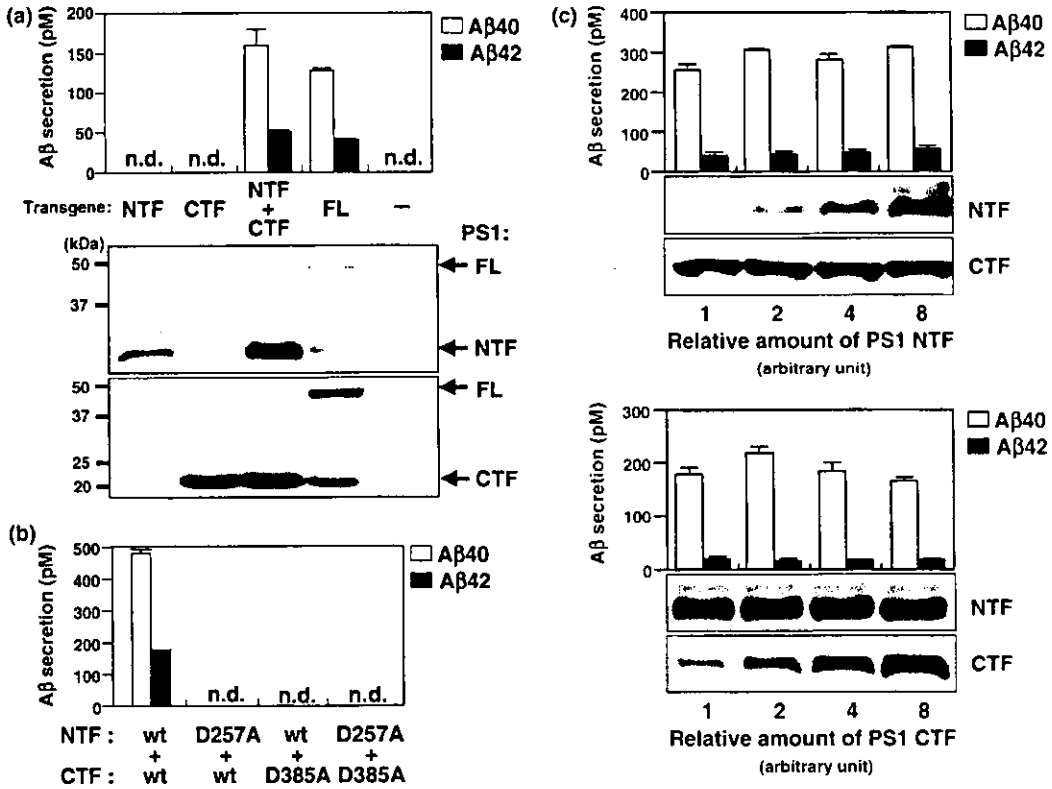

Fig. 3 Coexpression of presenilin (PS)1 N- and C-terminal fragments (NTF and CTF) restores amyloid β-protein (Aβ) generation in PS-null cells. (a) The indicated exogenous cDNAs and β-amyloid precursor protein (APP)695 were retrovirally expressed in PS-null cells. The Aβ40 and Aβ42 secreted from cells during a 48-h culture were quantified by ELISA. Ristocetin-induced platelet agglutination-solubilized lysates (10 μg) were immunoblotted with the anti-PS1 NTF antibody (for PS1 NTF detection) or anti-PS1 loop antibody (for PS1 CTF detection). FL, PS1 full-length; n.d., Aβ was not detected (< 10 pM). (b) Coexpression of PS1 NTF mutated at Asp257 and CTF or PS1 NTF and CTF mutated at Asp285 does not restore Aβ generation in PS-null cells. wt, wild-type PS1. (c) Effects of various expression levels of PS1 NTF and CTF on Aβ generation in PS-null cells. In the upper panel, using various concentrations (arbitrary units 1–8) of the virus solution containing PS1 NTF cDNA and a constant concentration (8 arbitrary units) of the virus solution containing PS1 CTF cDNA, PS1 NTF and CTF were retrovirally expressed in PS-null cells (2×10^5) expressing APP695. In the lower panel, using various concentrations (arbitrary units 1–8) of the virus solution containing PS1 CTF cDNA and a constant concentration (8 arbitrary units) of the virus solution containing PS1 NTF cDNA, PS1 NTF and CTF were retrovirally expressed in PS-null cells (2×10^5) expressing APP695. The Aβ40 and Aβ42 secreted from cells during a 48-h culture were quantified by ELISA. Note: as far as the concentration of the viral solution showing 100% infection efficiency was used, a decrease in Aβ generation was not observed, although the expression of NTF alone or CTF alone resulted in no detectable Aβ generation.

NTF/NTF dimer formation in PS-null cells coexpressing N-terminal HA-tagged PS1 NTF and wild-type PS1 NTF/CTF because it was previously reported that PS is likely to form NTF/NTF dimers (Schroeter *et al.* 2003). However, we failed to detect a PS1 NTF/NTF dimer even when we employed N-terminal HA-tagged PS1 holoprotein and PEN-2 did not enhance the formation of NTF/NTF dimer (data not shown).

PEN-2 interaction with N- and C-terminal fragments

To understand the mechanism underlying the increase in Aβ generation by PEN-2, as an initial step we determined which fragment, NTF or CTF, interacts with PEN-2. The anti-PS1 NTF antibody clearly coimmunoprecipitated PEN-2 and the anti-PS1 CTF antibody also coimmunoprecipitated PEN-2 (Fig. 5b, upper panel). The anti-FLAG antibody also coimmunorecipitated PS1 NTF and CTF (Fig. 5b, lower panel). Thus, our results show that PEN-2 binds to both NTF and CTF.

Effect of γ-secretase inhibitors on PEN-2-mediated increase in amyloid β-protein generation in presenilin-null cells coexpressing presenilin 1 N- and C-terminal fragments

To address the mechanism underlying the increase in Aβ generation by PEN-2 expression, we finally investigated whether PEN-2 expression alters the IC_{50} of a specific inhibitor of γ-secretase, L-685,458, which was previously

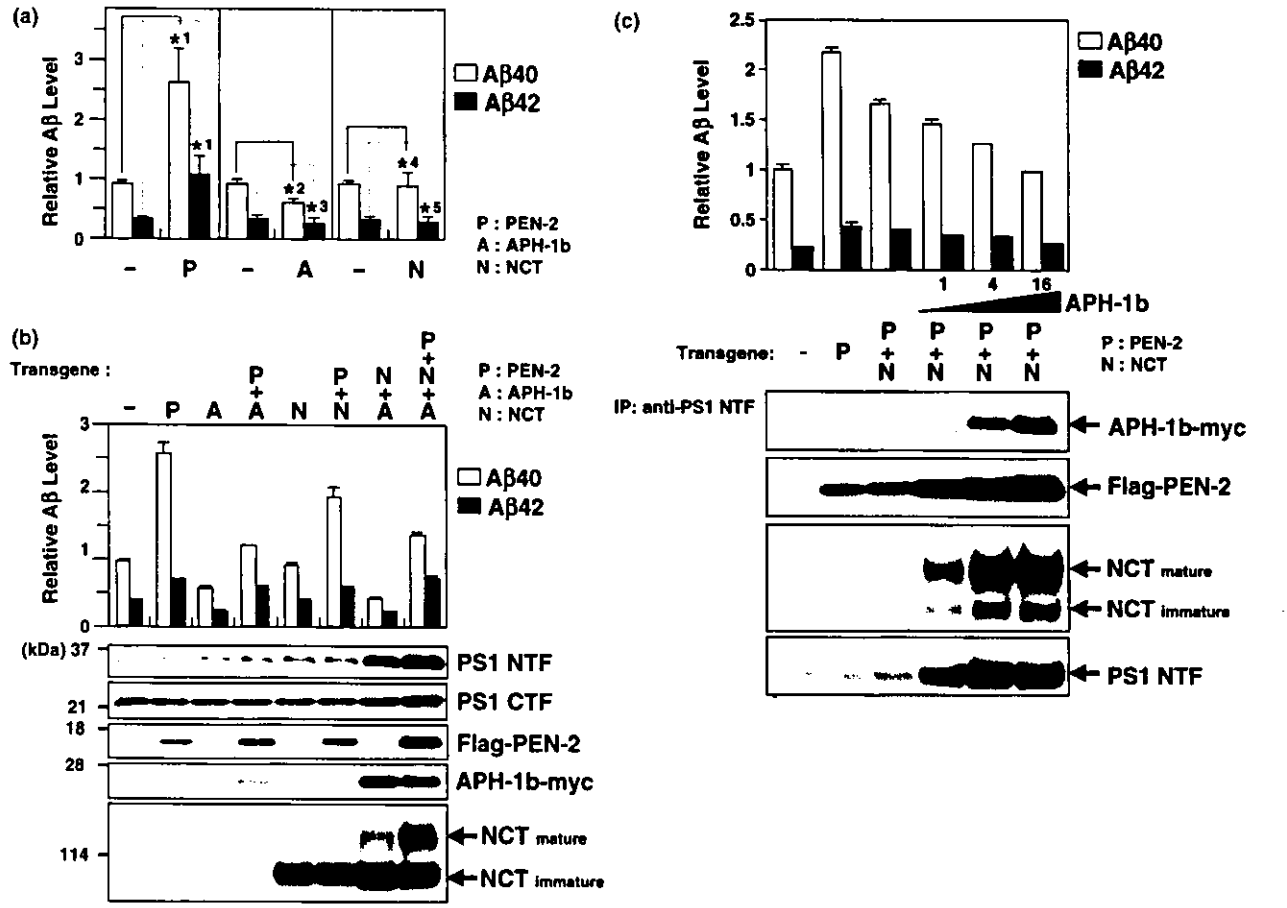

Fig. 4 Effects of expression of PEN-2, APH-1b and/or nicastrin (NCT) on amyloid β-protein (Aβ) generation in presenilin (PS)-null cells coexpressing PS1 N-/C-terminal fragment (NTF/CTF). (a) The indicated exogenous genes were retrovirally expressed in PS-null cells coexpressing PS1 NTF/CTF and expressing β-amyloid precursor protein (APP)695. The Aβ40 and Aβ42 secreted from 48-h cultured cells were quantified by ELISA. The Aβ levels were expressed as relative to basal Aβ40 levels obtained from PS-null cells expressing PS1 NTF and CTF. –, mock infection (retroviral vector, pMX, alone). P, FLAG-PEN-2; A, APH-1b-myc; N, NCT. Significant difference at $^{*1}p < 0.0001$ ($n = 16$), $^{*2}p < 0.001$ ($n = 8$), $^{*3}p < 0.05$ ($n = 8$), $^{*4}p > 0.5$ ($n = 10$) and $^{*5}p > 0.5$ ($n = 10$). (b) The indicated exogenous genes were retrovirally expressed in PS-null cells coexpressing PS1 NTF/CTF and expressing APP695. Values are means ± SD of two independent dishes ($n = 2$). The Aβ levels normalized to soluble APP were expressed as relative to basal Aβ40 levels obtained from PS-null cells expressing PS1 NTF and CTF as described in (a). Similar results were obtained from three independent experiments. The RIPA-solubilized lysates (10 μg) were immunoblotted with the anti-PS1 NTF antibody, anti-PS1 loop antibody, anti-NCT antibody, anti-FLAG antibody (for FLAG-tagged PEN-2) and anti-myc antibody (for myc-tagged APH-1b). Note that the long exposure revealed endogenous NCT in the first four lanes (data not shown). (c) APH-1b expression inhibits the PEN-2-induced enhancement of Aβ generation in PS-null cells coexpressing PS1 NTF and CTF. In the upper panel, the indicated exogenous genes were retrovirally expressed in PS-null cells coexpressing PS1 NTF/CTF and APP695. Using various concentrations of the virus solution containing APH-1b-myc cDNA (arbitrary units 1–16), APH-1b-myc was expressed at various levels. P, FLAG-PEN-2; A, APH-1b-myc; N, NCT. Note: the same results showing that APH-1b inhibits the PEN-2-mediated increase in Aβ generation were obtained from four independent experiments. In the lower panel, CHAPSO-solubilized lysates (1 mg) in the upper panel were immunoprecipitated (IP) with the anti-PS1 NTF antibody and then immunoblotted with the anti-myc antibody (for myc-tagged APH-1b), anti-FLAG antibody (for FLAG-tagged PEN-2), anti-NCT antibody or anti-PS1 NTF antibody.

characterized as a transition state analogue mimic at the catalytic site of an aspartyl protease (Shearman *et al.* 2000). It was also shown that the structurally related photoaffinity analogues of L-685,458 exclusively bind to PS NTF and CTF (Li *et al.* 2000b). As shown in Fig. 6(a), PEN-2 expression significantly reduced IC_{50} against Aβ formation compared with mock-transfected cells. In addition, we found that PEN-2 expression also reduced the IC_{50} of a non-transition state analogue inhibitor, DAPT (Dovey *et al.* 2001) (Fig. 6b), which was suggested to either directly bind

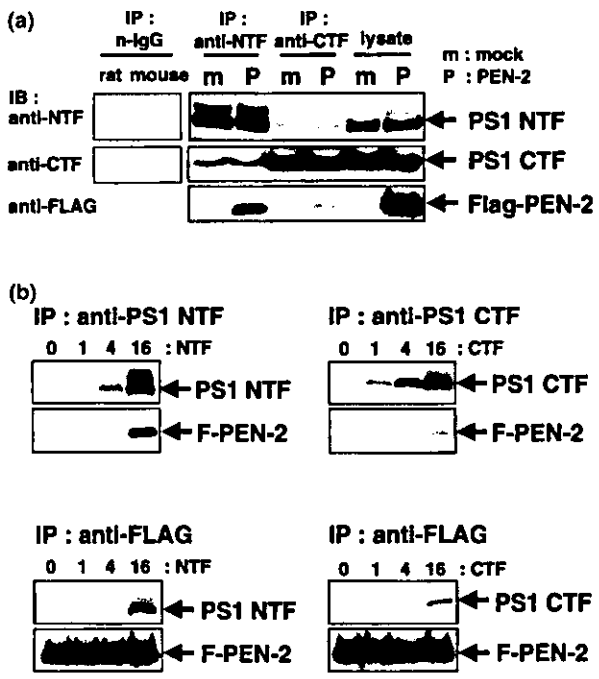

Fig. 5 Effect of PEN-2 expression on (a) presenilin (PS)1 N-/C-terminal fragment (NTF/CTF) heterodimer formation and (b) the interaction of PEN-2 with PS1 NTF and CTF. (a) CHAPSO-solubilized lysates (1 mg) from PS-null cells coexpressing PS1 NTF/CTF were immunoprecipitated (IP) with the anti-PS1 NTF antibody and then immunoblotted (IB) with the anti-PS1 NTF antibody, anti-PS1 loop antibody or anti-FLAG antibody (right panel, left two lanes). The same lysates were IP with the anti-PS1 loop antibody and then IB with the indicated antibodies (right panel, middle two lanes). The same lysates (10 μg) without immunoprecipitation were also IB with the indicated antibodies (right panel, right two lanes). In the left panel, the same lysates were IP with rabbit normal IgG (control for the anti-PS1 NTF antibody) or mouse normal IgG (control for the anti-PS1 loop antibody) and then IB with the anti-PS1 NTF antibody and anti-PS1 loop antibody. Note: the anti-PS1 loop antibody used in this study immunoprecipitates PS1 CTF but not PS1 NTF (data not shown). (b) In the upper two panels, a constant level of FLAG-PEN-2 and various levels (arbitrary units 0–16) of PS1 NTF (left panel) or CTF (right panel) were retrovirally expressed in PS-null cells. CHAPSO-solubilized lysates (1 mg) were IP with the anti-PS1 antibody (left panel) or anti-PS1 loop antibody (right panel). The immunoprecipitation products were then IB with the anti-FLAG antibody and anti-PS1 antibody (left panel) or the anti-PS1 loop antibody (right panel). In the lower two panels, the same lysates (1 mg) were IP with the anti-FLAG antibody. The immunoprecipitation products were then IB with the anti-FLAG antibody and anti-PS1 antibody (left panel) or the anti-PS1 loop antibody (right panel). F-PEN-2, FLAG-PEN-2.

