Nerve Growth Factor-Induced Neurite Sprouting in PC12 Cells Involves σ-1 Receptors: Implications for Antidepressants

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ABSTRACT

One theory concerning the action of antidepressants relates to the drugs’ ability to induce an adaptative plasticity in neurons such as neurite sprouting. Certain antidepressants are known to bind to σ-1 receptors (Sig-1R) with high affinity. Sig-1R are dynamic endoplasmic reticulum proteins that are highly concentrated at the tip of growth cones in cultured cells. We therefore tested the hypotheses that Sig-1R might participate in the neurite sprouting and that antidepressants with Sig-1R affinity may promote the neuronal sprouting via Sig-1R. The prototypic Sig-1R agonist (+)-pentazocine [(+)-PTZ], as well as the Sig-1R-active antidepressants imipramine and fluvoxamine, although ineffective by themselves, were found to enhance the nerve growth factor (NGF)-induced neurite sprouting in PC12 cells in a dose-dependent manner. A Sig-1R antagonist N,N-dipropyl-2-[4-methoxy-3-[2-phenylethoxy]phenyl]-ethylamine monohydrochloride (NE100) blocked the enhancements caused by these Sig-1R agonists. In separate experiments, we found that NGF dose and time dependently increased Sig-1R in PC12 cells. Chronic treatment of cells with [(+)-PTZ, imipramine, or fluvoxamine also increased Sig-1R. These latter results suggested that NGF induces the neurite sprouting by increasing Sig-1R. Indeed, the overexpression of Sig-1R per se in PC12 cells enhanced the NGF-induced neurite sprouting. Furthermore, antisense deoxyoligonucleotides directed against Sig-1R attenuated the NGF-induced neurite sprouting. Thus, when taken together, our results indicate that Sig-1R play an important role in the NGF-induced neurite sprouting and that certain antidepressants may facilitate neuronal sprouting in the brain via Sig-1R.

An emerging hypothesis suggests that the action of antidepressants likely involves neuroplasticity, i.e., antidepressants may exert their therapeutic effects by stimulating adaptive changes in the neuronal system. Indeed, stress as well as psychotropic drugs, including antidepressants, were reported to induce not only neurochemical alterations but also structural alterations and synaptic remodeling, including sprouting of neurites and changes of dendritic spines (Nakamura, 1991; McEwen, 2000; Norholm and Ouimet, 2001). Specifically, in animal studies, several classes of antidepressants could reverse the reductions in the density of dendritic spines in hippocampal neurons induced by stress or by depression (McEwen, 2000; Norholm and Ouimet, 2001). Additionally, chronic electroconvulsive shock, which is a treatment for severely depressed patients, induced the sprouting of hippocampal neurons (Gombos et al., 1999).

Antidepressants, including tricyclic antidepressants and selective serotonin reuptake inhibitors, possess moderate-to-high affinities at σ-1 receptors (Sig-1R) (Su, 1982; Narita et al., 1996; Shirayama et al., 1997). However, the physiological implication of the binding of these antidepressants to Sig-1R is unknown. Although most of antidepressants are known to inhibit serotonin and/or norepinephrine reuptake, the efficacy of these antidepressants cannot be solely explained by their actions on the monoaminergic system (Nestler et al., 2002).

Sig-1R are unique endoplasmic reticulum (ER) proteins that bind (+)-benzomorphans, neurosteroids, and psychotropic drugs, including antidepressants (Snyder and Largent, 1989). σ-Receptors are nonopioid, nonphencyclidine receptors that differ from the opioid/σ-receptors proposed by Martin et al. (1976). Studies using [(+)-pentazocine [(+)-PTZ] led to the suggestion of two subtypes of σ-receptors (Quirion et al., 1992). Sig-1R were cloned and were characterized to
contain one transmembrane region and a binding domain for sterols and to have a 33% identity to a yeast C8-C7 sterol isomerase (Hanner et al., 1996). However, mammalian Sig-1R do not possess the sterol isomerase activity, and the amino acid sequence of Sig-1R does not resemble that of any mammalian protein.

