# GINSENOSIDE $Rb_1$ AND NERVE GROWTH FACTOR

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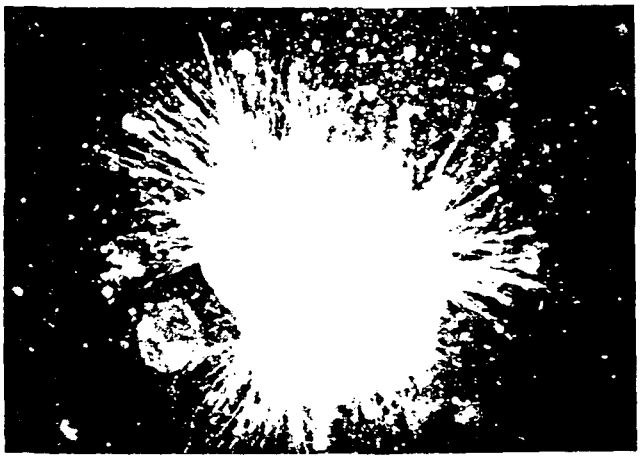
Sedative, antipyretic and anti-stress actions of ginsenoside $Rb_1$ (GRbI), 20S-protopanaxadiol glycoside contained in the root of Panax ginseng C.A. Meyer, were confirmed with selected special screenings. These actions of $GRb_1$ are not so potent as those of well-known drugs. I happened to use $GRb_1$ instead of nerve growth factor (NGF) or with NGF in culture of chick embryonic dorsal root ganglia and lumbar sympathetic ganglia. Stimulation of fiber outgrowth from chick embryonic dorsal root and sympathetic ganglia in 24 hr culture is one of the most frequently used criteria for estimating the biological activity of NGF. $GRb_1$ did not promote fiber production. On the other hand, I did find that the effect of NGF was markedly potentiated by $GRb_1$. The use of in vitro system for the study of neural tissues or cells has been an indispensable component of NGF investigations from the early days. The validity of these approaches for the understanding of in vivo situations, has been firmly established in recent years by the demonstration that the properties first noted and characterized in vitro, have been subsequently confirmed for the tissue in situ. NGF has been described as an agent promoting growth and differentiation in its target neurons. The recent results of studies on NGF have recognized an essential role of NGF for survival, regeneration and regulation of catecholaminergic neurons of brain and ganglion in adult animals. These findings suggest unrecognized facets of NGF's action and opens new problems to be dealt with. NGF was isolated as the 2.5S subunit from a 2 month-old mouse according to the procedure of Bocchini and Angelletti (1) and NGF-antibody from rabbit antiserum was produced according to the procedure of Stöckel et al (2). Incubation and observation of Ganglia were performed according to a modification of the procedure of Fenton (3). Numerical scores (index 0–8) of intensity were assigned to each ganglion.

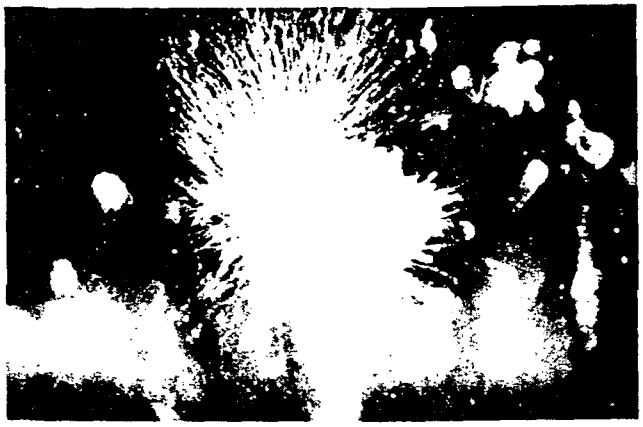
Optimal fiber outgrowth (dense halo of fibers and maximal fiber length) was designed as index 4 and corresponded to one biological unit of NGF. The optimal response was about 20 ng/ml of NGF, and 3.5 ng/ml of NGF with 30 $\mu$M of $GRb_1$. Supraoptimal concentrations resulted in increased density of fiber halo concomitant with a reduced fiber length (index 5–8). The appearance of fiber outgrowth produced by NGF is found about 9 hrs after the incubation. Figure 1 (a) shows fiber outgrowth by 10 ng/ml of NGF (index 3), and Fig 1 (b), by 0.6ng/ml with 30 $\mu$M of $GRb_1$ (index 3). $GRb_1$ also potentiated the NGF-mediated nerve fiber production in organ cultures of mouse embryonic dorsal root ganglia and superior cervical ganglia. Figure 2 (a) shows the culture of 16 day-old mouse embryo-