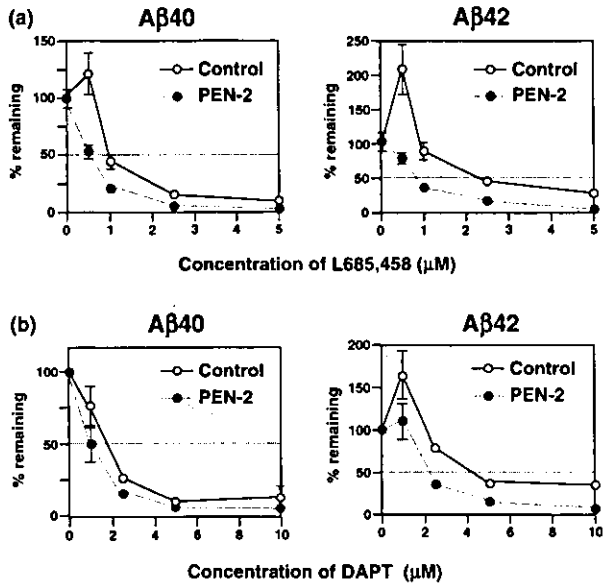

Fig. 6 Effects of γ-secretase inhibitors on PEN-2-mediated increase in amyloid β-protein (Aβ) generation in presenilin (PS)-null cells coexpressing PS1 N- and C-terminal fragments (NTF and CTF). Seventy-two hours after PS1 NTF/CTF and PEN-2 or mock was retrovirally coexpressed in PS-null cells (2×10^5) stably expressing APP695, the cells were treated with the indicated concentrations of (a) a transition state analogue, L-685,458, and (b) a non-transition state analogue, DAPT, in a fresh medium and incubated for 24 h. The Aβ40 and Aβ42 secreted from 24-h-cultured cells were quantified by ELISA. Data are expressed as a percentage of the mean Aβ level of untreated controls (vehicle-treated cells). ○, mock infection; ●, cells retrovirally expressing exogenous FLAG-PEN-2. Values are means ± SD of four independent dishes ($n = 4$). The IC_{50} of the inhibitors was as follows: L-685,458 (PEN-2 expression, mock expression), Aβ40 (0.5 μM, 1 μM), Aβ42 (0.8 μM, 2.2 μM); DAPT, Aβ40 (1 μM, 1.6 μM), Aβ42 (2 μM, 4 μM).

to the active site or alter it through an allosteric effect because these inhibitors prevent PS labelling with a transition state analogue affinity reagent (Kornilova *et al.* 2003). These results suggest that the expression of PEN enhances the direct affinity or the accessibility of the γ-secretase substrate to the catalytic site (see Discussion). Similar reductions in the IC_{50} of another transition state analogue inhibitor, WPE-III-31C (Esler *et al.* 2002), and another non-transition state analogue inhibitor, Compound E (Seiffert *et al.* 2000), were observed [IC_{50} of WPE-III-31C (PEN-2 expression, mock expression): Aβ40 (2 μM, 8 μM), Aβ42 (1.8 μM, 2.2 μM); Compound E: Aβ40 (0.5 nM, 0.8 nM), Aβ42 (2 nM, 7 nM)].

Discussion

It was previously shown that NCT, APH-1 and PEN-2 constitute the essential components of the functional PS complex (Francis *et al.* 2002; Lee *et al.* 2002; Steiner *et al.* 2002; Edbauer *et al.* 2003; Hu and Fortini 2003; Kim *et al.* 2003; Kimberly *et al.* 2003; Luo *et al.* 2003; Takasugi *et al.* 2003). Although it has been firmly established that the formation of the active γ-secretase requires all four components, PS, PEN-2, NCT and APH-1, the precise individual roles of the three cofactors with regard to Aβ generation are unclear because it is not known whether the cofactors are only necessary for PS endoproteolysis and the formation of the active γ-secretase or

whether they also play a role in Aβ generation after the formation of the γ-secretase complex. Our study, using a retrovirus expression system, of the effects of various expression levels of the three cofactors on Aβ generation clearly demonstrated that exogenous PEN-2 expression increases Aβ generation. However, this result was discrepant from earlier works showing that an enhanced γ-secretase activity was observed when all four components (PS, NCT, APH and PEN-2) are expressed using the stable transfectant (Kimberly *et al.* 2003; Takasugi *et al.* 2003). Although, at present, the exact reason for this discrepancy is not known, one possible explanation is that it is caused by the difference in exogenous PEN-2 level in the cells expressing PEN-2 alone between our study and other studies. As the intracellular levels of PEN-2 and APH-1 are tightly regulated, as previously reported (Gu *et al.* 2002; Lee *et al.* 2002; Steiner *et al.* 2002; Bergman *et al.* 2004; Crystal *et al.* 2004; Prokop *et al.* 2004), it will be difficult to obtain cells stably expressing PEN-2 or APH-1 at a high level because their intracellular levels could be under this tight regulation during a drug selection step for obtaining a stable transfectant. Indeed, in the earlier works, the PEN-2 level in cells stably expressing PEN-2 alone was definitely lower than that in cells expressing all four components (Kimberly *et al.* 2003; Takasugi *et al.* 2003). As suggested in our study (Fig. 4b), PEN-2 appears to be greatly stabilized when the other three components are coexpressed. Therefore, it will be difficult to compare the effect of PEN-2 on Aβ generation between PEN-2-expressing cells and three-cofactor-coexpressing cells when the PEN-2 expression level is different between them. The retroviral gene transfer system used in this study efficiently delivers gene stably into a target cell without drug selection during a short time. It allows the induction of a stable PEN-2 expression at a high level similar to that observed in three-cofactor-coexpressing cells and comparison between the effect of the expression of PEN-2 alone and that of the coexpression of PEN-2 with the other cofactors (Fig. 1a). Although we cannot completely exclude the unknown indirect effect of the overexpression of these cofactors, our present results strongly suggest that the level of PEN-2 or APH-1 affects γ-secretase activity even after PS endoproteolysis occurs.

Therefore, we next investigated whether γ-secretase activity can be reconstituted by the coexpression of PS NTF and CTF in PS-null cells, as the study of the coexpression of PS NTF and CTF in PS-null cells allows us to distinguish the roles of the cofactors in Aβ generation from the roles in PS endoproteolysis. Previously, it was shown that the coexpression of PS1 NTF and CTF rescues the *sel-12* egg-laying defect, suggesting that the coexpression of PS1 and CTF induces biologically active PS in the cells (Levitan *et al.* 2001). That study also showed that the coexpression of PS NTF and CTF in HEK293 cells does not further increase γ-secretase activity, suggesting that the coexpression of NTF and CTF does not reconstitute γ-secretase activity (Levitan *et al.* 2001). However, we assumed that this result only suggests that γ-secretase activity is saturable, instead of the coexpression of NTF and CTF failing to form the functional complex for γ-secretase activity in cells. Indeed, in the present study, we demonstrated that the coexpression of NTF and CTF induces γ-secretase activity similarly to the PS holoprotein in PS-null cells. In addition, our present study of the reconstitution of γ-secretase activity by NTF/CTF coexpression demonstrated that Asp257 or Asp385 in PS is required for γ-secretase activity. It was also noted that, in this reconstitution of γ-secretase activity in PS-null cells, γ-secretase activity is saturable at certain expression levels of NTF and CTF as observed in cells expressing the PS holoprotein.

In the reconstitution of γ-secretase activity by the coexpression of PS NTF and CTF in PS-null cells, we noted that PEN-2 expression increases Aβ generation but APH-1b expression has an inhibitory effect on PEN-2 stimulation of Aβ generation without inhibiting the binding of PEN-2 to PS. It was also noted that the coexpression of all cofactors in PS-null cells expressing PS1 NTF/CTF does not increase Aβ generation similarly to full-length PS (Figs 1a and b and 4b and c). A possible explanation for this is as follows. The binding of NCT and APH-1 (and PEN-2) to full-length PS is suggested to be a prerequisite of the formation of an active γ-secretase from full-length PS (Hu and Fortini 2003; LaVoie *et al.* 2003; Takasugi *et al.* 2003). However, in the reconstitution of γ-secretase by the coexpression of PS NTF and CTF, the situation may be slightly different because there is no full-length PS in PS-null cells. Therefore, the absence of the step prerequisite to the assembly of cofactors with full-length PS can lead to a smaller effect of all the coexpressed cofactors on γ-secretase activity restored by the expression of PS NTF/CTF than those on γ-secretase activity observed in full-length PS-expressing cells. Our results strongly suggest that the molecular ratio of APH-1 to PEN-2 bound to PS is critical for determining the degree of γ-secretase activity after the formation of a heterodimer complex.

Interestingly, the coexpression of APH-1, PEN-2 and NCT greatly stabilizes the expression levels of PS1 NTF and CTF, accompanied by the stimulation of NCT maturation. However, the stimulation of NCT maturation is not associated with the enhancement of Aβ generation. Recently, it has been shown that the expression of PS1 with a mutation at Asp257 or Asp385 also hastens NCT maturation, indicating that PS catalytic activity is separable from the transport of a γ-secretase complex (Nyabi *et al.* 2003). Our present data also agree with this interpretation. In addition, our study demonstrated that the enhancement of Aβ generation by PEN-2 expression is not associated with the increase in the level of PS1 NTF/CTF. Recent studies have strongly suggested that only a small portion of the PS complex is

involved in an active γ-secretase complex (Lai *et al.* 2003; Gu *et al.* 2004). Therefore, after the formation of an active γ-secretase complex, the activity could be regulated by losing the cofactor(s) from the PS complex or by further binding the cofactor(s) to the PS complex.

To address the mechanism underlying the increase in Aβ generation by PEN-2 expression, we investigated whether PEN-2 expression enhances the formation of PS1 NTF/CTF heterodimers in PS-null cells because PS NTF/CTF heterodimers are believed to constitute the active site in the γ-secretase complex (Esler *et al.* 2000; Li *et al.* 2000b). We found that PEN-2 expression does not enhance PS1 NTF/CTF heterodimer formation, probably because PEN-2 appears not to equivalently interact with both PS1 fragments (Fraering *et al.* 2004). At present, the precise mechanism underlying the increase in Aβ generation by PEN-2 expression has not been elucidated. However, PEN-2 expression caused a significant reduction in the IC_{50} of a transition state analogue γ-secretase inhibitor for Aβ generation, suggesting that PEN-2 expression enhances the direct affinity or the accessibility of the transition state analogue to the catalytic site. PEN-2 expression also reduced the IC_{50} of the non-transition state analogue inhibitors, which were suggested to act at partially overlapping sites where the transition state analogue inhibitors act because these inhibitors prevent PS labelling with a transition state analogue affinity reagent (Kornilova *et al.* 2003). Therefore, it is likely that PEN-2 expression enhances the direct affinity or the accessibility of these non-transition state analogue inhibitors to the overlapping sites where the transition state analogue acts. Although more precise studies using the inhibitors are required, we can speculate that the binding of PEN-2 to PS may enhance the direct affinity or the accessibility of the γ-secretase substrate to the catalytic site, resulting in an increase in γ-secretase activity.

Our present study strongly suggests that PEN-2 is not only a component of the functional PS complex but is also an enhancer of PS-mediated γ-cleavage after PS heterodimer formation. Our results also raise the possibility that PEN-2 regulates γ-secretase activity positively and APH-1 negatively after the functional PS complex is formed. Further studies on how PEN-2 and APH-1 regulate γ-secretase activity using an *in vitro* system will help clarify the molecular mechanism underlying PS-mediated γ-secretase activity.

Acknowledgements

This study was supported by a research grant for Longevity Sciences; Brain Science Research from the Ministry of Health, Labor and Welfare, Japan and the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research. We thank B. De Strooper (Katholieke Universiteit Leuven and Flanders Interuniversity Heveatraat, Belgium) for the supply of antibody B12/4, PS1/PS2 double-deficient fibroblasts and wild-type fibroblasts and S. Gandy for providing antibody 369.

Note added in proof

While this manuscript was being revised, a study on the coexpression of PS1 NTF and CTF in PS-null cells was published (Laudon *et al.* 2004). The authors showed that the coexpression of PS1 NTF and CTF restored γ-secretase activity similar to our results; however, the effects of PS cofactors on Aβ generation restored by the coexpression of NTF/CTF in PS-null cells were not examined in their study.

References

Asami-Odaka A., Ishibashi Y., Kikuchi T., Kitada C. and Suzuki N. (1995) Long amyloid β-protein secreted from wild-type human neuroblastoma IMR-32 cells. *Biochemistry* **34**, 10272–10278.

Bergman A., Hansson E. M., Pursglove S. E., Farmery M. R., Lannfelt L., Lendahl U., Lundkvist J. and Naslund J. (2004) Pen-2 is sequestered in the endoplasmic reticulum and subjected to ubiquitylation and proteasome-mediated degradation in the absence of presenilin. *J. Biol. Chem.* **279**, 16744–16753.

Buxbaum J. D., Gandy S. E., Cicchetti P., Ehrlich M. E., Czernik A. J., Fracasso R. P., Ramabhadran T. V., Unterbeck A. J. and Greengard P. (1990) Processing of Alzheimer β/A4 amyloid precursor protein: modulation by agents that regulate protein phosphorylation. *Proc. Natl Acad. Sci. USA* **87**, 6003–6006.

Crystal A. S., Morais V. A., Fortna R. R., Carlin D., Pierson T. C., Wilson C. A., Lee V. M. and Doms R. W. (2004) Presenilin modulates Pen-2 levels posttranslationally by protecting it from proteasomal degradation. *Biochemistry* **43**, 3555–3563.

De Strooper B., Simons M., Multhaup G., Van Leuven F., Beyreuther K. and Dotti C. G. (1995) Production of intracellular amyloid-containing fragments in hippocampal neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence. *EMBO J.* **14**, 4932–4938.

De Strooper B., Saftig P., Craessaerts K., Vanderstichele H., Guhde G., Annaert W., Von Figura K. and Van Leuven F. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387–390.

De Strooper B., Annaert W., Cupers P. *et al.* (1999) A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518–522.

Dovey H. F., John V., Anderson J. P. *et al.* (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J. Neurochem.* **76**, 173–181.

Edbauer D., Winkler E., Haass C. and Steiner H. (2002) Presenilin and nicastrin regulate each other and determine amyloid β-peptide production via complex formation. *Proc. Natl Acad. Sci. USA* **99**, 8666–8671.