Sig-1R exhibit a unique pattern of subcellular distribution in the brain and are localized in areas such as hippocampus, cortex layer, and olfactory bulb (Alonso et al., 2000). Sig-1R have been demonstrated to play important roles in learning and memory in animal models of amnesia (Maurice et al., 1999, 2001), as well as in behavioral models of depression, including the forced swimming test and the tail suspension test (Matsuno et al., 1996; Urani et al., 2001). Specifically, Sig-1R agonists were demonstrated to have beneficial effects on the amnesia caused by scopolamine or dizocilpine (Maurice et al., 1999). Furthermore, Sig-1R agonists such as (+)PTZ possess antidepressant-like effects that were antagonized by Sig-1R antagonists such as NE100 or BD1047 (Matsuno et al., 1996; Urani et al., 2001). These reports strongly suggest that Sig-1R play a role in learning and memory and in the mood alteration processes that are known to involve neuronal plasticity.

Our laboratory has reported that following the activation by Sig-1R agonists, Sig-1R dissociate as a Sig-1R-cytoskeletal adaptor protein (ankyrin) complex from the ER and that the Sig-1R-ankyrin complex translocates to the nucleus and the plasma membrane in NG-108 cells (Hayashi et al., 2000; Hayashi and Su, 2001). These findings suggest that Sig-1R affect the dynamics and rearrangement of cytoskeletal proteins and that Sig-1R might thus cause structural alterations of cells (Su and Hayashi, 2001). In fact, we found that Sig-1R and ankryns are highly concentrated at the growth cone of NG-108 cells (Hayashi and Su, 2001). The growth cone, located at the tip of neurite outgrowth, is related to neurite sprouting, extension, and guidance. Therefore, we hypothesized that Sig-1R may be involved in the morphological change of cells, specifically in the initiation of neurite outgrowth or sprouting. In this study, we examined this hypothesis by using PC12 cells, which have been used extensively for examining the neurite outgrowth in response to nerve growth factor (NGF) treatment (Greene and Tischler, 1976). PC12 cells express Sig-1R (Sagi et al., 1996). We also tested whether certain antidepressants increase the neurite sprouting via Sig-1R.

Materials and Methods

Materials. Rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (Manassas, VA). RPMI 1640 medium, fetal calf serum, horse serum, glutamine, penicillin, streptomycin, and laminin were purchased from Invitrogen (Carlsbad, CA). NGF, adenosine-3',5'-cyclic monophosphate, N\textsuperscript{6}-O\textsuperscript{-} dibutyryl, sodium salt (3',5'-cyclicAMP), and Nonidet P-40 were from Calbiochem (San Diego, CA). Fluvoxamine was from Tocris-Cookson (Ballwin, MO). Imipramine was from Sigma-Aldrich (St. Louis, MO). (+)PTZ was from the Division of Basic Research, National Institute on Drug Abuse.

Cell Culture and Experimental Treatments. PC12 cells were cultured at 37°C, 5% CO\textsubscript{2} with RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, and 10% heat-inactivated horse serum, 4 mM glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. The medium was changed two or three times a week. The cells were routinely grown in culture flasks with collagen coating. For passaging and subculturing, the cells were mechanically detached by pipetting with phosphate-buffered saline. For experiments on the quantification of neurite outgrowth, the cells were plated at a density of 1 x 10\textsuperscript{4}/cm\textsuperscript{2} on a 12-well plate precoated with 1 µg/cm\textsuperscript{2} poly-d-lysine and 1 µg/cm\textsuperscript{2} laminin. The coating with laminin was used to protect against aggregations and detachments of PC12 cells. The medium was replaced with a medium containing a minimal level of serum (0.5% fetal calf serum) that is known to contain steroid hormones. Progesterone and other neurosteroidspossess affinities at Sig-1R (Su et al., 1988).