(a)



score 3, NGF 10 ng/ml in culture medium

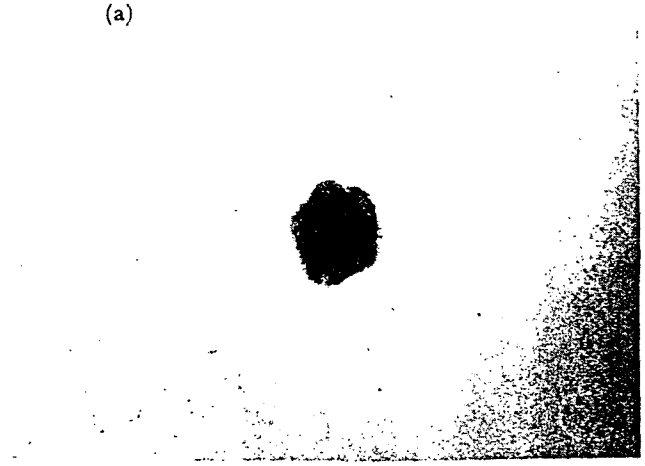
(b)



score 3, NGF 0.6 ng/ml with 30 $\mu$M of GRbl in culture medium

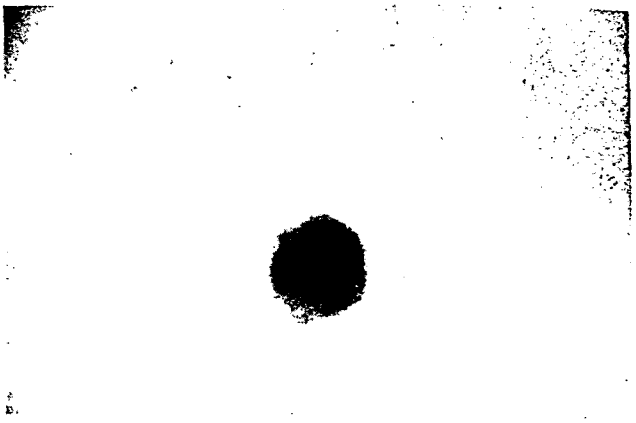
**Fig. 1.** Effect of NGF on neurite outgrowth in chick embryonic dorsal root ganglia in organ culture.

(a)



score 4, NGF 2.5 ng/ml in culture medium

(b)



score 4, NGF 0.6 ng/ml with 30 $\mu$M of $GRb_1$ in culture medium

**Fig. 2.** *Effect of NGF on neurite outgrowth in mouse* embryonic dorsal root ganglia in organ culture.

nic dorsal root ganglion with 2.5 ng/ml of NGF (index 4), and Fig. 2 (b), with 0.6 ng/ml of NGF with 30 $\mu$M of $GRb_1$ (index 4).