Edbauer D., Winkler E., Regula J. T., Pesold B., Steiner H. and Haass C. (2003) Reconstitution of γ-secretase activity. *Nat. Cell Biol.* **5**, 486–488.

Esler W. P., Kimberly W. T., Ostaszewski B. L., Diehl T. S., Moore C. L., Tsai J. Y., Rahmati T., Xia W., Selkoe D. J. and Wolfe M. S. (2000) Transition-state analogue inhibitors of γ-secretase bind directly to presenilin-1. *Nat. Cell Biol.* **2**, 428–434.

Esler W. P., Kimberly W. T., Ostaszewski B. L., Ye W., Diehl T. S., Selkoe D. J. and Wolfe M. S. (2002) Activity-dependent isolation

of the presenilin-gamma-secretase complex reveals nicastrin and a γ substrate. *Proc. Natl Acad. Sci. USA* **99**, 2720–2725.

Fraering P. C., LaVoie M. J., Ye W., Ostaszewski B. L., Kimberly W. T., Selkoe D. J. and Wolfe M. S. (2004) Detergent-dependent dissociation of active γ-secretase reveals an interaction between Pen-2 and PS1-NTF and offers a model for subunit organization within the complex. *Biochemistry* **43**, 323–333.

Francis R., McGrath G., Zhang J. *et al.* (2002) aph-1 and pen-2 are required for Notch pathway signaling, γ-secretase cleavage of βAPP, and presenilin protein accumulation. *Dev. Cell* **3**, 85–97.

Goutte C., Tsunozaki M., Hale V. A. and Priess J. R. (2002) APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos. *Proc. Natl Acad. Sci. USA* **99**, 775–779.

Gu Y., Misonou H., Sato T., Dohmae N., Takio K. and Ihara Y. (2001) Distinct intramembrane cleavage of the β-amyloid precursor protein family resembling γ-secretase-like cleavage of Notch. *J. Biol. Chem.* **276**, 35 235–35 238.

Gu Y., Chen F., Sanjo N. *et al.* (2002) APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin-nicastrin complexes. *J. Biol. Chem.* **278**, 7374–7854.

Gu Y., Sanjo N., Chen F. *et al.* (2004) The presenilin proteins are components of multiple membrane-bound complex which have different biological activities. *J. Biol. Chem.* in press.

Herreman A., Serneels L., Annaert W., Collen D., Schoonjans L. and De Strooper B. (2000) Total inactivation of γ-secretase activity in presenilin-deficient embryonic stem cells. *Nat. Cell Biol.* **2**, 461–462.

Hu Y. and Fortini M. E. (2003) Different cofactor activities in gamma-secretase assembly: evidence for a nicastrin-Aph-1 subcomplex. *J. Cell Biol.* **161**, 685–690.

Kim S. H., Ikeuchi T., Yu C. and Sisodia S. S. (2003) Regulated hyperaccumulation of presenilin-1 and the 'γ-secretase' complex. Evidence for differential intramembranous processing of transmembrane subatrates. *J. Biol. Chem.* **278**, 33 992–34 002.

Kimberly W. T., LaVoie M. J., Ostaszewski B. L., Ye W., Wolfe M. S. and Selkoe D. J. (2003) γ-Secretase is a membrane protein complex comprised of presenilin, nicastrin, Aph-1, and Pen-2. *Proc. Natl Acad. Sci. USA* **100**, 6382–6387.

Komano H., Shiraishi H., Kawamura Y., Sai X., Suzuki R., Serneels L., Kawaichi M., Kitamura T. and Yanagisawa K. (2002) A new functional screening system for identification of regulators for the generation of amyloid beta-protein. *J. Biol. Chem.* **277**, 39 627–39 633.

Kornilova A. Y., Das C. and Wolfe M. S. (2003) Differential effects of inhibitors on the γ-secretase complex. Mechanistic implications. *J. Biol. Chem.* **278**, 16 470–16 473.

Lai M. T., Chen E., Crouthamel M. C. *et al.* (2003) Presenilin-1 and presenilin-2 exhibit distinct yet overlapping gamma-secretase activities. *J. Biol. Chem.* **278**, 22 475–22 481.

Lammich S., Okochi M., Takeda M., Kaether C., Capell A., Zimmer A. K., Edbauer D., Walter J., Steiner H. and Haass C. (2002) Presenilin-dependent intramembrane proteolysis of CD44 leads to the liberation of its intracellular domain and the secretion of an Abeta-like peptide. *J. Biol. Chem.* **277**, 44 754–44 759.

Laudon H., Mathews P. M., Karlstrom H. *et al.* (2004) Co-expressed presenilin 1 NTF and CTF form functional γ-secretase complexes in cells devoid of full-length protein. *J. Neurochem.* **89**, 44–53.

LaVoie M. J., Fraering P. C., Ostaszewski B. L., Ye W., Kimberly W. T., Wolfe M. S. and Selkoe D. J. (2003) Assembly of the γ-secretase complex involves early formation of an intermediate subcomplex of Aph-1 and nicastrin. *J. Biol. Chem.* **278**, 37 213–37 222.

Lee H. J., Jung K.M., Huang Y.Z , Bennet L.B , Lee J.S., Mei L. and Kim T.W. (2002) Presenilin-dependent γ-secretase-like intramembrane cleavage of ErbB4. *J. Biol. Chem.* **277**, 6318–6323.

Lee S. F., Shah S., Li H., Yu C., Han W. and Yu G. (2002) Mammalian APH-1 interacts with presenilin and nicastrin and is required for intramembrane proteolysis of amyloid-β precursor protein and notch. *J. Biol. Chem.* **277**, 45 013–45 019.

Levitan D., Lee J., Song L., Manning R., Wong G., Parker E. and Zhang L. (2001) PS1 N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling. *Proc. Natl Acad. Sci. USA* **98**, 12 186–12 190.

Li Y. M., Lai M. T., Xu M., Huang Q., DiMuzio-Mower J., Sardana M. K., Shi X. P., Yin K. C., Shafer J. A. and Gardell S. J. (2000a) Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state. *Proc. Natl Acad. Sci. USA* **97**, 6138–6143.

Li Y. M., Xu M., Lai M. T. *et al.* (2000b) Photoactivated γ-secretase inhibitors directed to the active site covalently label presenilin 1. *Nature* **405**, 689–694.

Luo W. J., Wang H., Li H., Kim B. S., Shah S., Lee H. J., Thinakaran G., Kim T. W., Yu G. and Xu H. (2003) PEN-2 and APH-1 coordinately regulate proteolytic processing of presenilin 1. *J. Biol. Chem.* **278**, 7850–7854.

Marambaud P., Shioi J., Serban G. *et al.* (2002) A presenilin-1/γ-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J.* **21**, 1948–1956.

Marambaud P., Wen P. H., Dutt A., Shioi J., Takashima A., Siman R. and Robakis N. K. (2003) A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell* **114**, 635–645.

Murakami D., Okamoto I., Nagano O., Kawano Y., Tomita T., Iwatsubo T., De Strooper B., Yumoto E. and Saya H. (2003) Presenilin-dependent γ-secretase activity mediates the intramembranous cleavage of CD44. *Oncogene* **22**, 1511–1516.

Ni C. Y., Murphy M. P., Golde T. E. and Carpenter G. (2001) γ-Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science* **294**, 2179–2181.

Nyabi O., Bentahir M., Horre K., Herreman A., Gottardi-Littell N., Van Broeckhoven C., Merchiers P., Spittaels K., Annaert W. and De Strooper B. (2003) Presenilins mutated at Asp-257 or Asp-385 restore Pen-2 expression and Nicastrin glycosylation but remain catalytically inactive in the absence of wild type Presenilin. *J. Biol. Chem.* **278**, 43 430–43 436.

Onishi M., Kinoshita S., Morikawa Y., Shibuya A., Phillips J., Lanier L. L., Gorman D. M., Nolan G. P., Miyajima A. and Kitamura T. (1996) Applications of retrovirus-mediated expression cloning. *Exp. Hematol.* **24**, 324–329.

Prokop S., Shirotani S., Edbauer D., Haass C. and Steiner H. (2004) Requirement of PEN-2 for stabilization of the presenilin NTF/CTF heterodimer within the gamma-secretase complex. *J. Biol. Chem.* **279**, 23 255–23 261.

Ratovitski T., Slunt H. H., Thinakaran G., Price D. L., Sisodia S. S. and Borchelt D. R. (1997) Endoproteolytic processing and stabilization of wild-type and mutant presenilin. *J. Biol. Chem.* **272**, 24 536–24 541.

Sai X., Kawamura Y. H,, Kokame K..*et al* (2002) Endoplasmic reticulum stress-inducible protein, Herp, enhances presenilin-mediated generation of amyloid {beta} protein . *J. Biol. Chem.* **277**, 12 915–12 920.

Sastre M., Steiner H., Fuchs K., Capell A., Multhaup G., Condron M. M., Teplow D. B. and Haass C. (2001) Presenilin-dependent γ-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep.* **2**, 835–841.

Schroeter E. H., Ilagan M. X., Brunkan A. L. *et al.* (2003) A presenilin dimer at the core of the γ-secretase enzyme: insights from parallel analysis of Notch 1 and APP proteolysis. *Proc. Natl Acad. Sci. USA* **100**, 13 075–13 080.

Seiffert D., Bradley J. D., Rominger C. M. *et al.* (2000) Presenilin-1 and -2 are molecular targets for γ-secretase inhibitors. *J. Biol. Chem.* **275**, 34 086–34 091.

Selkoe D. J. (1999) Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* **399**, A23–A31.

Shearman M. S., Beher D., Clarke E. E., Lewis H. D., Harrison T., Hunt P., Nadin A., Smith A. L., Stevenson G. and Castro J. L. (2000) L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid β-protein precursor gamma-secretase activity. *Biochemistry* **39**, 8698–8704.

Steiner H., Kostka M., Romig H. *et al.* (2000) Glycine 384 is required for presenilin-1 function and is conserved in bacterial polytopic aspartyl proteases. *Nat. Cell Biol.* **2**, 848–851.

Steiner H., Winkler E., Edbauer D., Prokop S., Basset G., Yamasaki A., Kostka M. and Haass C. (2002) PEN-2 is an integral component of the γ-secretase complex required for coordinated expression of presenilin and nicastrin. *J. Biol. Chem.* **277**, 39 062–39 065.

Sudoh S., Kawamura Y., Sato S., Wang R., Saido T. C., Oyama F., Sakaki Y., Komano H. and Yanagisawa K. (1998) Presenilin 1 mutations linked to familial Alzheimer's disease increase the intracellular levels of amyloid β-protein 1–42 and its N-terminally truncated variant(s) which are generated at distinct sites. *J. Neurochem.* **71**, 1535–1543.

Takasugi N., Tomita T., Hayashi I., Tsuruoka M., Niimura M., Takahashi Y., Thinakaran G. and Iwatsubo T. (2003) The role of presenilin cofactors in the γ-secretase complex. *Nature* **422**, 438–441.

Thinakaran G., Borchelt D. R., Lee M. K. *et al.* (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron* **17**, 181–190.

Thinakaran G., Harris C. L., Ratovitski T., Davenport F., Slunt H. H., Price D. L., Borchelt D. R. and Sisodia S. S. (1997) Evidence that levels of presenilins (PS1 and PS2) are coordinately regulated by competition for limiting cellular factors. *J. Biol. Chem.* **272**, 28 415–28 422.

Vassar R., Bennett B. D., Babu-Khan S. *et al.* (1999) β-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* **286**, 735–741.

Weidemann A., Eggert S., Reinhard F. B., Vogel M., Paliga K., Baier G., Masters C. L., Beyreuther K. and Evin G. (2002) A novel epsilon-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing. *Biochemistry* **41**, 2825–2835.

Weihofen A., Binns K., Lemberg M. K., Ashman K. and Martoglio B. (2002) Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science* **296**, 2215–2218.

Wolfe M. S., Xia W., Ostaszewski B. L., Diehl T. S., Kimberly W. T. and Selkoe D. J. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity. *Nature* **398**, 513–517.

Yang D. S., Tandon A., Chen F. *et al.* (2002) Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins. *J. Biol. Chem.* **277**, 28 135–28 142.

Yu G., Chen F., Nishimura M. *et al.* (2000a) Mutation of conserved aspartates affects maturation of both aspartate mutant and endogenous presenilin 1 and presenilin 2 complexes. *J. Biol. Chem.* **275**, 27 348–27 353.

Yu G., Nishimura M., Arawaka S. *et al.* (2000b) Nicastrin modulates presenilin-mediated notch/glp-1 signal transduction and βAPP processing. *Nature* **407**, 48–54.

Zhang Z., Nadeau P., Song W., Donoviel D., Yuan M., Bernstein A. and Yankner B. A. (2000) Presenilins are required for γ-secretase cleavage of β-APP and transmembrane cleavage of Notch-1. *Nat. Cell Biol.* **2**, 463–465.

The tissue plasminogen activator–plasmin system participates in the rewarding effect of morphine by regulating dopamine release

Taku Nagai*†, Kiyofumi Yamada*†‡§, Masako Yoshimura*, Kazuhiro Ishikawa*, Yoshiaki Miyamoto*, Kazuki Hashimoto‡, Yukihiro Noda*, Atsumi Nitta*, and Toshitaka Nabeshima*§

*Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan; and ‡Laboratory of Neuropsychopharmacology, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa 920-0934, Japan

Edited by Solomon H. Snyder, John Hopkins University School of Medicine, Baltimore, MD, and approved January 8, 2004 (received for review October 11, 2003)

Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of plasminogen (plg) to plasmin, which in turn functions to degrade extracellular matrix proteins in the central nervous system. The tPA-plasmin system plays a role in synaptic plasticity and remodeling. Here we show that this protease system participates in the rewarding effects of morphine by acutely regulating morphine-induced dopamine release in the nucleus accumbens (NAcc). A single morphine treatment induced tPA mRNA and protein expression in a naloxone-sensitive manner, which was associated with an increase in the enzyme activity in the NAcc. The acute effect of morphine in inducing tPA expression was diminished after repeated administration. Morphine-induced conditioned place preference and hyperlocomotion were significantly reduced in $tPA^{-/-}$ and $plg^{-/-}$ mice, being accompanied by a loss of morphine-induced dopamine release in the NAcc. The defect of morphine-induced dopamine release and hyperlocomotion in $tPA^{-/-}$ mice was reversed by microinjections of either exogenous tPA or plasmin into the NAcc. Our findings demonstrate a previously undescribed function of the tPA-plasmin system in regulating dopamine release, which is involved in the rewarding effects of morphine.

Extracellular proteases are expressed by neurons in the central nervous system (1–4), and their function can vary from potentiating neurotransmitter receptor function (5) to structural alterations associated with long-lasting forms of synaptic plasticity (6, 7). Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of plasminogen (plg) to plasmin and plays a role in fibrinolysis. In addition, tPA is abundantly expressed in the central nervous system (8–10), where this protease is stored in synaptic vesicles (11, 12), released into the extracellular space by a depolarization stimulus (11, 12), and then the expression of its mRNA is up-regulated (8, 12). Recent studies have demonstrated that tPA regulates a cascade of extracellular proteolytic activities involved in neurite outgrowth (13), cell migration (14, 15), long-term potentiation and depression (6, 16–18), learning and memory (9, 17, 18), excitotoxic cell death (19, 20), and regeneration or recovery from injury in the nervous system (21). These findings suggest that tPA is involved in the regulation of numerous aspects of synaptic plasticity and remodeling.

