EFFECTS OF NEREISTOXIN AND ITS DERIVATIVES ON THE SPINAL CORD AND MOTOR NERVE TERMINALS

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Nereistoxin is a potent neuroactive substance isolated first by Nitta (1) from the Japanese species of annelid, Lumbriconereis heteropoda Marenz. In our preliminary pharmacological study of synthetic nereistoxin (2), it has been shown in the cat with chronically implanted electrodes that the intraperitoneal injection of 5 mg/kg produced the muscle relaxing signs due to the moderate impairment of the neuromuscular transmission. At the higher dose level than 10 mg/kg, however, this toxin caused salivation, mydriasis, panting, tremor and tonic convulsion which accompanied the spike and dome pattern in electroencephalogram (EEG). Components of this convulsion, tremor and twitching of the limb and body trunk muscles in addition to the EEG seizure patterns were also caused by this toxin in unanesthetized encéphale isolé cats. This indicates that the sites of the convulsive action of this toxin exist in both the supraspinal and spinal levels.

The present study was carried out in an attempt to clarify the mechanisms in the spinal cord and at the motor nerve terminal for producing convulsion by nereistoxin. Comparative study of this toxin with the derivatives in the potency was also done.

METHODS

A total of 44 adult cats of both sexes, weighing from 2.2 to 4.4 kg, were used. Spinal cats were prepared by transecting the spinal cord at CI level under ether anesthesia and maintained on artificial respiration. The animals were immobilized with the intramuscular injection of gallamine (10 mg/kg), except when the electromyographic (EMG) recording was required. Some cats anesthetized with alpha chlortalose (50 mg/kg, i.v.) and urethane (500 mg/kg, i.p.) were also used.

1. Procedures in the spinal cord

The whole length of the spinal cord between L1 and S2 segments were exposed by laminectomy and covered with mineral oil maintained at the body temperature. The central cut end of the L7 dorsal root or gastrocnemius nerve was stimulated by monophasic rectangular pulses, and the resultant mono- and polysynaptic reflex potentials were recorded with bipolar silver electrodes placed on the cut end of the ventral root of the same segment. The dorsal root reflex potentials evoked by the same stimuli were also

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recorded in the L6 dorsal root. The stimulus was 0.4 msec in pulse duration, 0.3 c/s in frequency and of a voltage to produce a maximal response. The stimuli were interrupted for 1 or 2 minutes at the interval of 10 minutes in order to observe the spontaneous discharges of the ventral roots. The activity of the EMG was taken with the bipolar silver tiny disk electrodes placed on the gastrocnemius muscle. Blood pressure was measured from the femoral artery with a pressure transducer in all experiments. In 3 preparations, sections of all ventral and dorsal roots arising from the segments between L1 and S2 in order to exclude the nervous connection with muscles, and additional transection of the spinal cord at L1 as well as S2 levels to reduce the number of the connected segments were carried out.

2. Procedures for antidromic activity of motor nerve

The preparation for recording the ventral root potentials was done according to the methods described by Riker et al. (3) and Werner (4). The spinal cord and gastrocnemius nerve were exposed at the popliteal cavity in the spinal cats prepared as described before. The peripheral cut end of the gastrocnemius nerve was stimulated using the bipolar silver electrode, with rectangular pulses of 0.4 msec. pulse duration, of 0.3 c/s in frequency and of supramaximal intensity. The resultant antidromic electrical activities were recorded with the bipolar silver electrode in the peripheral cut end of the ventral filament. At the same time, the action potentials of the gastrocnemius muscle were also recorded using the bipolar tiny silver disk electrode placed on the gastrocnemius muscle. A loose ligature was placed around the popliteal artery and was used to occlude the artery only at the moment of the close-arterial injection of the drug.

| TABLE 1. Chemical structures of nereistoxin (NT) and its derivatives. |
|-------------------------|------------------|------------------|
| NT | CH₉₋₁₀ N-C Hopkins CH₂₋₁₀ S \( \text{COOH}_₂ \) |
| NTD-1 | CH₂₋₁₀ CH₉₋₁₀ N-C Hopkins CH₂₋₁₀ S \( \text{COOH}_₂ \) |
| NTD-2 | CH₉₋₁₀ N-C Hopkins CH₂₋₁₀ S-CO\( \text{NH}_₂ \) · HCl |

Nereistoxin (NT) and the derivatives with the chemical structures shown in Table 1 were synthesized in this Chemical Laboratory. The following agents were used for studying the interaction and comparison with the effects of nereistoxin: picrotoxin (Merck), strychnine nitrate (Nishin), nicotine tartrate (Wako Junyaku), cysteamine hydrochloride (Tokyo Kasei), guanidine sulfate (Tokyo Kasei), pentobarbital sodium (Nembutal®, Abbott), mephasenin (Myoserol®, Sankyo), atropine sulfate (Merck), physostigmine salicylate (Merck), neostigmine methylsulfate (Vagostigmine®, Shionogi), tetrodotoxin (Tetrodotoxin® Sankyo), d-tubocurarine chloride (Amelizol®, Yoshitomi), gallamine triethiodide (Flaxedil®, C.H. Boehringer) and succinylcholine chloride (Succin®, Yamanouchi). The other agents used were tremorine, nitrazepam and d-penicillamine synthesized in this Chemical Laboratory. Nitrazepam was dissolved in 20% glycofurol solution and me-
phenesin in 10% propyleneglycol solution for injection. Other agents were dissolved in physiological saline.