**Potentiation mechanism of $GRb_1$ on NGF-mediated fiber outgrowth in organ cultures of chick embryonic dorsal root and sympathetic ganglia.**

A figure to study the influence of $GRb_1$ on the NGF-receptor binding site and the process of fiber production by NGF is shown in Fig. 3. It is under discussion that increase of cAMP level, RNA synthesis, protein synthesis and tubulin assembly were occurred in the process of fiber production by NGF. In each experiment we used the same ganglia in the opposite side of the embryo as control. The antagonism between neurite

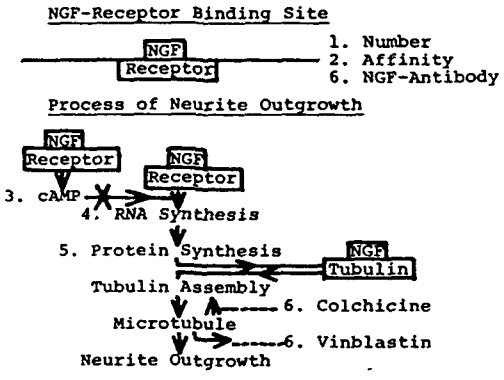



**Fig. 3.** Summary of the process of neurite outgrowth by NGF

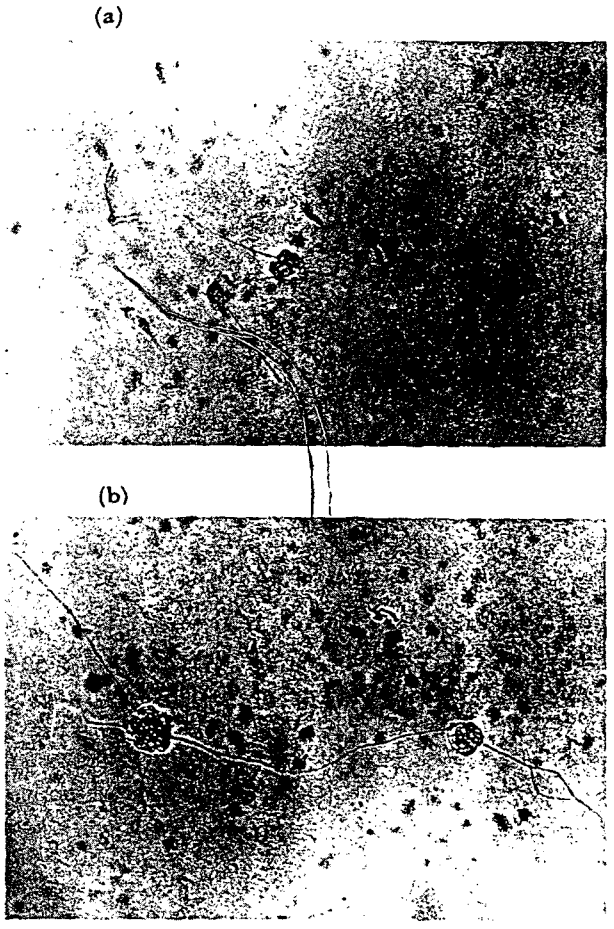
**Table 1.** Outline of the experiments for the process of neurite outgrowth by NGF

| Experiments | Methods | Samples |
|---|---|---|
| NGF binding assay | $^{125}$I labeled NGF (Fabricant et al., 1977)<br>Specific binding assay<br>(modification of Herrup and Shooter, 1975) | DRG (8 days old)<br>1 x $10^5$ cells/tube |
| Cyclic AMP level | Radioimmunoassay<br>(modification of Steiner, 1972) | 20 DRG, 4 SympG chains<br>2 SCG (rat) |
| RNA synthesis | $^3$H-Uridine incorporation<br>(Mans and Novelli, 1960) | 10 DRG<br>2 SympG chains |
| Protein synthesis | $^3$H-Leucine incorporation<br>(Mans and Novelli, 1960) | 10 DRG<br>2 SympG chains |
| The inhibition of neurite outgrowth by some drugs | NGF antibody<br>Colchicine<br>Vinblastin<br>Cytochalasin B<br>(Fenton, 1970) | DRG (8 days old) |