It is well known that drugs of abuse, including morphine, acutely modulate the activity of mesolimbic dopaminergic neurons, projecting from the ventral tegmental area (VTA) of the midbrain to the nucleus accumbens (NAcc) (22–24). Morphine increases dopaminergic neurotransmission in the NAcc via the activation of dopamine cells in the VTA, an area that possesses a high density of μ-opioid receptors. This activation results mainly from the disinhibition of inhibitory GABAergic (GABA, γ-aminobutyric acid) interneurons in the VTA (25, 26). The rewarding effects of morphine that are associated with enhanced dopamine release in the NAcc are related to its abuse. It has been proposed that activity-dependent synaptic plasticity and remodeling of the mesolimbic dopaminergic system play a crucial role in the development of drug dependence (27).

In the present study, we examined the role of the tPA-plasmin system in the rewarding effects of and dependence on morphine in mice with a targeted deletion of the tPA ($tPA^{-/-}$ mice) (28) and plg ($plg^{-/-}$ mice) genes (29). Our findings suggest that the tPA-plasmin system participates in the rewarding effects of morphine by acutely regulating morphine-induced dopamine release in the NAcc.

Materials and Methods

Animals. Male Wistar rats (7 weeks old) were obtained from Charles River Breeding Laboratories (Yokohama, Japan). Wild-type (C57BL/6J), $tPA^{-/-}$, and $plg^{-/-}$ mice were from The Jackson Laboratory. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Nagoya University.

Real-Time RT-PCR. For the single morphine treatment, animals were given morphine hydrochloride (Shionogi Pharmaceutical, Osaka) at a dose of 10 mg/kg s.c., whereas for repeated treatment, they were subjected to a 5-day regimen in which increasing doses of morphine (10, 20, 30, 40, and 50 mg/kg s.c.) were injected twice a day and then challenged with morphine (10 mg/kg s.c.) on day 6. The levels of tPA mRNA were determined by real-time RT-PCR by using an ABI PRISM 7700 sequencer detector (PE Applied Biosystems). The primers used were as follows: 5′-AAGGAGGCTCACGTCAGACTGTA-3′ (forward), 5′-CCTGCACACAGCATGTTGCT-3′ (reverse), TaqMan probe, 5′-CAGCCGCTGTACCTCACAGCATCTGTTTAA-3′.

Immunoprecipitation and Immunoblot. Brain tissues were homogenized, and the supernatants were incubated with polyclonal goat anti-tPA antibodies (Santa Cruz Biotechnology), followed by protein G-Sepharose. The resulting immune complexes were resuspended in Laemli sample buffer. Immunoblots were probed with polyclonal rabbit anti-tPA antibodies (American Diagnostica, Greenwich, CT), followed by horseradish peroxidase-linked

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: plg, plasminogen; tPA, tissue plg activator; NAcc, nucleus accumbens; VTA, ventral tegmental area.

†T.N. and K.Y. contributed equally to this work.

§To whom correspondence may be addressed. E-mail: kyamada@p.kanazawa-u.ac.jp or tnabeshi@med.nagoya-u.ac.jp.

www.pnas.org/cgi/doi/10.1073/pnas.0306587101

anti-rabbit secondary antibodies. The enhanced chemiluminescence method (Amersham Pharmacia Biosciences) was used.

Zymography. Gel zymography was adapted from a procedure described previously (30). Ten percent polyacrylamide SDS gels were copolymerized with casein (1 mg/ml; Sigma) and plasminogen (15 μg/ml; Chromogenix, Molndal, Sweden). Brain tissues were homogenized in lysis buffer (50 mM Tris·HCl, pH 6.8/0.05% Triton X-100/2 mM EDTA) and assayed for protein content. Serial concentrations of the homogenate were loaded onto the gels and electrophoresed. After electrophoresis, the SDS was extracted from the gel by using 2.5% Triton X-100, and the gel was incubated for 1 h in 0.1 M Tris·HCl, pH 8.1, at 37°C, followed by staining with 0.125% Coomassie brilliant blue in 50% methanol/10% acetic acid. Destaining with the same solvent revealed a transparent zone of lysis against the dark protein background at 65 kDa corresponding to tPA. The enzymatic activity of tPA was analyzed with the ATTO Densitograph Software Library Lane Analyzer (Atto Instruments, Tokyo).

***In Situ* Hybridization.** Frozen sections (10 μm) were thawed on coverslips and used for *in situ* hybridization (31). pAtlas1A plasmid with rat tPA cDNA (BD Biosciences, Palo Alto, CA) was linearized with *Sac*I or *Kpn*I and used as a template for production of digoxigenin-labeled antisense cRNA (335 base) or sense cRNA (328 base) with T3 or T7 RNA polymerase (Promega).

Immunohistochemistry. Sections (14 μm) were incubated with polyclonal rabbit anti-tPA (1:1,000, Molecular Innovations, Southfield, MI) and monoclonal mouse antimicrotubule-associated protein 2 (MAP2) antibodies (1:5,000, Sigma), in blocking serum containing goat anti-rabbit Alexa Fluor 546 (1:500, Molecular Probes) and goat anti-mouse Alexa Fluor 488 (1:1,000, Molecular Probes). Samples were observed with AXIOVISION 3.0 systems (Zeiss).

***In Vivo* Microdialysis.** Animals were anesthetized with sodium pentobarbital, and a guide cannula (AG-8, EICOM, Kyoto) was implanted in the NAcc (AP +1.1, ML +1.0 from bregma, DV −3.6 from the skull) according to the atlas (32). Two days after the operation, a dialysis probe (AI-8–1; 1-mm membrane length, EICOM) was inserted through a guide cannula and perfused with an artificial cerebrospinal fluid (aCSF; 147 mM NaCl/4 mM KCl/2.3 mM $CaCl_2$) at a flow rate of 1.0 μl/min. The outflow fractions were collected every 20 min. After the collection of three baseline fractions, mice were treated with morphine (10 mg/kg s.c.). For depolarization stimulation, 60 mM KCl containing aCSF was delivered through the dialysis probe for 20 min. Dopamine levels in the dialysates were analyzed as described (33). For the rescue study with tPA and plasmin, a dialysis probe equipped with a microinjection tube (MIA-8–1; 1 mm membrane length, EICOM) was used (34). After the collection of baseline fractions, a 100-ng dose of human recombinant tPA (provided by Eisai, Tokyo) or 0.01 unit dose of human plasmin (Chromogenix) dissolved in 1 μl of aCSF solution was injected during a 10-min period through the microinjection tube into the NAcc. Ten minutes after the microinjection, mice were treated with morphine.

Behavioral Analysis. For the acute locomotor-stimulating effect, $tPA^{-/-}$ and wild-type mice were injected with saline or morphine (10 mg/kg s.c.) on day 1, and the locomotor activity was measured for 180 min. For the sensitization, mice were injected with morphine (10 mg/kg s.c.) twice per day for 5 days from days 2 to 6, and morphine-induced locomotor activity was measured on day 16. For the rescue study with tPA and plasmin, a guide cannula was implanted in the NAcc (AP +1.1, ML ±1.0 from bregma, DV −3.6 from the skull). After recovery from the operation, tPA (100 ng) or human plasmin (0.01 units) was injected through the microinjection tube into the NAcc. Ten minutes after the microinjection, mice were treated with morphine (10 mg/kg s.c.), and the locomotor activity was measured for 180 min.

For the conditioned place preference test (35), a mouse was allowed to move freely between transparent and black boxes for 15 min once per day for 3 days (days 1–3) in the preconditioning. On day 3, the time the mouse spent in each box was measured. On days 4, 6, and 8, the mouse was treated with morphine and confined in either the transparent or black box for 30 min. On days 5, 7, and 9, the mouse was given saline and placed opposite to the morphine-conditioning box for 30 min. On day 10, the postconditioning test was performed without drug treatment, and the time the mouse spent in each box was measured for 15 min.

For the hot-plate test (36), nociceptive latency was assessed as the response time to the hot plate (55 ± 1°C). To avoid tissue damage, an artificial maximum time for exposure was imposed, which prevented the animal from making contact with the plate for >30 sec.

Statistical Analysis. Statistical analysis was performed by using ANOVA and the Bonferroni test. The Mann–Whitney *U* test was used to compare two sets of data. Data were expressed as the mean ± SE. *P* values of <0.05 were considered statistically significant.

Results

Morphine-Induced tPA mRNA Expression in the Brain. The effects of single and repeated administration of morphine on the expression of tPA mRNA in various regions of the rat brain were measured 2 h after the final morphine treatment by a real-time RT-PCR method. Single morphine treatment remarkably induced the tPA mRNA expression compared with saline treatment in the frontal cortex (FC, 257% of control, $P < 0.01$), NAcc (772%, $P < 0.01$), striatum (STR, 535%, $P < 0.01$), hippocampus (HIP, 799%, $P < 0.01$), VTA (337%, $P < 0.01$), and amygdala (AMY, 332%, $P < 0.01$) (Fig. 1*A*). Although the effect of morphine on tPA mRNA expression was significantly reduced after repeated treatment, expression levels were significantly higher in the repeated morphine-treated group than control group in the FC (146%, $P < 0.05$), NAcc (150%, $P < 0.05$), STR (143%, $P < 0.05$), and HIP (248%, $P < 0.01$) (Fig. 1*A*). Because it is known that there are some species differences in the effect of morphine, we also examined the effect of morphine on tPA expression in mice. The levels of tPA mRNA were significantly increased in the NAcc of mice 1 h (158% of control, $P < 0.01$), 2 h (179%, $P < 0.01$), and 6 h (140%, $P < 0.05$) after morphine treatment and then returned to the control value 24 h later [$F_{(6, 35)} = 4.401$, $P < 0.01$, Fig. 1*B*]. The protein levels of tPA determined by Western blotting were also significantly increased 6 h (147%, $P < 0.05$), 24 h (155%, $P < 0.01$), and 48 h (143%, $P < 0.05$) after single morphine treatment and then returned to the control value 1 week after the treatment [$F_{(6, 42)} = 3.384$, $P < 0.01$, Fig. 1*C*]. The enzymatic activity of tPA, which was assayed by gel zymography (30), was significantly increased compared with the saline-treated group 1 h (145%, $P < 0.01$), 2 h (139%, $P < 0.01$), 6 h (126%, $P < 0.05$), and 48 h (128%, $P < 0.05$) after the morphine treatment [$F_{(6,28)} = 3.733$, $P < 0.01$, Fig. 1*D*]. No enzymatic activity of tPA was detected in $tPA^{-/-}$ mice (Fig. 1*D*). Morphine-induced tPA activity was completely inhibited by pretreatment with naloxone (1 mg/kg i.p.), although naloxone itself had no effect, suggesting the involvement of opioid receptors in the morphine-induced increase in tPA activity (Fig. 1*E*).

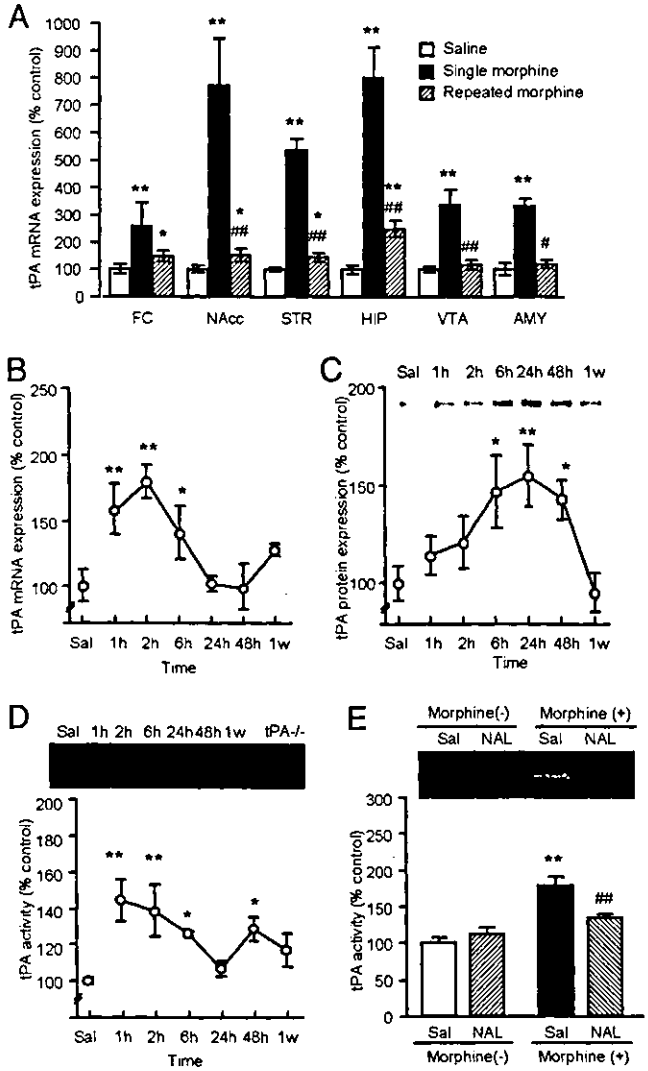

Fig. 1. Increased tPA expression after morphine treatment. (*A*) Changes in tPA mRNA expression after single (10 mg/kg s.c.) and repeated (10–50 mg/kg × 2 per day s.c.) morphine treatment in the rat brain. (*B*) Time course changes in tPA mRNA expression after single morphine (10 mg/kg s.c.) treatment in the NAcc of mice. (*C*) Immunoprecipitation/immunoblot analysis of tPA protein. (*D*) Zymographic analysis of tPA activity. (*E*) Effect of naloxone on single morphine-induced tPA activity. Values indicate means ± SE (n = 9–10 for *A*, n = 6 for *B*, n = 7 for *C*, n = 6 for *D*, and n = 4 for *E*). *, $P < 0.05$ and **, $P < 0.01$ compared with the saline (Sal)-treated group. #, $P < 0.05$ and ##, $P < 0.01$ compared with the single morphine-treated group.

Morphine-Induced tPA Is Produced in Neurons of the NAcc. It has been reported that tPA mRNA is present in both neurons and microglia but not oligodendrocytes or astrocytes (10, 19). To determine the cell types in which tPA expression is induced by acute morphine treatment, *in situ* hybridization with antisense tPA digoxigenin-labeled RNA probes as well as immunohistochemistry with specific tPA antibodies was performed (Fig. 2). The tPA mRNA was detected in cells of the NAcc in both saline- and morphine-treated wild-type mice (Fig. 2 *A* and *B*), but the signals in morphine-treated animals were apparently more intense than those in saline-treated animals. No signals were detected in the brain sections of $tPA^{-/-}$ mice (Fig. 2*C*). Immunohistochemistry revealed that tPA immunoreactivity was localized to cells positive for MAP2, a marker of neuronal cells (Fig. 2 *D–F*), indicating that tPA is produced in neuronal cells after morphine treatment.