Stable Overexpression of Sig-1R in PC12 Cells. PC12 cells were cultured on collagen-coated 100-mm dishes at a density of 1.5 x 10\textsuperscript{5}/ml 1 day before transfection and were incubated in an RPMI 1640 medium containing 15% serum without penicillin and streptomycin (transfection medium). Rat Sig-1R was cloned from NG-108 cells as described previously (Hayashi and Su, 2001). The polymerase chain reaction products were subcloned into the TA cloning vector (pcDNA3.1 vector; Invitrogen). The pcDNA3.1 vector encoding the whole coding region of Sig-1R cDNA from 22 to 940 base pairs was transfected. Forty-five microliters of LipofectAMINE 2000 (Invitrogen, Eragny, France) was placed into 1.5 ml of serum-free Dulbecco's modified Eagle's medium (Opti-MEM I medium; Invitrogen), mixed with 1.5 ml of serum-free Dulbecco's modified Eagle's medium containing 30 µg of Sig-1R vectors, and kept for 20 min at room temperature. The solution was added to the plated cells, which were then incubated at 37°C for 19 h. Afterward, the medium was replaced with the transfection medium every 24 h. After 2 days, the cells were grown in the transfection medium containing 0.5 mg/ml gentamicin (G418 sulfate; Invitrogen) for 4 weeks. The medium containing genetin was exchanged every 2 to 3 days. Transfected cells produced colonies after approximately 3 weeks. The colonies were subcloned in 24-well plates, grown until they reached confluence, and passaged two to three times in six-well plates. MT40 was thus selected for this study, which showed a moderately high level of overexpressed Sig-1R (approximately 4-fold) as judged by Western blotting (Fig. 6A).

Quantification of Neurite Sprouting. Neurite sprouting was estimated by using phase-contrast microscopy and was quantified as the percentage of cells bearing neurite processes of >1 cell diameter in length. The magnification of the phase-contrast microscopy was 100-fold. Cells with neurites were counted every 100 cells per field with three fields in each well. The field was randomly selected from the entire culture area (upper, lower, and left), and more than three wells (i.e., >900 cells) were routinely used for each experimental condition. Cells in clusters were not included. A cell with more than two neurites was counted as one. Dead cells, distinguished by their “phase dark” appearance under the microscope, were not counted.

Immunodetection of Sig-1R by Western Blotting. The anti-Sig-1R polyclonal antibody was raised in rabbits by cDNA immunization (QED/Advanced Research Technologies, San Diego, CA). The pcDNA3.1 vector encoding the Sig-1R cDNA was prepared as described in “Stable Overexpression of Sig-1R in PC12 Cells”. The vector was dissolved in phosphate-buffered saline at a concentration of approximately 1 to 2 µg/ml. Each rabbit received an injection (i.p.) of 100 µg of the vector. The sera from two rabbits were collected before immunization, and every 2 to 3 weeks after the injection (total of three collections). Sera were screened for antibodies by Western blotting using the antibody against guinea pig Sig-1R (Hayashi et al., 2000). For the Western blotting experiments, except those on the time-course studies, PC12 cells were cultured at a density of 30 x 10\textsuperscript{6}/cm\textsuperscript{2} on a six-well plate precoated with 1 µg/cm\textsuperscript{2} poly-d-lysine in 0.5% serum with or without test drugs for 2 days. For the time-course studies, the cells were plated at a density of 3 x 10\textsuperscript{5}/cm\textsuperscript{2} on 140-mm (for 1-day treatment studies) or 100-mm (for >3-day studies) dishes. The RPMI 1640 medium containing 0.5% serum was replaced every other day. The cells were solubilized in the sample buffer (100 mM...
Tris-HCl, pH 6.8, 20% glycerol, and 4% SDS), and proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred without methanol to polyvinylidene difluoride membranes. The nonspecific sites were blocked with 10% (w/v) nonfat milk for 7 h at room temperature. The resulting bands were detected by using enhanced chemiluminescence detection kit (Amer sham Biosciences).

**Antisense Oligodeoxynucleotide Transfection.** The 21-mer antisense oligodeoxynucleotide (AS ODN) described previously (King et al., 1997) was synthesized (Hayashi et al., 2000) and used in this study: 5’-GAGTCCCCAGCCACACCGA3’. Three base pairs in the antisense sequence were reversed to obtain the following mismatched oligodeoxynucleotide (MS ODN): 5’-GAGGTCCCCAGCCACACCGA-3’. This antisense sequence protected least significant difference post hoc test. Student’s t test was used in Figs. 2 and 8, B to D, as indicated by symbols. The two-way ANOVA was used in Figs. 2 and 8, B to D. The significance levels were set at p < 0.05.