outgrowth by NGF and drugs was also studied. NGF-antibody, colchicine and vinblastin were a few which antagonized the NGF effect. Table 1 shows experiments we had. $GRb_1$ had no influence on affinity and number of NGF-receptor binding site. Cyclic AMP levels were measured by the radio immunoassay procedure. NGF and $GRb_1$ did not alter the levels of cAMP. RNA and protein synthesis were measured using $^3$H-urisine and $^3$H-leucine. Supraoptimal concentrations of NGF had marked effect on the incorporation of $^3$H-urisine and $^3$H-leucine more than optimal concentration NGF which promoted optimal neurite outgrowth. Suboptimal concentration of NGF with 30 $\mu$M of $GRb_1$ promoted optimal neurite outgrowth but did not alter the incorporations. $GRb_1$ did neither alter the incorporation nor potentiate the original effects of NGF. It appears that increase of RNA and protein synthesis by NGF may have no direct corelation with neurite outgrowth by NGF. We studied the antagonism between the neurite outgrowth by NGF and drugs. $GRb_1$ protected the NGF-effect from colchicine in both ganglia. Vinblastin did not alter the dense halo of fiber by NGF, but produced shortening of the length of fibers. $GRb_1$ potentiated the inhibitory effect of vinblastin on the NGF-effect in both ganglia.

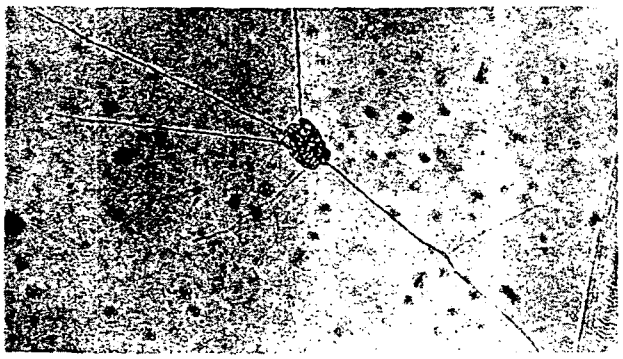
**Effect of $GRb_1$ on the NGF-mediated nerve fiber production in cell cultures of chick embryonic dorsal root and lumbar sympathetic neurons.**

Dissociation, incubation and observation of neurons were performed according to a modification of the procedure of Nakai (4). $GRb_1$ also potentiated the NGF-effect in both neurons and results were shown in Fig. 5 (a,b,c,d,e). Figure 5 shows the 24 hr culture of 9 day-old chick embryonic lumbar sympathetic neurons. Figure 5 (a) shows neurite outgrowth without NGF in culture medium, and Fig. 5 (b), with 30 $\mu$M of $GRb_1$. A few axons are found. $GRb_1$ did not alter the number of axons, but it appears that $GRb_1$-treated neurons have more axons whose terminals were divided into branches than control. Figure 5 (c) shows neurite outgrowth with 50 ng/ml of NGF, and Fig. 5 (d) and (e), with 50 ng/ml of NGF and 30 $\mu$M of $GRb_1$. Many large axons were found. $GRb_1$ increased the number of axons, and axons whose terminals were divided into

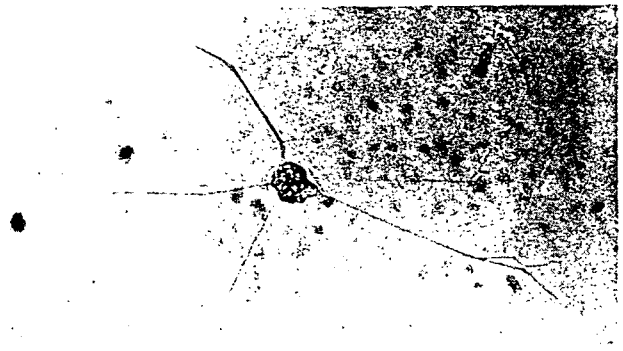



(a) No NGF in culture medium. (b) $GRb_1$ 30 $\mu$M; (c) NGF 50 ng/ml; (d & e) NGF 50 ng/ml with $GRb_1$ 30$\mu$M.