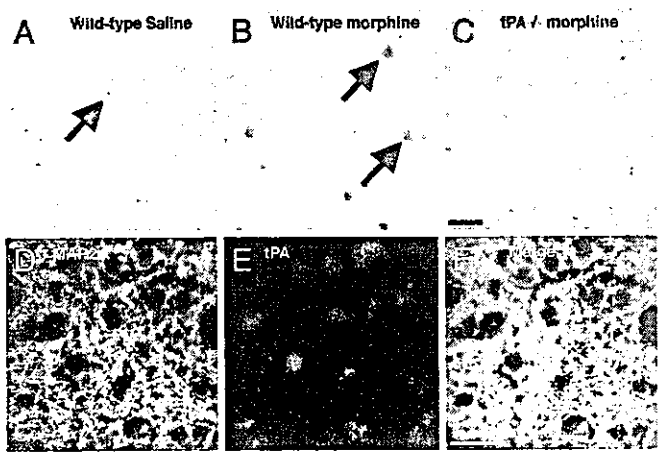

Fig. 2. tPA expression in the NAcc after morphine treatment. *In situ* hybridization analysis of tPA mRNA expression in the NAcc of saline-treated wild-type mice (*A*), morphine-treated wild-type mice (*B*), and morphine-treated $tPA^{-/-}$ mice (*C*). (*D–F*) Immunohistochemical analysis of morphine-induced tPA expression in the NAcc of mice. (*D*) MAP2. (*E*) tPA. (*F*) Merge. (Bar = 20 μm.)

No Changes in Antinociceptive Effects of Morphine and Tolerance in $tPA^{-/-}$ Mice. To investigate the physiological significance of the morphine-induced increase in tPA expression, we examined the antinociceptive effect of morphine and the development of its tolerance (36) in $tPA^{-/-}$ mice. There were no differences in the basal antinociceptive threshold between wild-type and $tPA^{-/-}$ mice in the hot-plate test (wild-type, 4.4 ± 0.5 sec; $tPA^{-/-}$, 4.6 ± 0.5 sec). No differences were observed either in the acute morphine-induced antinociceptive effect between wild-type and $tPA^{-/-}$ mice. Repeated injections of morphine (10 mg/kg × 2 per day s.c.) for 5 days resulted in a gradual loss of the antinociceptive effects of morphine in both wild-type and $tPA^{-/-}$ mice, and there were no differences at all in the time course (Fig. 3*A*). These results suggest that tPA has little effect on morphine-induced antinociception and its tolerance after repeated administration.

Morphine-Induced Behavioral Effects in $tPA^{-/-}$ and $plg^{-/-}$ Mice. Because we observed marked changes in tPA expression and activity in the NAcc, a brain area important for the rewarding effects of drugs of abuse (24, 27), we focused on the role of the tPA-plasmin system in the rewarding effects of morphine, which can be assessed by using the conditioned place-preference test (35). Morphine induced a dose-dependent conditioned place preference in wild-type mice, whereas saline treatment had no effect on place preference [$F_{(2, 48)} = 11.811$, $P < 0.01$, Fig. 3*B*]. Interestingly, the rewarding effects of morphine were markedly reduced in $tPA^{-/-}$ mice at the conditioning doses of 3 and 10 mg/kg ($P < 0.05$, Fig. 3*B*). Moreover, morphine (10 mg/kg s.c.) failed to induce place preference in $plg^{-/-}$ mice ($P < 0.05$, Fig. 3*C*), suggesting a role for the tPA-plasmin system in the rewarding effects of morphine.

The NAcc is involved not only in rewarding effects but also in locomotor-stimulating effects of morphine (22). Therefore, we measured the locomotor-stimulating effects of morphine in $tPA^{-/-}$ mice. Single morphine (10 mg/kg s.c.) treatment induced a hyperlocomotion in wild-type mice and $tPA^{-/-}$ mice, but the magnitude was significantly reduced in $tPA^{-/-}$ mice compared with wild-type mice ($P < 0.05$, Fig. 3*D*). Although repeated morphine treatment significantly potentiated the locomotor-stimulating effects of morphine in wild-type mice ($P < 0.01$), it failed to potentiate the hyperlocomotion in $tPA^{-/-}$ mice (Fig. 3*D*). We also examined whether exogenous tPA and plasmin can reverse the defect of loco-

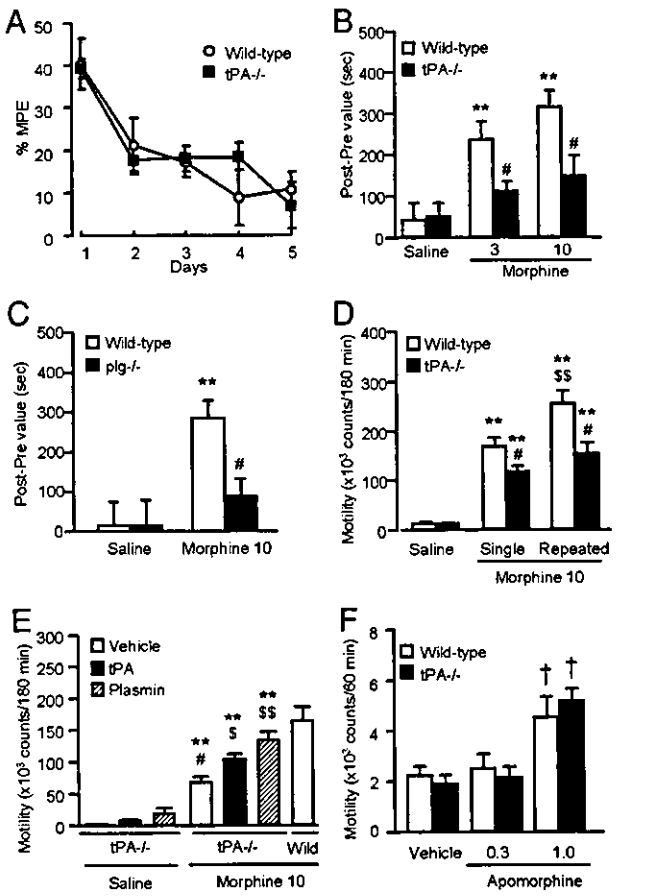

Fig. 3. Morphine-induced behavioral effects in $tPA^{-/-}$ and $plg^{-/-}$ mice. (*A*) Development of tolerance to the antinociceptive effect of morphine (10 mg/kg s.c.) in $tPA^{-/-}$ mice. (*B* and *C*) Morphine (3 and 10 mg/kg s.c.)-induced place preference in $tPA^{-/-}$ mice (*B*) and $plg^{-/-}$ mice (*C*). (*D*) Locomotor activity and locomotor sensitization induced by morphine (10 mg/kg s.c.) treatment. (*E*) Effect of tPA and plasmin microinjection into the NAcc on locomotor activity induced by morphine (10 mg/kg s.c.) in the $tPA^{-/-}$ mice. (*F*) Apomorphine (0.3 and 1.0 mg/kg s.c.)-induced hyperlocomotion. Values indicate means ± SE (n = 4–5 for *A*, n = 8–21 for *B*, n = 6–12 for *C*, n = 10 for *D*, n = 8 for *E*, and n = 8 for *F*). *, $P < 0.05$ and **, $P < 0.01$ compared to corresponding saline-treated mice. #, $P < 0.05$ compared to corresponding morphine-treated wild-type mice. $, $P < 0.05$ and $$, $P < 0.01$ compared to vehicle plus morphine-treated $tPA^{-/-}$ mice. †, $P < 0.05$ compared to corresponding vehicle-treated mice.

motor-stimulating effect of morphine in $tPA^{-/-}$ mice. The attenuation of hyperlocomotion in $tPA^{-/-}$ mice was significantly reversed by microinjections of either exogenous tPA (100 ng, $P < 0.05$) or plasmin (0.01 units, $P < 0.01$) into the NAcc [$F_{(6, 49)} = 32.824$, $P < 0.01$, Fig. 3*E*].

The reduction of these behavioral effects of morphine in $tPA^{-/-}$ and $plg^{-/-}$ mice might be due to the alteration of dopamine and/or opioid receptor sensitivity. To test this possibility, we studied locomotor responses to different doses of apomorphine, a direct dopamine D1/D2 receptor agonist. In wild-type mice, apomorphine (0.3 and 1.0 mg/kg s.c.) significantly increased locomotor activities in a dose-dependent manner [$F_{(2, 21)} = 4.389$, $P < 0.05$], and there were no differences in apomorphine-induced hyperlocomotion between wild-type and $tPA^{-/-}$ mice (Fig. 3*F*). We also measured dopamine- and morphine-induced stimulation of [^{35}S]GTPγS binding in membrane preparation *in vitro*. The dopamine- and morphine-induced [^{35}S]GTPγS binding in $tPA^{-/-}$ mice did not differ from those in wild-type mice (Fig. 6, which is published as supporting information on the PNAS web site). These results indicate that the tPA-plasmin system plays a crucial role in morphine-induced rewarding and locomotor stimulating effects, and that these behavioral changes in $tPA^{-/-}$ mice are not due to alterations of dopamine and opioid receptor sensitivity.

Role of tPA in Morphine-Induced Dopamine Release in the NAcc. It has been suggested that the enhancement of dopamine release in the NAcc is an essential process related to the morphine-induced rewarding effect (37). To clarify the mechanisms by which $tPA^{-/-}$ mice exhibited the reduced morphine-induced rewarding and locomotor-stimulating effects, we measured morphine-induced dopamine release in the NAcc. *In vivo* microdialysis revealed that basal levels of dopamine in the NAcc were not different between wild-type and $tPA^{-/-}$ mice (wild-type, 0.41 ± 0.13 nM, n = 4; $tPA^{-/-}$, 0.53 ± 0.01 nM, n = 4). The dopamine levels in the NAcc were markedly increased by s.c. injection of morphine at 10 mg/kg in wild-type mice (Fig. 4*A*). This morphine-induced dopamine release was markedly diminished in $tPA^{-/-}$ mice [$F_{(1, 6)} = 13.061$, $P < 0.05$, Fig. 4*A*]. Similarly, although basal levels of dopamine in the NAcc of $plg^{-/-}$ mice did not differ from those in wild-type mice (wild-type, 0.38 ± 0.10 nM, n = 6; $plg^{-/-}$, 0.38 ± 0.05 nM, n = 6), morphine-induced dopamine release in the NAcc was significantly reduced in $plg^{-/-}$ mice compared to wild-type mice [$F_{(1, 10)} = 25.147$, $P < 0.01$, Fig. 4*B*].

We investigated the mechanisms underlying the defect of morphine-induced dopamine release in $tPA^{-/-}$ mice and $plg^{-/-}$ mice. Microinjection of tPA (100 ng) into the NAcc slightly but significantly increased basal levels of extracellular dopamine in $tPA^{-/-}$ mice [$F_{(1, 8)} = 7.941$, $P < 0.05$, Fig. 4*C*]. Further, this pretreatment dramatically increased morphine-induced dopamine release in $tPA^{-/-}$ mice as observed in wild-type mice [$F_{(1, 8)} = 5.428$, $P < 0.05$, Fig. 4*D*]. In contrast, microinjection of tPA (100 ng) into the VTA failed to increase morphine-induced dopamine release in the NAcc of $tPA^{-/-}$ mice (Fig. 7, which is published as supporting information on the PNAS web site). These results suggest that the defect of morphine-induced dopamine release in $tPA^{-/-}$ mice is due to the deficiency of tPA in the NAcc, not to a developmental malfunction. Microinjection of plasmin (0.01 units) into the NAcc caused a transient but significant increase in basal dopamine levels in $tPA^{-/-}$ mice [$F_{(1, 8)} = 6.612$, $P < 0.05$, Fig. 4*E*]. Moreover, this pretreatment markedly increased morphine-induced dopamine release in $tPA^{-/-}$ mice [$F_{(1, 8)} = 13.121$, $P < 0.01$, Fig. 4*F*]. These results suggest that tPA modulates morphine-induced dopamine release probably by converting plg to plasmin in the NAcc.

Because tPA is released into the extracellular space by a depolarization stimulus (11, 12), we investigated the depolarization (60 mM KCl)-evoked dopamine release in the NAcc of $tPA^{-/-}$ mice. The lack of tPA significantly attenuated the depolarization-evoked dopamine release in the NAcc [$F_{(1, 10)} = 6.846$, $P < 0.05$, Fig. 5*A*]. There were no differences in the protein content of tyrosine hydroxylase in the NAcc and lower midbrain, a rate limiting enzyme of dopamine synthesis, between $tPA^{-/-}$ and wild-type mice (Fig. 5*B*).

Discussion

It has been demonstrated that tPA takes part in the proteolysis of extracellular matrix proteins in the central nervous system and is involved in neuronal plasticity. In the present study, we demonstrated a previously undescribed function of the tPA-plasmin system in regulating dopamine release in the NAcc. Furthermore, the tPA-plasmin system is involved in the rewarding effects of morphine by regulating dopamine release.

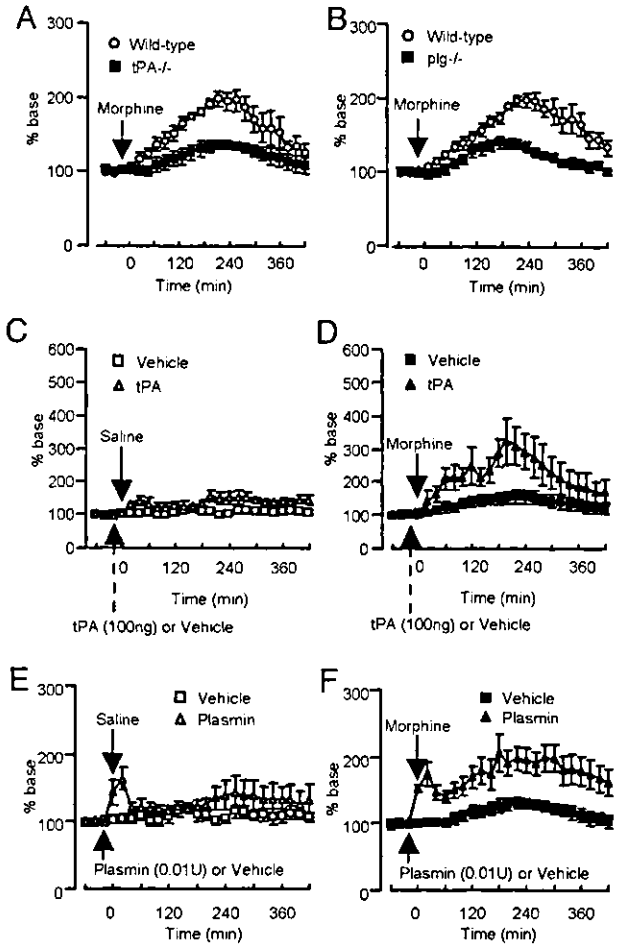

Fig. 4. Defect of morphine-induced dopamine release in $tPA^{-/-}$ mice and $plg^{-/-}$ mice. (*A*) Basal levels of dopamine in the NAcc did not differ between wild-type and $tPA^{-/-}$ mice (wild-type, 0.41 ± 0.13 nM, $n = 4$; $tPA^{-/-}$, 0.53 ± 0.01 nM, $n = 4$). Morphine (10 mg/kg s.c.)-induced dopamine release was markedly diminished in $tPA^{-/-}$ mice [$F_{(1, 6)} = 13.061$, $P < 0.05$]. (*B*) Basal levels of dopamine in the NAcc of $plg^{-/-}$ mice did not differ from those in wild-type mice (wild-type, 0.38 ± 0.10 nM, $n = 6$; $plg^{-/-}$, 0.38 ± 0.05 nM, $n = 6$). Morphine-induced dopamine release in the NAcc was significantly reduced in $plg^{-/-}$ mice compared to wild-type mice [$F_{(1, 10)} = 25.147$, $P < 0.01$]. (*C* and *D*) Effect of tPA on dopamine release in the NAcc of $tPA^{-/-}$ mice. Microinjection of tPA significantly increased basal levels of extracellular dopamine [$F_{(1, 8)} = 7.941$, $P < 0.05$] and morphine-induced dopamine release [$F_{(1, 8)} = 5.428$, $P < 0.05$) in $tPA^{-/-}$ mice]. (*E* and *F*) Effect of plasmin on dopamine release in the NAcc of $tPA^{-/-}$ mice. Microinjection of plasmin significantly increased basal levels of extracellular dopamine [$F_{(1, 8)} = 6.612$, $P < 0.05$] and morphine-induced dopamine release [$F_{(1, 8)} = 13.121$, $P < 0.01$] in $tPA^{-/-}$ mice. Values indicate means ± SE ($n = 4$ for *A*, $n = 6$ for *B*, and $n = 5$ for 4 *C–F*).