**Results**

Examples of cells with neurite sprouting are shown in Fig. 1. The formation of neurite was a small, fiber-like process at the initiation point of the neurite outgrowth (Fig. 1, A–C). As cells differentiated further, the process grew longer as shown in Fig. 1D. Over a course of 2 days, under a reduced serum condition (0.5%) that was used in this study to minimize the endogenous sterols that might be present in the serum (0.5%), 1 to 20 ng/ml NGF dose dependently induced the neurite sprouting in PC12 cells (Fig. 2A, solid line). At 50 ng/ml NGF, the neurite sprouting was slightly attenuated. (+)PTZ, a Sig-1R agonist without an effect on the neurite sprouting when administered by itself, potentiated the neurite-sprouting induced by NGF and shifted the dose-response curve of the NGF to the left (shown herein at 100 nM (+)PTZ; dashed line, Fig. 2A; 1 ng/ml NGF, p = 0.0001; 3 ng/ml NGF, p = 0.014; 10 ng/ml NGF, p = 0.003; Student’s t test). However, at 20 ng/ml NGF, (+)PTZ did not potentiate the effect of NGF (p = 0.57; Student’s t test, Fig. 2A). The potentiation by (+)PTZ was affected by the concentration of NGF (p = 0.03; two-way ANOVA). The neurite sprouting induced by cAMP in PC12 cells (Young et al., 1994) was not affected by (+)PTZ.
When cells were cultured in the presence of 15% serum, the maximal effect of NGF in inducing the neurite sprouting was almost double (data not shown). (+)PTZ, however, it did not potentiate the effect of NGF when cells were cultured in the presence of 15% serum (data not shown).

To mimic the physiological condition, we chose a low concentration of (+)PTZ for the experiments. The characterization of (+)PTZ in potentiating the NGF-induced neurite sprouting is shown in Fig. 3. (+)PTZ by itself without NGF had no effect on neurite sprouting. B, effect of (+)PTZ (1–300 nM) on neurite sprouting in the presence of 1 ng/ml NGF for 2 days. *p < 0.05; ***, p < 0.001 compared with the control without (+)PTZ. C and D, effects of NE100 (0.1 nM–1 μM) on neurite sprouting in the absence of NGF (C) or in the presence of NGF (D) for 2 days. Each value represents the mean from three to four separate experiments. E, antagonism by NE100 of the (+)PTZ potentiation of the NGF-induced neurite sprouting. Percentage of cells bearing neurites was estimated after 2 days. NGF, 1 ng/ml; (+)PTZ, 100 nM; NE100, 1 μM. Each value represents the mean from four separate experiments. ***, p < 0.001 compared with controls containing only NGF; ###, p < 0.01 between the two indicated groups.
concentration of NGF (1 ng/ml) to examine in detail the (+)PTZ effect on the NGF-induced neurite sprouting. The observations were made 2 days after cells were exposed to NGF and (+)PTZ. As mentioned above, (+)PTZ alone without NGF was ineffective in potentiating the NGF-induced neurite sprouting (Fig. 3A). In the presence of 1 ng/ml NGF, (+)PTZ dose dependently potentiated the neurite sprouting caused by NGF (Fig. 3B). The maximal effect of (+)PTZ was seen at 100 nM (Fig. 3B). At 30 μM or higher, (+)PTZ inhibited the effect caused by NGF (data not shown). Regardless of whether NGF was present, Sig-1R antagonist NE100 did not affect the neurite sprouting at all test concentrations (Fig. 3, C and D). The potentiation by (+)PTZ was antagonized by 1 μM NE100 (##p < 0.005; Fig. 3E). These data indicate that (+)PTZ enhances the NGF-induced neurite sprouting and that this enhancement is mediated via Sig-1R.

Next, we investigated the effects of antidepressants that possess affinity at Sig-1R on the NGF-induced neurite sprouting. The experiments were carried out in the same manner as described above for (+)PTZ. Imipramine was ineffective on its own (Fig. 4A). Imipramine at 100 nM and 1 μM potentiated the effect of 1 ng/ml NGF (100 nM, p = 0.011; 100 nM–1 μM, p < 0.0001; 3 μM, p = 0.005; Fig. 4B). The potentiation by FVX was inhibited by NE100 (p < 0.0001; Fig. 5C). Because selective serotonin reuptake inhibitors increase serotonin in the extracellular space through an inhibition of serotonin transporters (Goodnick and Goldstein, 1998), we examined whether the potentiation by FVX was mediated via serotonin. No concentration of serotonin tested (1 nM–10 μM) affected the neurite sprouting caused by NGF (Fig. 5D). Furthermore, as a negative control, we investigated the effect of sulpiride on neurite sprouting caused by 1 ng/ml NGF. Sulpiride is a selective dopamine D2 receptor antagonist without affinity for Sig-1R. Sulpiride has been used not only as a neuroleptic but also as an antidepressant in several countries, including Europe and Asia (Ruther et al., 1999). Sulpiride (1 nM–10 μM) did not affect the neurite sprouting caused by NGF (Fig. 5E).