(c)



(d)



(e)

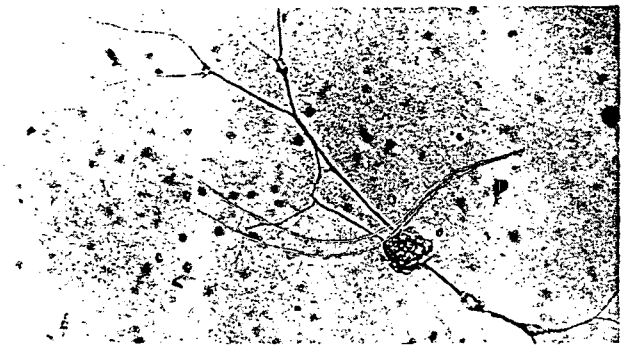


**Fig. 5.** Effects of NGF on neurite outgrowth in chick embryonic lumbar sympathetic neurons in culture

branches, and thick axons were often found in $GRb_1$-treated neurons (e). There are many problems for us to tackle now on.

## Effect of $GRb_1$ on the activity of tyrosine hydroxylase (TH) in superior cervical ganglion (SCG) and submaxillary gland (SubG) in irradiated mice (5).

In senescent mice, NGF content in SubG was reduced significantly and TH activity in SubG was slightly reduced, but TH activity in SCG

**Table 2.**

NGF Content of Submandibular Gland in Irradiated Mice

| Drugs Treated (Dose; mg/kg) | Body Weight (g) | SubG Weight (mg) | Protein (mg/SubG) | Total Activity (Unit$\times 10^3$/SubG) | Specific Activity (Unit/mg Protein) |
|---|---|---|---|---|---|
| Non-irradiated Mice | 27.3 ± 0.3 | 121.3 ± 5.0 | 11.9 ± 1.6 | 8.1 ± 1.5 | 807 ± 161 |
| Irradiated Mice with Saline | 20.3 ± 0.4** | 93.2 ± 6.7** | 6.3 ± 0.4** | 2.1 ± 0.6** | 327 ± 76* |
| Irradiated Mice with $GRb_1$ (30) | 19.0 ± 0.6 | 83.5 ± 4.4 | 5.7 ± 0.5 | 3.6 ± 2.1 | 613 ± 289 |
| Irradiated Mice with $GRb_1$ (100) | 19.5 ± 0.4 | 79.7 ± 6.2 | 5.2 ± 0.6 | 2.3 ± 0.6 | 396 ± 100 |
| Irradiated Mice with $GRb_1$[a] | 20.0 ± 0.1 | 88.5 ± 3.8 | 6.8 ± 0.7 | 2.7 ± 0.6 | 417 ± 71 |
| Irradiated Mice with Testosterone | 19.6 ± 0.5 | 89.5 ± 7.2 | 4.0 ± 1.0 | 8.4 ± 1.2** | 1478 ± 309** |

After a 600 R irradiation, 4 week-old male mice (ddy-strain) in groups of 6 were given saline, $GRb_1$ (30 & 100 mg/kg) or testosterone (100 mg/kg) subcutenously once a day for a week, then SubG were measured NGF content according to the method of Fenton.
a) $GRb_1$ (300 mg/kg) was given orally once a day for a week.
**: Symbols indicate the significant difference between Non-irradiated and irradiated mice (Student's t-test $p<0.01$) and *: ($p<0.05$).
**: Symbols indicate the significant difference between irradiated control and drug tested mice ($p<0.01$).