Single morphine treatment induced tPA mRNA expression in various regions of the rat brain. Although repeated morphine treatment reduced the ability of morphine to induce tPA mRNA expression in the brain, levels of tPA mRNA remained significantly higher in the frontal cortex, NAcc, striatum, and hippocampus. It is reported that the level of tPA is regulated by cAMP response element-binding protein (CREB) (8), and that CREB levels are reduced in the NAcc after chronic morphine treatment (38). Therefore, the reduced ability of morphine after repeated treatment to induce tPA mRNA expression in the brain may be due at least in part to the decrease in CREB levels.

Morphine-induced conditioned place preference was significantly attenuated in $tPA^{-/-}$ and $plg^{-/-}$ mice as compared with

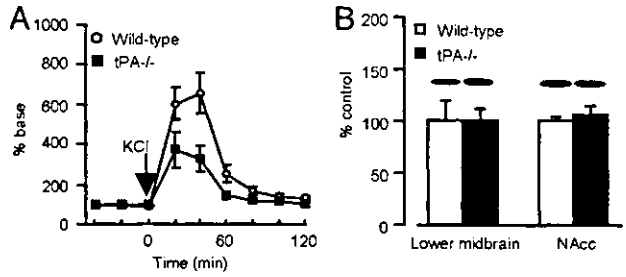

Fig. 5. Depolarization-evoked dopamine release and tyrosine hydroxylase content of $tPA^{-/-}$ mice. (*A*) High KCl (60 mM)-induced dopamine release in the NAcc of wild-type and $tPA^{-/-}$ mice. The lack of tPA significantly attenuated the depolarization-evoked dopamine release in the NAcc [$F_{(1, 10)} = 6.846$, $P < 0.05$]. (*B*) Immunoblot analysis of tyrosine hydroxylase of $tPA^{-/-}$ mice. There were no differences in the protein content of tyrosine hydroxylase in the lower midbrain and NAcc between $tPA^{-/-}$ and wild-type mice. Values indicate means ± SE ($n = 6$ for *A*, and $n = 4$ for *B*).

wild-type mice, suggesting that the tPA-plasmin system participates in the rewarding effects of morphine. However, because $tPA^{-/-}$ mice show impaired learning and memory in the context fear conditioning and two-way active avoidance test (18), we cannot exclude the possibility that $tPA^{-/-}$ mice failed to associate morphine-induced rewarding effects with the context during the conditioning. The mesolimbic dopaminergic pathway projecting from the VTA to the NAcc is thought to play a major role in mediating the rewarding effects of many stimuli, such as electrical brain stimulation and drugs of abuse (24). This dopamine system is important not only for rewarding effects but also locomotor-stimulating effects of morphine, the behavior being unaffected by the learning and memory function. The attenuation of both morphine-induced place preference and hyperlocomotion in $tPA^{-/-}$ and $plg^{-/-}$ mice suggests that the tPA-plasmin system plays a role in morphine-induced dopamine release in the NAcc.

Electrophysiological, biochemical, and behavioral data have suggested that tPA interacts extensively with dopamine D1 receptor-mediated responses in the brain. For instance, inactivation of the gene encoding tPA prevents the electrophysiological effects of D1 receptor agonists and mimics the effects of D1 receptor antagonists on the late phase of CA1 hippocampal long-term potentiation (39). The sensitivity of striatal cholinergic interneurons to dopamine D1 receptor stimulation is lost in $tPA^{-/-}$ mice (40). In contrast, we found that there were no differences in dopamine and morphine-induced increases in [^{35}S]GTPγS binding between wild-type and $tPA^{-/-}$ mice. No differences were evident in apomorphine-induced hyperlocomotion and tyrosine hydroxylase protein levels between the two types of mice. Accordingly, it is unlikely that the alterations of rewarding and locomotor-stimulating effects of morphine in $tPA^{-/-}$ mice are mainly due to the dysfunction of dopamine and opioid receptors in $tPA^{-/-}$ mice.

In vivo microdialysis and electrophysiological studies have provided evidence that the enhancement of dopamine release in the NAcc may be an essential process related to the morphine-induced rewarding effect (37, 41). In the present study, we demonstrated that morphine-induced dopamine release was attenuated in the NAcc of $tPA^{-/-}$ and $plg^{-/-}$ mice, and that microinjection of either exogenous tPA or plasmin into the NAcc, but not into the VTA, restored the morphine-evoked dopamine release in $tPA^{-/-}$ mice. The reduction of morphine-induced hyperlocomotion in $tPA^{-/-}$ mice was also reversed by microinjections of either exogenous tPA or plasmin into the NAcc. Western blotting of tyrosine hydroxylase contents of the brain revealed there are no differences be-

tween tPA$^{-/-}$ and wild-type mice, which is consistent with the immunohistochemical data (40). Therefore, plasmin that is converted from plg by tPA may have a role in regulating morphine-induced dopamine release in the NAcc.

The molecular mechanisms by which the tPA-plasmin system regulates morphine-induced dopamine release in the NAcc remain to be determined. However, it is known that the tPA-plasmin system degrades several extracellular matrix proteins (42), including laminin (43). Laminin in the synaptic cleft localizes calcium channels to the sites of active zones (44) and induces a small but significant increase in calcium levels in ciliary ganglion neurons when applied in soluble form to the culture medium (45). Accordingly, it is possible that a defect of the tPA-plasmin system may result in a malfunction of calcium channel activity, which leads to the reduction of depolarization-evoked dopamine release.

Conclusion

We have demonstrated that morphine increases tPA expression, and its enzyme activity in neuronal cells of the NAcc, by activating opioid receptors. The tPA-plasmin system plays a crucial role in regulating morphine-induced dopamine release in the NAcc and thereby is involved in the rewarding and locomotor-stimulating effects, without affecting the antinocipeptive effects.

We thank Eisai Co. Ltd. for providing us with human recombinant tPA. This study was supported in part by a Grant-in-Aid for Science Research (no. 14658249) and Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program, from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Grant-in-Aid for Health Sciences Research from the Ministry of Health, Labour and Welfare of Japan; and by the Fujisawa Foundation.

1. Sappino, A. P., Madani, R., Huarte, J., Belin, D., Kiss, J. Z., Wohlwend, A. & Vassalli, J. D. (1993) *J. Clin. Invest.* **92,** 679–685.
2. Chen, Z. L., Yoshida, S., Kato, K., Momota, Y., Suzuki, J., Tanaka, T., Ito, J., Nishino, H., Aimoto, S. & Kiyama, H. (1995) *J. Neurosci.* **15,** 5088–5097.
3. Backstrom, J. R., Lim, G. P., Cullen, M. J. & Tokes, Z. A. (1996) *J. Neurosci.* **16,** 7910–7919.
4. Hoffman, K. B., Larson, J., Bahr, B. A. & Lynch, G. (1998) *Brain. Res.* **811,** 152–155.
5. Gingrich, M. B., Junge, C. E., Lyuboslavsky, P. & Traynelis, S. F. (2000) *J. Neurosci.* **20,** 4582–4595.
6. Baranes, D., Lederfein, D., Huang, Y. Y., Chen, M., Bailey, C. H. & Kandel, E. R. (1998) *Neuron* **21,** 813–825.
7. Neuhoff, H., Roeper, J. & Schweizer, M. (1999) *Eur. J. Neurosci.* **11,** 4241–4250.
8. Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R. & Kuhl, D. (1993) *Nature* **361,** 453–457.
9. Seeds, N. W., Williams, B. L. & Bickford, P. C. (1995) *Science* **270,** 1992–1994.
10. Ware, J. H., Dibenedetto, A. J. & Pittman, R. N. (1995) *Brain Res. Bull.* **37,** 275–281.
11. Gualandris, A., Jones, T. E., Strickland, S. & Tsirka, S. E. (1996) *J. Neurosci.* **16,** 2220–2225.
12. Parmer, R. J., Mahata, M., Mahata, S., Sebald, M. T, O'Connor, D. T. & Miles, L. A. (1997) *J. Biol. Chem.* **272,** 1976–1982.
13. Krystosek, A. & Seeds, N. W. (1981) *Science* **213,** 1532–1534.
14. Seeds, N. W., Basham, M. E. & Haffke, S. P. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 14118–14123.
15. Moonen, G., Grau-Wagemans, M. P. & Selak, I. (1982) *Nature* **298,** 753–755.
16. Frey, U., Muller, M. & Kuhl, D. A. (1996) *J. Neurosci.* **16,** 2057–2063.
17. Madani, R., Hulo, S., Toni, N., Madani, H., Steimer, T., Muller, D. & Vassalli, J. D. (1999) *EMBO J.* **18,** 3007–3012.
18. Calabresi, P., Napolitano, M., Centonze, D., Marfia, G. A., Gubellini, P., Teule, M. A., Berretta, N., Bernardi, G., Frati, L., Tolu, M., *et al.* (2000) *Eur. J. Neurosci.* **12,** 1002–1012.
19. Tsirka, S. E., Gualandris, A., Amaral, D. G. & Strickland, S. (1995) *Nature* **377,** 340–344.
20. Nicole, O., Docagne, F., Ali, C., Margaill, I., Carmeliet, P., MacKenzie, E. T., Vivien, D. & Buisson, A. (2001) *Nat. Med.* **7,** 59–64.
21. Siconolfi, L. B. & Seeds, N. W. (2001) *J. Neurosci.* **21,** 4336–4347.
22. Koob, G. F. (1992) *Trends. Pharmacol. Sci.* **13,** 177–184.
23. Wise, R. A. (1996) *Curr. Opin. Neurobiol.* **6,** 243–251.
24. Koob, G. F., Sanna, P. P. & Bloom, F. E. (1998) *Neuron* **21,** 467–476.
25. Johnson, S. W. & North, R. A. (1992) *J. Neurosci.* **12,** 483–488.
26. Bonci, A. & Williams, J. T. (1997) *J. Neurosci.* **17,** 796–803.
27. Nestler, E. J. (2001) *Nat. Rev. Neurosci.* **2,** 119–128.
28. Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., De Vos, R., van den Oord, J. J., Collen, D. & Mulligan, R. C. (1994) *Nature* **368,** 419–424.
29. Bugge, T. H., Flick, M. J., Daugherty, C. C. & Degen, J. L. (1995) *Genes Dev.* **9,** 794–807.
30. Heussen, C. & Dowdle, E. B. (1980) *Anal. Biochem.* **102,** 196–202.
31. Nitta, A., Ito, M., Fukumitsu, H., Ohmiya, M., Ito, H., Sometani, A., Nomoto, H., Furukawa, Y. & Furukawa, S. (1999) *J. Pharmacol. Exp. Ther.* **291,** 1276–1283.
32. Franklin, J. B. J. & Paxinos, G. T. (1997) *The Mouse Brain: In Stereotaxic Coordinates* (Academic, New York).
33. Miyamoto, Y., Yamada, K., Noda, Y., Mori, H., Mishina, M. & Nabeshima, T. (2002) *J. Neurosci.* **22,** 2335–2342.
34. Shintani, F., Kanba, S., Nakaki, T., Nibuya, M., Kinoshita, N., Suzuki, E., Yagi, G., Kato, R. & Asai, M. (1993) *J. Neurosci.* **13,** 3574–3581.
35. Noda, Y., Miyamoto, Y., Mamiya, T., Kamei, H., Furukawa, H. & Nabeshima, T. (1998) *J. Pharmacol. Exp. Ther.* **286,** 44–51.
36. Mamiya, T., Noda, Y., Nishi, M., Takeshima, H. & Nabeshima, T. (1998) *Brain Res.* **783,** 236–240.
37. Matthews, R. T. & German, D. C. (1984) *Neuroscience* **11,** 617–625.
38. Widnell, K. L., Self, D. W., Lane, S. B., Russell, D. S., Vaidya, V. A., Miserendino, M. J., Rubin, C. S., Duman, R. S. & Nestler, E. J. (1996) *J. Pharmacol. Exp. Ther.* **276,** 306–315.
39. Huang, Y. Y., Bach, M. E., Lipp, H. P., Zhuo, M., Wolfer, D. P., Hawkins, R. D., Schoonjans, L., Kandel, E. R., Godfraind, J. M., Mulligan, R., *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8699–8704.
40. Centonze, D., Napolitano, M., Saulle, E., Gubellini, P., Picconi, B., Martorana, A., Pisani, A., Gulino, A., Bernardi, G. & Calabresi, P. (2002) *Eur. J. Neurosci.* **16,** 713–721.
41. Narita, M., Funada, M. & Suzuki, T. (2001) *Pharmacol. Ther.* **89,** 1–15.
42. Schnaper, H. W. (1995) *Pediatr. Nephrol.* **9,** 104–111.
43. Goldfinger, L. E., Jiang, L., Hopkinson, S. B., Stack, M. S. & Jones, J. C. (2000) *J. Biol. Chem.* **275,** 34887–34893.
44. Sunderland, W. J., Son, Y. J., Miner, J. H., Sanes, J. R. & Carlson, S. S. (2000) *J. Neurosci.* **20,** 1009–1019.
45. Bixby, J. L., Grunwald, G. B. & Bookman, R. J. (1994) *J. Cell Biol.* **127,** 1461–1475.

0026-895X/04/6505-1293–1301$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2004 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 65:1293–1301, 2004

Vol. 65, No. 5
3028/1149379
Printed in U.S.A.

Regulations of Methamphetamine Reward by Extracellular Signal-Regulated Kinase 1/2/ets-Like Gene-1 Signaling Pathway via the Activation of Dopamine Receptors

Hiroyuki Mizoguchi, Kiyofumi Yamada, Makoto Mizuno, Tomoko Mizuno, Atsumi Nitta, Yukihiro Noda, and Toshitaka Nabeshima

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan (H.M., K.Y., M.M., T.M., A.N., Y.N., T.N.); and Laboratory of Neuropsychopharmacology, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan (K.Y.)

Received October 13, 2003; accepted February 17, 2004

This article is available online at http://molpharm.aspetjournals.org

0026-895X/04/6505-1293–1301$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 65:1293–1301, 2004

Vol. 65, No. 5
3028/1149379
Printed in U.S.A.