In separate experiments, the effects of the NGF treatment and the Sig-1R ligand treatments on the level of Sig-1R in PC12 cells were examined. We speculated that the level of Sig-1R may be critical in the neurite sprouting inasmuch as Sig-1R are enriched at the tip of the growth cones of NG-108 cells (Hayashi and Su, 2001). Treatments of cells with various amounts of NGF for 2 days, even without the exogenously added Sig-1R agonists, significantly increased Sig-1R in a
dose-dependent manner (10 ng/ml NGF, \( p < 0.039 \); 20 ng/ml NGF, \( p < 0.049 \); 50 ng/ml NGF, \( p < 0.048 \); Fig. 6A). This effect of NGF was also time-dependent (Fig. 6B). Treatment of cells with 100 nM (\( \text{H}^{+} \))PTZ alone for 2 days tended to increase Sig-1R. This increase by (\( \text{H}^{+} \))PTZ, however, did not reach significance (\( p < 0.08 \), Fig. 7A; \( p < 0.12 \), Fig. 7B).

Cotreatment of cells with 100 nM (\( \text{H}^{+} \))PTZ and 1 ng/ml NGF for 2 days caused a significant increase of Sig-1R (\( p < 0.001 \); Fig. 7A). The effects of (\( \text{H}^{+} \))PTZ and NGF (at 1 ng/ml) on Sig-1R levels are apparently additive. However, (\( \text{H}^{+} \))PTZ did not increase the level of Sig-1R when tested in the presence of 20 ng/ml NGF (Fig. 7B). These results (Fig. 7B) suggest a maximal up-regulation of Sig-1R in PC12 cells, which is in agreement with the results seen in the dose-response curves of NGF in increasing Sig-1R. NGF at 20 ng/ml caused a maximal increase of Sig-1R (Fig. 6A). Treatments of cells with 1 \( \mu \)M imipramine or 100 nM fluvoxamine alone for 2 days also increase Sig-1R. Notably, this concentration of FVX

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**Fig. 5.** Characterization of the fluvoxamine potentiation of the NGF-induced neurite sprouting. Figure legends in A to C are the same as in Fig. 3 except that fluvoxamine was tested in this figure. Note: * \( p < 0.05 \) compared with the NGF control in C. D, lack of the effect of serotonin on the 1 ng/ml NGF-induced neurite sprouting. \( n = 4 \) independent determinations for each experiment in this figure. E, lack of the effect of sulpiride on the 1 ng/ml NGF-induced neurite sprouting (\( n = 4 \)).
(100 nM) alone seemed to cause a maximal up-regulation of Sig-1R (IMP, \( p = 0.052 \); FVX, \( p = 0.01 \); Fig. 7, C and D). A cotreatment of cells with 1 ng/ml NGF and imipramine or fluvoxamine also caused a significant up-regulation of Sig-1R (\( p = 0.025 \), Fig. 7C; \( p = 0.02 \), Fig. 7D). The effects of 1 \( \mu \)M imipramine and 1 ng/ml NGF on Sig-1R levels are apparently additive. Serotonin (100 nM) and 100 nM sulpiride, however, failed to up-regulate Sig-1R (Fig. 7, D and E). It has to be mentioned that the exact reason for the apparent maximal up-regulation of Sig-1R caused by NGF or by Sig-1R ligands is unknown at present.

The above-mentioned results on the neurite sprouting and the up-regulation of Sig-1R suggest a possibility that the increase of Sig-1R, whether caused by NGF or Sig-1R ligands, may play an important role in the neurite sprouting. In fact, a strong correlation (correlation coefficient = 0.822; Pearson-Spearman rank order test) exists between the increase of neurite sprouting (solid line, Fig. 2A) and the increase of Sig-1R (Fig. 6A) induced by NGF. Therefore, when taken together, a hypothesis can be formulated: NGF induces neurite sprouting via an increase of Sig-1R. To provide evidence for a causal role of Sig-1R implicated in this hypothesis, two strategies were used: 1) overexpressing Sig-1R in PC12 cells, and 2) inactivating Sig-1R expression by using the antisense oligodeoxynucleotides directed against Sig-1R.