**Table 3.**

Effect of Ginsenoside $Rb_1$ on Tyrosine Hydroxylase Activity in Irradiated Mice

| Drugs Treated | TH Activity (nmole DOPA Formed/hr/Pairs of Organ) SCG | Submandibular Gland |
|---|---|---|
| Non-irradiated Mice | 1.82 ± 0.20 | 0.346 ± 0.017 |
| Irradiated Mice with Saline | 1.82 ± 0.18 | 0.239 ± 0.035[a] |
| Irradiated Mice with Saline and NGF* | 2.23 ± 0.15 | 0.219 ± 0.018[b] |
| Irradiated Mice with $GRb_1$ | 1.93 ± 0.10 | 0.366 ± 0.041[b] |
| Irradiated Mice with $GRb_1$ and NGF* | 2.20 ± 0.09 | 0.386 ± 0.021[c] |
| Irradiated Mice with Testosterone | 1.59 ± 0.14 | 0.420 ± 0.031[c] |
| Irradiated Mice with $GRb_1$ | 1.55 ± 0.04 | 0.401 ± 0.053[b] |

After a 600 R irradiation, male mice (ddy-strain) in groups of 6 were given saline, $GRb_1$ (100 mg/kg) or testosterone (100 mg/kg) subcutenously once a day for a week, then SCG and submandibular gland were removed and measured TH activity by the method of Nagatsu et al.
*: NGF (10 mg/kg) was given subcutenously 48 hr before sacrifice.
**: $GRb_1$ (300 mg/kg) was given orally once a day for a week.
a) Symbols indicate the significant difference of TH activity between non-irradiated and irradiated mice (Student's t-test, $p<0.05$), b) between irradiated control and drug tested, ($p<0.05$), and c) ($p<0.01$). 4 week-old mice were irradiated 600 R (200 KVP, 20 mA, 100-110 R/min in air, thickness of filter; 0.5 mm Cu & 0.5 mm Al).

remained unchanged. The purpose of this experiments is to know whether irradiated mice can be utilized as substitute for senescent mice on research of degeneration of sympathetic neurons, and to know whether $GRb_1$ has any protective role against the degeneration of sympathetic neurons in irradiated mice. TH activity was measured according to the procedure of Nagatsu et al (6). A 600 R irradiation was performed with an X-ray machine to 4 week-old male mice. One week after an irradiation TH activity and NGF content were measured.

As shown in Table 2 and 3, Body weight, protein content, NGF content and TH activity in SubG were significantly reduced. NGF was given S.C. (10 mg/kg) immediately after an irradiation and sacrificed one week later. TH activity in SCG and SubG remained unchanged. When NGF was given S.C. (10 mg/kg) 48 hrs before sacrifice, TH activity in SCG remained unchanged but TH activity in SubG were reduced.

$GRb_1$ and testosterone were given S.C. or

p.o. once a day for a week after an irradiation. NGF content and TH activity in SubG were not reduced in the testosterone-treated mice. TH activity in SubG remained unchanged, but NGF content were reduced in the $GRb_1$-treated mice. It was estimated that exposure to an irradiation led to a gradual destruction of the sympathetic nerve terminals but irradiated mice were not utilized as substitute for senescent mice.

It is very difficult to give any definite conclusion on the results of these experiments, but they may indicate that NGF is required for fiber production and maintenance of the sympathetic nerve terminals and $GRb_1$ and testosterone play an important role in the NGF-effects with their different actions.

I would like to give my sincere thanks to Prof. S. Shibata, Meiji College of Pharmacy, Prof. O. Tanaka, School of Medicine, Hiroshima University, Prof. J. Shoji, Showa University, Prof. K. Takagi, Tokyo Science University, Prof. H. Thoenen, Max Planck Institute, München and my young co-workers.

**Chairman:** Now the time is open to discussion.

**Questioner:** Thank you very much for your interesting paper. I also examined $Rb_1$ effects on the HDH induced in isolated system. $Rb_1$ inhibited HDH induced and HDH calcium ion is essential for the HDH induced by action. Therefore, I assume that calcium $Rb_1$ might moderate calcium ion metabolism. Is there any possibility that $Rb_1$ might associate with the calcium ion metabolism in your system?

**Saito:** No, it is impossible to use our system to do such experiment. This is a problem and I have to solve this. But the most important thing to do is to make a preparation. We are not trying to make a pure preparation. Then I can do it.

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