Regulations of Methamphetamine Reward by Extracellular Signal-Regulated Kinase 1/2/ets-Like Gene-1 Signaling Pathway via the Activation of Dopamine Receptors

Hiroyuki Mizoguchi, Kiyofumi Yamada, Makoto Mizuno, Tomoko Mizuno, Atsumi Nitta, Yukihiro Noda, and Toshitaka Nabeshima

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan (H.M., K.Y., M.M., T.M., A.N., Y.N., T.N.); and Laboratory of Neuropsychopharmacology, Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Kanazawa University, Kanazawa, Japan (K.Y.)

Received October 13, 2003; accepted February 17, 2004

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Little is known about molecular mechanisms for long-lasting neuroadaptation related to the rewarding effects of methamphetamine (MAP). In the present study, we examined the intracellular signaling that is associated with the expression of conditioned place preference (CPP) induced by MAP in rats. Rats were given MAP or saline (control group) for conditioning to the CPP test. MAP-treated and control animals were killed immediately after the CPP test [CPP$^+$]. Some of the MAP-treated rats were killed without the CPP test [CPP$^-$]. Hyperphosphorylation of mitogen-activated protein kinase (MAPK) ERK1/2, but not p38 and c-Jun N-terminal kinase/stress-activated protein kinase, was found in the nucleus accumbens (NAc) and striatum but not in other brain areas of MAP-treated CPP$^+$ animals. No such phosphorylation was seen in control and MAP-treated CPP$^-$ animals. Moreover, the transcription factor ets-like gene-1 (Elk-1), but not cAMP response element-binding protein, also showed a similar hyperphosphorylation in the same regions of MAP-treated CPP$^+$. Tyrosine kinase receptors, including tyrosine kinase B, were not activated in any brain regions examined in all groups. Both the dopamine D1 receptor antagonist *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine (SCH23390) and the D2 receptor antagonist raclopride inhibited the expression of CPP as well as the activation of ERK1/2 in MAP-treated CPP$^+$ animals, when they were injected before the CPP test. The microinjection of 2′-amino-3′-methoxyflavone (PD98059), a selective MAPK kinase inhibitor, into the NAc before the test, abolished the MAP-induced ERK1/2 activation and decreased the expression of MAP-induced CPP. These results suggest the importance of the ERK1/2 signaling pathway through activation of dopamine D1 and D2 receptors in the expression of CPP induced by MAP.

The mesolimbic dopaminergic projection to the nucleus accumbens (NAc) or striatum is thought to mediate the reinforcing effects of drugs of abuse through activation of dopamine receptors on NAc or striatal neurons (Koob, 1992; Self et al., 1998). Dopamine signals are mediated by two major classes of dopamine receptors, termed D1 and D2 receptors, that are distinguishable by their structural heterogeneity and action on the cAMP/protein kinase A (PKA) system (Silbley et al., 1993; Zanassi et al., 2001). Despite these opposing actions on cellular signaling via cAMP/PKA, previous studies have found that both dopamine D1 and D2 receptors can mediate reinforcing signals of abuse, because amphetamine-induced conditioned place preference (CPP) is blocked by either D1 or D2 receptor antagonists (Hiroi and White, 1991) and selective dopamine D1 and D2 receptor agonists were self-administered by rats (Self et al., 1996).

Psychostimulants act to enhance memory consolidation in general and facilitate the learning of specific behaviors un-

This study was supported in part by grants-in aid for Health Sciences Research from the Ministry of Health, Labor and Welfare, Japan, Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and an SRF grant for Biomedical Research.

ABBREVIATIONS: NAc, nucleus accumbens; MAP, methamphetamine; CPP, conditioned place preference; CREB, cAMP response element-binding protein; Elk-1, ets-like gene-1; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase 1/2; NMDA, *N*-methyl-D-aspartate; PKA, protein kinase A; MEK, MAPK kinase; St, striatum; VTA, ventral tegmental area; Trk, tyrosine kinase; JNK, c-Jun N-terminal kinase/stress-activated protein kinase; BDNF, brain-derived neurotrophic factor; DARPP-32, dopamine and cAMP-regulated phosphoprotein of M_r 32,000; PD98059, 2′-amino-3′-methoxyflavone; SCH23390, *R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine; CGS19755, [[(±)-2-carboxypiperidin-4-yl]methyl]-phosphonic acid; SL327, (*Z*)- & (*E*)-α-(Amino-((4-aminophenyl)thio)methylene)-2-(trifluoromethyl)benzeneacetonitrile.

related to drug intake. For example, systemic injections of amphetamine after training can enhance the learning of discrimination or avoidance takes (Berke and Hyman, 2000). Thus, the learning/memory mechanisms are considered to overlap with and be involved in the development of drug dependence that occurs on chronic administration of drugs of abuse (Berke and Hyman, 2000; Ammassari-Teule, 2001). Several lines of evidence suggest an important role for the intracellular signal transduction pathways in the mechanism of neural plasticity in response to drugs of abuse (Nestler, 2001). One of these signal transduction pathways is the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade, a member of the mitogen-activated protein kinase (MAPK) family. ERK1/2 activation can phosphorylate tyrosine hydroxylase and stimulate dopamine synthesis in the brain (Lindgren et al., 2002). After activation, ERK1/2 proteins are translocated to the nucleus, resulting in phosphorylation and activation of transcription factors such as cAMP response element-binding protein (CREB) and Elk-1. These nuclear events would initiate cell-specific gene expression programs necessary for synaptic remodeling and long-term changes in synaptic efficacy. Recent evidence has demonstrated that the ERK signaling pathway is involved in the sensitization induced by cocaine (Valjent et al., 2000) and that ERK1 mutant mice have enhanced behavioral responses to the rewarding properties of morphine (Mazzucchelli et al., 2002), indicating that ERK may be involved in the response to drugs of abuse. However, still very little is known about the intracellular mechanisms leading to synaptic plasticity in reinforcing effects of drugs of abuse.

In the present study, we investigated intracellular signaling mechanisms that are associated with the expression of the MAP-induced CPP response in rats. The CPP response is a behavior that is developed by the association of reinforcing effects of drugs with the context in which animals have previously obtained positive reinforcing effects. Thus, it is considered that an understanding of the cellular signaling associated with the expression of MAP-induced CPP will provide insights into the mechanism of long-lasting neuroadaptation related to MAP dependence and drug-seeking behavior.

Materials and Methods

Animals. Male Wistar rats (8 weeks old; Charles River Japan, Yokohama, Japan) weighing 300 ± 20 g at the beginning of experiments were used in the study. They were housed three per cage with ad libitum access to food and water under controlled laboratory conditions (a 12-h light/dark cycle with lights on at 9:00 A.M., 23 ± 0.5°C, 50 ± 0.5% humidity). All experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University School of Medicine, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Drug Treatment. A specific dopamine D1 receptor antagonist, *R*-(+)-SCH23390 (Sigma-Aldrich, St. Louis, MO), at 0.03 and 0.1 mg/kg; dopamine D2 receptor antagonist, *S*-(−)-raclopride (Sigma-Aldrich), at 0.3 and 1 mg/kg; and competitive NMDA receptor antagonist CGS19755 (Novartis, Basel, Switzerland), at 0.3 and 1 mg/kg, respectively, were intraperitoneally injected 30 min before the CPP test. For the microinjection of the specific MAPK kinase (MEK) inhibitor PD98059 (Sigma-Aldrich) into the NAc, rats were anesthetized with pentobarbital (50 mg/kg i.p.) and placed in a stereotaxic apparatus. It is reported that PD98059 at 2 μg specifically inhibits MAPK phosphorylation, but not the phosphorylation of stress-activated protein kinase isoforms, related MAPK family members, by inhibiting MEK, and the second-messenger activities of calcium/calmodulin-dependent protein kinase II, PKA, and protein kinase C are unchanged by the infusion of PD98059 at 2 μg/side into the hippocampus, indicating that the dosage of drug used selectively inhibits the MAPK cascade (Blum et al., 1999). PD98059 has no significant effect on MAP kinase itself (Dudley et al., 1995). In addition, 2 μg/side of PD98059 infused into the hippocampus resulted in an equilibrium concentration of ~37.5 μM, although the concentration was likely to be higher immediately surrounding the infusion site (Blum et al., 1999). A guide cannula (0.4 × 0.5 mm in diameter; Eicom, Kyoto, Japan) was implanted bilaterally into the NAc (+1.2 mm anterior to bregma, ±1.9 mm lateral, and −7.0 mm for NAc ventral to dura), according to the atlas of Paxinos and Watson (1982). A dummy cannula (0.3 mm in diameter; Eicom) cut to extend 1.0 mm beyond the guide cannula was left in place throughout the experiment. PD98059 (2 μg/side) or vehicle (60% dimethyl sulfoxide-saline) was injected bilaterally through a 28 gauge injection cannula (Eicom) in a volume of 1.5 μl/side over a 4-min period, 20 min before the CPP test in the NAc of the rats.

Conditioned Place Preference (CPP). The apparatus used for the place conditioning task consisted of two compartments: a black Plexiglas box and a transparent Plexiglas box (both 27 × 22 × 26 cm high) with a metal grid floor. To enable the rat to distinguish easily the transparent box from the black one, the floors of the transparent and black boxes were covered with white plastic mesh and with black frosting Plexiglas, respectively. Each box could be divided by a sliding door (10 × 26 cm high).

The place conditioning paradigm was performed according to the method of Kitaichi et al. (1996), with a minor modification. In the preconditioning test, the sliding door was opened and the rat was allowed to move freely between both boxes for 15 min once a day for 3 days. On the third day of the preconditioning test, we measured the time that the rat spent in the black and transparent boxes by using Scanet SV-10 LD (Melquest, Toyama, Japan). The box in which the rat spent the most time was referred to as the "preferred side," and the other box as the "nonpreferred side."

Conditioning was performed during 6 successive days. Rats were given drugs or vehicle in the apparatus with the sliding door closed. That is, a rat was subcutaneously given saline or MAP at 2 mg/kg and put in its nonpreferred side for 30 min. The next day, the rat was given saline and placed opposite the drug conditioning site for 30 min. These treatments were repeated for three cycles (6 days). In the postconditioning test, the sliding door was opened, and we measured the time that the rat spent in the black and transparent boxes for 15 min, using the Scanet SV-10 LD.

Place conditioning behaviors were expressed by Post-Pre, which was calculated as: [(postvalue) − (prevalue)], where post- and prevalues were the difference in time spent in the drug conditioning and the saline conditioning sites in the postconditioning and preconditioning tests, respectively.

Animals were killed by rapid decapitation as described previously (Berhow et al., 1996; Atkins et al., 1998; Cammarota et al., 2000). Saline-treated animals received saline during the 6 days of conditioning phase (control). MAP-treated animals were injected with MAP three times during the conditioning phase, and they were divided into CPP^+ and CPP^- groups. CPP^+ and control animals were killed immediately after the CPP test, whereas CPP^- rats were killed without the CPP test on the postconditioning day. Various brain regions including frontal cortex, NAc, striatum (St), hippocampus, ventral tegmental area (VTA), and amygdala were dissected out from control, CPP^+, and CPP^- animals and immediately frozen and stored at −80°C until assayed.

Western Blotting and Immunoprecipitation. Brain tissues were homogenized in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM sodium orthovanadate, 2 mM EDTA, 50 mM NaF, 0.1% SDS, 1%

Nonidet P-40, 1% sodium deoxycholate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, pH 7.4), and microwaved for 15 s according to the protocol for immunoblotting with monoclonal antibodies. The homogenate was centrifuged at 10,000*g* for 10 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid Kit (Wako Pure Chemicals, Osaka, Japan). The sample was boiled in a sample buffer [0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol, and 20% 2× TBE (90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.4)] and electrophoresed by SDS-polyacrylamide gel electrophoresis on a 4.75% stacking gel and 10% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). The same concentration (20 or 50 μg) of protein per lane was located in all Western blotting. The membrane was incubated in the blocking solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 2 h at room temperature and then incubated with primary antibodies. After washing, blots were incubated with the secondary antibodies. Immunoreactive materials on the membrane were detected using the ECL Western blotting detection reagents (Amersham Biosciences Inc., Piscataway, NJ) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry. To calculate the amount of phosphorylated form versus total protein, the same membranes were stripped with a stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) at 50°C for 20 min, incubated with primary antibodies for total protein, and detected as described above.

For phosphorylation analysis of TrkB, protein A Sepharose (Amersham Biosciences Inc.) was incubated with monoclonal anti-TrkB antibody for 6 h and then with each lysate (0.5 mg of protein) overnight. The immunoprecipitate was boiled in Laemmli sample buffer, separated on a 7.5% polyacrylamide gel, and subsequently transferred to a polyvinylidene difluoride membrane. The membranes were blocked and probed with anti-phosphotyrosine antibody (1:1000; Upstate Biotechnology, Lake Placid, NY), and detected as described above. To confirm equal loading of each protein, membranes were stripped with the stripping buffer, incubated with anti-TrkB antibody, and detected as described above. For the quantification of protein phosphorylation, the mean values in the control group were converted to 100%, and then individual data including those of control groups, were recalculated as percentages of the mean values. All the data in Western blotting are expressed as a percentage of the control.

The primary monoclonal mouse antibodies used in the present study were anti-P-ERK (1:1000; Cell Signaling Technology Inc., Beverly, MA), P-Elk-1 (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), P-Pan-Trk (1:1000; Santa Cruz Biotechnology, Inc.), Pan-Trk (1:1000; Santa Cruz Biotechnology, Inc.), P-JNK (c-Jun N-terminal kinase/stress-activated protein kinase) (1:1000; Santa Cruz Biotechnology, Inc.), P-p38 (1:1000; Santa Cruz Biotechnology, Inc.), and CREB (1:1000; Santa Cruz Biotechnology, Inc.). The primary polyclonal mouse or rabbit antibodies were anti-Elk-1 (1:1000; Santa Cruz Biotechnology, Inc.), JNK (1:1000; Santa Cruz Biotechnology, Inc.), and P-CREB (1:1000; Santa Cruz Biotechnology, Inc.). The primary polyclonal rabbit antibodies were anti-p38 (1:1000; Santa Cruz Biotechnology, Inc.), TrkB (1:1000; Santa Cruz Biotechnology, Inc.), and ERK (1:2000; Upstate Biotechnology). The secondary antibodies, used at a 1:2000 or 1:5000 dilution, were horseradish peroxidase-linked anti-mouse or anti-rabbit IgG (Kirkegaard and Perry Laboratories).

Statistical Analyses. Results are expressed as the mean ± S.E. The significance of differences was determined by one-way analysis of variance, followed by the Student-Newman-Keuls test for multigroup comparisons. Student's *t* test was used for two-group comparisons in Fig. 1A.