A PC12 cell line stably overexpressing Sig-1R was thus established and termed the MT40 cell line. MT40 cells were used to investigate whether the increase of Sig-1R may affect the efficacy of NGF in causing the neurite sprouting. MT40 cells expressed Sig-1R at a level approximately 4-fold higher than that in the wild-type PC12 cells (Fig. 8A). In MT40 cells, NGF dose dependently induced a higher degree of neurite sprouting compared with that seen in the wild-type cells (Fig. 8B). At all NGF doses tested, the degree of neurite sprouting was significantly higher in MT40 cells than in wild-type cells (Fig. 8B). Apparently, NGF was both more potent and more efficacious in MT40 cells than in wild-type cells in inducing the neurite sprouting (Fig. 8B). Furthermore, the increased effects of NGF in inducing the neurite sprouting in MT40 cells, compared with the wild type, were seen at almost all observation time points in the present study (Fig. 8, C and D). The poteniations seen in MT40 cells were significantly affected by concentrations of NGF, but not by the treatment days (\( p < 0.0001 \), Fig. 8B; \( p = 0.99 \), Fig. 8C; \( p = 0.30 \), Fig. 8D; two-way ANOVA).

The AS ODN directed against Sig-1R reduced the expression of Sig-1R in wild-type PC12 cells (Fig. 9A). The MS ODN reduced the expression of Sig-1R, apparently to a lesser degree compared with the effect caused by AS ODN (Fig. 9A). The AS ODN treatment in PC12 cells signifi-
cantly reduced the degree of neurite sprouting caused by NGF \( (p < 0.0001) \), whereas the MS ODN treatment did not \( (p = 0.56; \text{Fig. 9B}) \). It has to be mentioned that the effects of AS ODN and MS ODN on the NGF-induced neurite sprouting were examined using a culture medium without penicillin and streptomycin. The lack of the two antibiotics is essential for the transfection experiment. Thus, in Fig. 9B, NGF at 10 ng/ml caused a degree of neurite sprouting (about 24%), which was significantly higher than that seen in other experiment in the study (e.g., 15% in Fig. 8B; \( p < 0.01 \)). The two antibiotics obviously inhibit the neurite sprouting in PC12 cells.

### Discussion

This study constitutes the first report that Sig-1R are related to the neurite sprouting and that antidepressants with Sig-1R affinity may exert their clinical effects by causing the neuronal sprouting via Sig-1R and NGF. Our negative results with sulphiride (Figs. 5E and 7E), however, suggest that sulphiride may exert its antidepressant effect through other mechanisms unrelated to neuronal sprouting.

Because a strong correlation exists between the NGF-induced increase of neurite sprouting and the NGF-induced increase of Sig-1R, we proposed in this study that NGF

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**Fig. 7.** Effects of (+)PTZ, IMP, FVX, and sulphiride (SUL), with or without NGF, on the level of Sig-1R. Typical examples of immunoblotting are also shown. Rest of the figure legends is similar to those described in Fig. 6. Note: treatment days = 2; NGF1, 1 ng/ml NGF; NGF20, 20 ng/ml NGF; (+)PTZ, 100 nM; IMP, 1 µM; FVX, 100 nM; 5HT, serotonin 100 nM; SUL, 100 nM; \( n = 7 \) in A, \( n = 6 \) in B to E.
Fig. 8. Effects of the overexpression of Sig-1R in PC12 cells on the NGF-induced neurite sprouting. A, quantitation of Sig-1R by Western blotting: comparison of Sig-1R levels in MT40 cells and in wild-type cells (Wt). B, neurite sprouting induced by NGF in Sig-1R overexpressing MT40 cells are more efficacious than in wild-type PC12 cells. PC12 cell line stably overexpressing Sig-1R (MT40) was established as described under Materials and Methods. After cells were exposed to 1 to 50 ng/ml NGF for 2 days, percentage of cells bearing neurites was estimated. Solid line, wild type; dashed line, MT40 cells. Each point represents the mean value from three separate experiments. ##, p < 0.01 between the values from wild-type cells and MT40 cells. C and D, extended observations (day 2–7) on the 1 or 10 ng/ml NGF-induced neurite sprouting in wild-type PC12 cells (solid lines) and in MT40 cells (dashed line). The medium containing 0.5% serum and NGF was replaced every other day. Each point represents the mean value from three separate experiments. *, p < 0.05; **, p < 0.01 comparing the data point between two groups on the same observation day.