Results

Hyperphosphorylation of ERK1/2 Was Observed Specifically in the NAc and St after the Expression of MAP-Induced CPP. MAP (2 mg/kg)-treated rats spent significantly more time in the drug-paired compartment than did control rats ($P < 0.05$ by *t* test), indicating rewarding effects of MAP (Fig. 1A). To examine the intracellular signaling associated with the expression of MAP-induced CPP, MAP-treated rats were killed immediately after the CPP test, and the phosphorylation of various signaling molecules was examined by Western blotting. Hyperphosphorylation of ERK1/2 was found specifically in the NAc [$F(2,18) = 7.33$; $P < 0.005$, and $P < 0.05$ by post hoc] and St [$F(2,21) = 11.5$; $P < 0.001$, and $P < 0.05$ by post hoc], but not in other brain areas, of MAP-treated CPP$^+$ animals, compared with control and CPP$^-$ animals ($P < 0.0001$, and $P < 0.05$ by post hoc). Importantly, no such phosphorylation was seen in the control and the MAP-treated CPP$^-$ animals (Fig. 1B). Accordingly, it is suggested that activation of ERK1/2 in the NAc and St is associated with the exposure of MAP-treated rats to the environment in which they had previously received the drug treatment, but not with the drug treatment itself.

No Changes in the Phosphorylation of Other MAPKs, JNK, and p38 on MAP-Induced CPP. Three members of the MAP kinase family have been identified: ERK, JNK, and p38, which are activated by stress stimuli. In this study, no changes in phosphorylated levels of JNK and p38 MAPK were observed in any brain areas in MAP-treated CPP$^+$ and

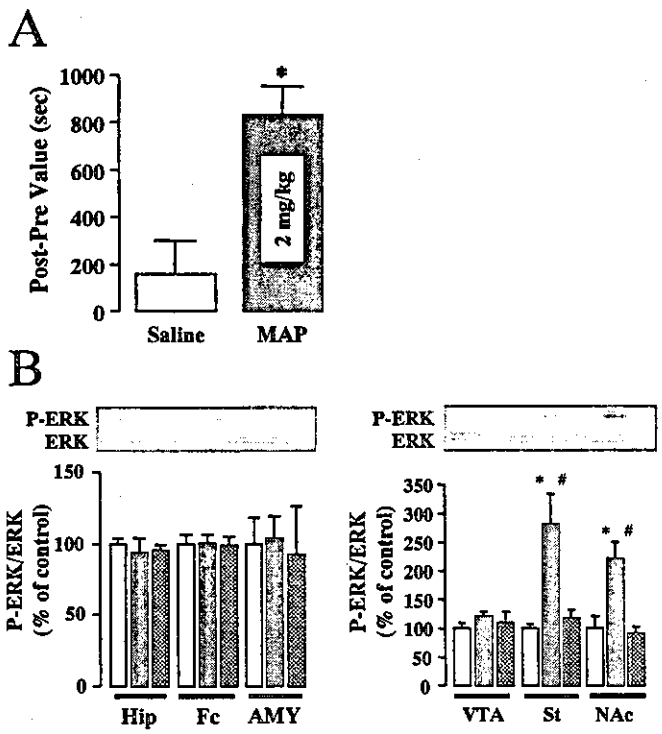

Fig. 1. A, MAP-induced CPP in rats; B, ERK1/2 activation associated with the expression of MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning to the CPP test and were killed immediately after the test. Open column, saline-treated CPP$^+$ rats; closed column, MAP-treated CPP$^+$ rats; hatched column, MAP-treated CPP$^-$ rats. Data are presented as the mean ± S.E. (n = 10~12 for A; n = 7~8 for B). *, $P < 0.05$ vs. saline-treated CPP$^+$ rats; #, $P < 0.05$ vs. MAP-treated CPP$^-$ rats.

CPP^- animals (Fig. 2). These results suggest that the three MAPKs are specifically and differentially activated by exposure to the context associated with the rewarding effects of MAP, and that the ERK1/2, but not JNK and p38 MAPK, phosphorylation may be related to drug-seeking/relapse behavior.

No Changes in the Phosphorylation of Tyrosine Kinase Receptors, Including TrkB, on MAP-Induced CPP. To examine whether Trk receptors are upstream of the hyperphosphorylation of ERK1/2 evoked by MAP-induced CPP, we investigated Trk receptor phosphorylation. Phosphorylated Trk receptors were detected with P-Pan-Trk antibodies, which recognize the phosphorylated form of TrkA, TrkB, and TrkC. Phosphorylated TrkB levels were also measured by immunoprecipitation with TrkB antibodies followed by Western blotting with anti-phosphotyrosine antibodies. There were no changes in phosphorylated levels of Pan-Trk and TrkB in any brain areas of MAP-treated CPP^+ and CPP^- animals (Fig. 3).

Hyperphosphorylation of Elk-1 Is Evoked after the Expression of MAP-Induced CPP. Transcription factors such as CREB and Elk-1 are the nuclear targets of ERK1/2, and their activation by ERK1/2 is observed in various model systems (Davis et al., 2000). For instance, activation of ERK1/2 and Elk-1 has been reported in cocaine responses (Valjent et al., 2000). As illustrated in Fig. 4A, hyperphosphorylation of Elk-1 was specifically found in the NAc of MAP-treated CPP^+ animals without any changes in the St [$F(2,12) = 8.37$; $P < 0.01$ compared with saline-treated animals or MAP-treated CPP^-, and $P < 0.05$ by post hoc comparison]. No such phosphorylation of CREB was seen in either the NAc or St of MAP-treated CPP^- animals (Fig. 4B).

Involvement of Dopamine Receptors in MAP-Induced CPP and Activation of ERK1/2. We then evaluated the involvement of dopamine receptors in the expression of MAP-induced CPP and the ERK activation evoked by MAP-induced CPP in the NAc and striatum. Both SCH23390 (dopamine D1 receptor antagonist) and raclopride (dopamine D2 receptor antagonist) dose dependently abolished the expression of MAP-induced CPP without affecting the behavior of control animals [Fig. 5B, $F(3,51) = 4.68$, $P < 0.05$, and $P < 0.05$ by post hoc comparison; Fig. 6A, $F(3,42) = 3.99$, $P < 0.05$, and $P < 0.05$ by post hoc comparison]. We also examined the involvement of NMDA receptors in the expression of MAP-induced CPP. CGS19755 at 1 mg/kg, which inhibits NMDA receptor function (Mori et al., 2001), failed to affect the expression of MAP-induced CPP (Fig. 5A), suggesting that the activation of NMDA receptors may not be critical to the expression of MAP-induced CPP. To further analyze the role of ERK1/2 activation in the expression of CPP, the effects of dopamine receptor antagonists on ERK1/2 phosphorylation were measured. The blockade of dopamine D1 receptors by administration of SCH23390 (0.1 mg/kg) resulted in the inhibition of ERK1/2 activation evoked by MAP-induced CPP in both NAc and St [Fig. 5C; $F(3,19) = 4.60$, $P < 0.05$, and $P < 0.05$ by post hoc comparison in the NAc, and $F(3,21) = 12.5$, $P < 0.0001$, and $P < 0.05$ by post hoc comparison in the St), whereas the treatment had no effect on the phosphorylation of ERK1/2 in control animals. These results suggest that dopamine D1 receptors are activated when MAP-treated an-

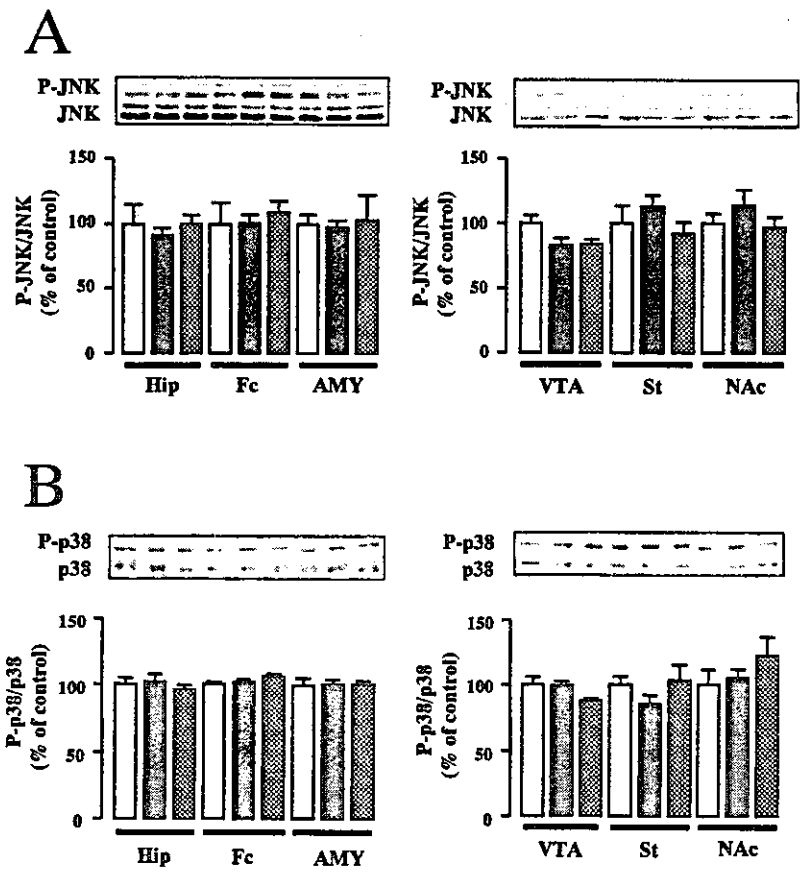

Fig. 2. No changes in JNK (A) and p38 (B) phosphorylation associated with the expression of MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for conditioning and were killed immediately after the test. Open column, saline-treated CPP^+ rats; closed column, MAP-treated CPP^+ rats; hatched column, MAP-treated CPP^- rats. Data are presented as the mean ± S.E. ($n = 5$).

imals are exposed to the context in which they had previously received MAP, and that the activation is crucial for the expression of CPP and activation of ERK1/2 in the NAc and St.

The possible involvement of the dopamine D2 receptor subtype in ERK activation evoked by MAP-induced CPP was also analyzed. Raclopride (1 mg/kg) failed to block the ERK activation evoked by MAP-induced CPP in the NAc but significantly decreased it in the St [Fig. 6B; $F(3,17) = 12.3$, $P < 0.001$, and $P < 0.05$ by post hoc comparison]. Taken together, these results show that dopamine D1 receptor-mediated ERK activation in the NAc and St is attributable to the expression of MAP-induced CPP, whereas the contribution of dopamine D2 receptors seems to be restricted to the St.

Effect of MEK Inhibitor PD98059 on MAP-Induced CPP Expression. Because both the MAP-induced CPP expression and ERK activation in the NAc were completely prevented by the dopamine D1 antagonist, we then tested the causal relation between MAP-induced CPP expression and ERK activation in the NAc. For this purpose, we assessed the effect of microinjection of PD98059, a selective MEK inhibitor, into the NAc. Bilateral microinjection of PD98059 into the NAc (2 μg/side) significantly inhibited the expression of MAP-induced CPP [Fig. 7A; $F(3,39) = 4.28$, $P < 0.05$, and $P < 0.05$ by post hoc comparison]. The ERK activation evoked by MAP-induced CPP in the NAc was significantly abolished by PD98059 treatment [Fig. 7B; $F(3,20) = 4.65$, $P < 0.05$, and $P < 0.05$ by post hoc comparison]. These results suggest a critical role for the ERK1/2 signaling cascade in the expression of CPP in MAP-treated animals.

Discussion

In the present study, we found that the hyperphosphorylation of ERK1/2, but not JNK and p38, in the NAc and St was associated with the expression of MAP-induced CPP. The transcription factor Elk-1, a nuclear target of ERK1/2, was also activated in the NAc by the expression of MAP-induced CPP. Moreover, the blockade of both D1 and D2 receptors by administration of SCH23390 and raclopride inhibited the expression of CPP induced by MAP. Administration of SCH23390 resulted in the inhibition of ERK1/2 hyperphosphorylation evoked by MAP-induced CPP in both NAc and St, whereas raclopride inhibited the hyperphosphorylation of ERK1/2 in the St but not the NAc. These findings suggest a role for the ERK1/2/Elk-1 signaling pathway via the activation of dopamine receptors in events underlying the expression of CPP induced by MAP. We suggest that the signaling pathway is crucial at least in part to the long-lasting neuroadaptation induced by MAP, which may be related to its abuse properties.

The ERK pathway is a signaling cascade, controlled by the Ras family of small GTPases, which plays a vital role in a variety of cell-regulatory events (Orban et al., 1999; Pearson et al., 2001). The role of the Ras/ERK pathway in long-term synaptic changes and behavior is well established (Orban et al., 1999; Mazzucchelli and Brambilla, 2000). There is an increasing amount of evidence that indicates the participation of a Ras/MEK/ERK/Elk-1 signaling pathway during the formation of new memories (Atkins et al., 1998; Cammarota

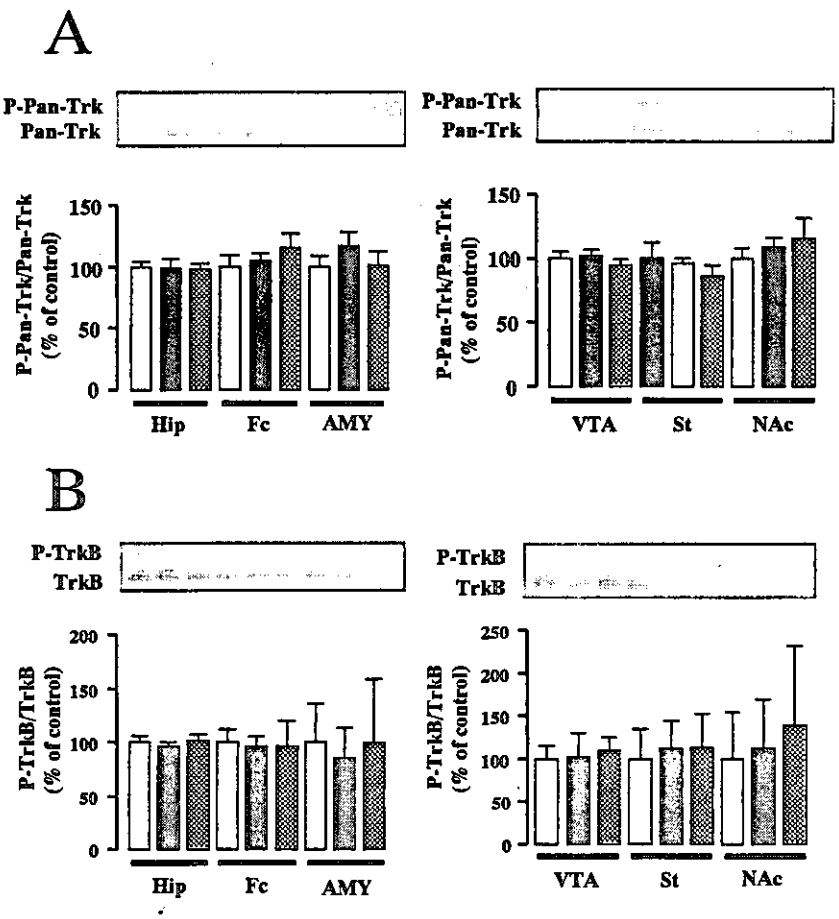

Fig. 3. No changes in Pan-Trk (A) and TrkB (B) phosphorylation on MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning and were killed immediately after the CPP test. Open column, saline-treated CPP^+ rats; closed column, MAP-treated CPP^+ rats; hatched column, MAP-treated CPP^- rats. Data are presented as the mean ± S.E. ($n = 5$).