Fig. 9. Effects of the treatment of cells with either the AS ODN directed against Sig-1R or the MS ODN on Sig-1R and on the neurite sprouting induced by 10 ng/ml NGF. A, Western blotting of Sig-1R 2 days after the transfection of ODN. B, percentage of cells bearing neurites was estimated after 2 days. Each column represents the mean value from seven separate experiments. **, p < 0.01 compared with the control group treated with 10 ng/ml NGF alone (NGF). ##, p < 0.01 compared with the group treated with MS ODN and 10 ng/ml NGF (MS + NGF).
induces the neurite sprouting by first increasing the concentration of Sig-1R and then corroborating with Sig-1R to achieve the goal. Furthermore, because Sig-1R ligands (+)PTZ, imipramine, and fluoxetine enhanced the NGF-induced neurite sprouting, and concomitantly, caused an increase of Sig-1R, we suggested that the primary role of Sig-1R ligands in enhancing the NGF-induced neurite sprouting is to increase the level of Sig-1R in the cell. Two experiments were thus performed. The results indeed provide evidence for a causal role of Sig-1R in the NGF-induced neurite sprouting: 1) overexpression of Sig-1R increased the efficacy of NGF-induced neurite sprouting (Fig. 8), and 2) the aberrance of Sig-1R by the antisense treatment dampened the effect of NGF (Fig. 9). Thus, Sig-1R seem to be intrinsic molecules involved in the signaling pathway(s) evoked by NGF. However, two points need to be addressed and clarified, at least to some extent, regarding this hypothesis. First, in some experimental conditions when both Sig-1R levels and NGF concentrations are high, the NGF effect on neurite sprouting seemed to be reduced. This may be explained by a possibility that the neurite sprouting induced by NGF at high concentrations may involve not only Sig-1R but also the low-affinity NGF receptor (p75 neurotrophin receptor). The low-affinity NGF receptor was reported to exert an opposite action against that induced by the high-affinity NGF receptor tropomyosin-related kinase (Trk) A (Kohn et al., 1999). Second, low concentrations of NGF tend to cause a nonsignificant increase of Sig-1R. Will low concentrations of NGF then induce only a low level of neurite sprouting? Our data seemed to indicate so. Low concentrations of NGF apparently caused only moderate increase of the neurite sprouting. It is conceivable that more studies are needed to render support to the hypothesis proposed in the present study.

It is not known at present exactly how Sig-1R can enhance the action of NGF. The enhancement caused by (+)PTZ is NGF-dependent, but not cAMP-dependent (Figs. 2B and 3B). The NGF-induced neurite outgrowth is known to involve the activation of the NGF receptor, i.e., the TrkA, and the subsequent signaling cascades, including the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase/Akt kinase pathway, and the phospholipase C (PLC)-γ1 pathway (Kaplan and Miller, 2000). Vossler et al. (1997) reported that CAMP induced neuronal differentiation via an activation of the small G protein Rap1 and MAPK in PC12 cells, suggesting that the CAMP-activated intracellular signaling may overlap with that activated by NGF. Our results therefore suggest that the action site of Sig-1R may be upstream of MAPK and Rap1 on one of the NGF-signaling pathways or at certain other loci related to the two other NGF pathways (i.e., phosphatidylinositol 3-kinase/Akt and PLC). Sig-1R-related signaling may be related to the action of NGF. For example, Sig-1R activate PLC and protein kinase C via an unknown mechanism (Morin-Surun et al., 1999). Also, the intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) has been known to play an important role in regulating the neurite outgrowth in many types of cells, including PC12 cells (Furukawa et al., 1998). Indeed, NGF increases the intracellular calcium concentration in PC12 cells (Furukawa et al., 1998), and Sig-1R are known to increase the [Ca\(^{2+}\)]\(_i\), by causing the dissociation of ankyrin from inositol 1,4,5-trisphosphate receptors at the ER (Hayashi and Su, 2001). Therefore, [Ca\(^{2+}\)]\(_i\), might be related to the action of Sig-1R in affecting the NGF-induced neurite sprouting. Although Sig-1R are ER proteins, they can translocate from the ER to the plasma membrane (Hayashi and Su, 2001). Sig-1R have recently been shown to be present at the plasma membrane as a regulatory subunit on the voltage-gated K\(^{+}\) channel (Aydar et al., 2002). Therefore, Sig-1R might also affect the action of NGF at the plasma membrane.

The mechanism of up-regulation of Sig-1R is unknown at present. Hellewell and Bowen (1990) reported that a 100 ng/ml NGF treatment for 7 days in PC12 cells induced a small but not significant increase of the binding of [\(^{3}\)H]1,3-di-o-tolylguanidines to \(\sigma\)-receptors. [\(^{3}\)H]1,3-Di-o-tolylguanidine is a nonselective ligand for Sig-1R and \(\sigma\)-2 receptors (Quirion et al., 1992). Our data by using Sig-1R Western blotting clearly demonstrate the increase of Sig-1R by NGF. The promoter region of the Sig-1R gene contains putative binding sites for activator protein (AP)-1, AP-2 and steroid receptors (Seth et al., 1997). Because NGF is known to activate the AP-1 family of transcription factors in PC12 cells (Furukawa et al., 1998), the AP-1 family of transcription factor may be involved in the up-regulation of Sig-1R by NGF. We do not know at present exactly how chronic treatments of Sig-1R ligands regulate the expression of Sig-1R. Results on the Sig-1R ligands on the expression of Sig-1R were less consistent in the literature. Zamanillo et al. (2000) reported that a chronic treatment of E-5842, one of Sig-1R ligands, increased the Sig-1R mRNA in the rat brain. Shirayama et al. (1993) reported a decrease of \(\sigma\) binding sites in the rat brain after a chronic imipramine treatment. Chronic haloperidol treatment was shown to cause a reduction of Sig-1R binding sites (Inoue et al., 2000). The apparent discrepancies may be due to different methodologies used, including in vivo versus in vitro tests and binding assays versus immunodetections. Our results with a Sig-1R agonist up-regulating Sig-1R are in contrast to that expected from the conventional receptor theory. More understanding on the molecular nature of Sig-1R is certainly required to clarify this apparent paradox.

Stress, depression, and psychotropic drugs were found to induce structural alterations and synaptic remodeling (McEwen, 2000; Norrholm and Ouimet, 2001; Nestler et al., 2002). A reduction in volume was found in the hippocampi of certain patients with depression (Bremner et al., 2000). These results suggest a linkage between morphological alteration in the brain of depressed patients and the effect of antidepressants. However, no direct evidence has been established in humans. Serotonin is known to regulate many aspects of neuronal development, including the neurite outgrowth (Lauder 1993). FVX binding to Sig-1R (36 nM) is about 10 times less potent than that to the serotonin reuptake site (3.8 nM) (Narita et al., 1996; Goodnick and Goldstein, 1998). In this study, however, we found that the enhancement of the NGF-induced neurite sprouting by FVX was mediated via Sig-1R, not via the action of serotonin (Fig. 6D). Sulpiride was also ineffective in causing the neurite sprouting and Sig-1R increase. Thus, sulpiride may work as an antidepressant through a different mechanism. It is worth noting that sulpiride is usually used for relatively mild depression (Ruther et al., 1999). Furthermore, considering that the enhancement of NGF-induced neurite sprouting by antidepressants and by (+)PTZ could only be seen at low-serum (0.5%) and low-NGF (<20 ng/ml) conditions, the results of this study may have a bearing with the clinical observation.
that antidepressants work for depressed patients, but not for healthy humans. The present study certainly warrants further investigations using other Sig-1R-active antidepressants.

In conclusion, Sig-1R may play an important role in neuronal plasticity, including the initiation of neurite sprouting. Sig-1R-active ligands, including certain antidepressants, may thus be able to affect the neuronal remodeling via Sig-1R that may subsequently help improve clinical symptoms of depression.

References
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