RESULTS

1. Effects on spinal cord

The intravenous injection of 10 mg/kg of nereistoxin produced an immediate fall in blood pressure ranging from 20 to 30 mmHg followed by a gradual recovery. The mono- and polysynaptic reflex potentials were not affected or sometimes rather augmented during this hypotension.

In 9 of ten spinal animals, however, the spinal reflex and dorsal root reflex potentials were progressively depressed from 20 to 30 minutes after the injection but the former potentials were sometimes augmented when they were superimposed on the increased spontaneous discharges of the ventral root described later (Fig. 1). Sixty to 90 minutes following nereistoxin, both potentials were almost or completely inhibited whenever they were superimposed on the increased ventral discharges. The spontaneous discharges of the ventral root increased in characteristic of the irregular, repetitive appearances of short

CONTROL    NT.10mg/kg. 30min.

A)

1)SR. \[\text{Spinal reflex potentials}\]

2)DRR. \[\text{Dorsal root reflex potentials}\]

3)SD. \[\text{Spontaneous discharges of the ventral root}\]

B)

FIG. 1. A) : Effect of nereistoxin (10 mg/kg, i.v.) on spinal reflex potentials (SR.), dorsal root reflex potentials (DRR.) and spontaneous discharges of the ventral root (SD.). B) : Augmentation of spinal reflex potentials when they were superimposed on the increased spontaneous discharges of the ventral root.
train of discharges lasting for 0.5 to 1.0 second and longer train of 10 to 15 seconds, which generally accompanied a transient rise in blood pressure, 20 to 30 minutes after the injection. Generally, the longer was the duration of one train, the following pause was longer. The pause progressively disappeared and the train of discharges eventually became continuous 60 to 90 minutes following the injection. Synchronized with these spontaneous ventral discharges, tremor and twitching of the muscles of the body trunk and especially the limbs were observed as indicated by the increase in EMG activity of the gastrocnemius muscle (Fig. 2). Autonomic changes such as lacrimation, salivation and mydriasis were also observed. Depression of the spinal and dorsal root reflex potentials were still observed even after the increased ventral discharges disappeared 2 hours or more later. The effects of NTD-1 and NTD-2 were similar to that of nereistoxin, and the relative potencies of NTD-1, nereistoxin and NTD-2 were 100, 78 and 53 respectively, as compared in those minimum effective doses.

![Fig. 2. EMG., ventral root discharges (VRD.), spinal reflex potentials (indicated as vertical line on ventral root discharges) and blood pressure recorded on polygraph. Note that increase in ventral root discharges was associated with activation of EMG.](image)

Similarly, the above-mentioned spinal effects of nereistoxin were also observed in the anesthetized or gallamine-immobilized intact cats, although the effects appeared to be less in the latter preparations. Nereistoxin did not affect the nerve conduction because the action potential of the L7 dorsal root evoked by stimulating the central cut end of the gastrocnemius nerve was never changed despite the marked depression of the spinal and dorsal root reflex potentials. The increased spontaneous discharges of the ventral roots and the depressed spinal polysynaptic reflex potentials evoked by stimulation of the L7 dorsal root were obtained by nereistoxin not only in L7 level but also in L4, L5, L6 and S1 levels. On the other hand, the spontaneous discharges of the dorsal root were not affected by nereistoxin at any of the above-mentioned segments.

The bilateral section of all ventral and dorsal roots of the segments from L1 to S2 did not modify the spinal effects of nereistoxin. The additional transection of the spinal cord at L1 and S2 levels, however, resulted in the moderate but significant recovery from the increased spontaneous discharges of the ventral root and the depressed polysynaptic
FIG. 3. Influence of reduction of the number of connected spinal cord segments on spinal effect of nereistoxin. The moderate but significant recovery from the depression of spinal polysynaptic reflex potentials and the increase in spontaneous discharges of the ventral root without any change in the depressed spinal monosynaptic reflex potentials.

It was also studied whether the spinal actions of nereistoxin were modified by the drugs such as picrotoxin, strychnine, pentobarbital, mephenesin, nitrazepam, physostigmine, atropine and penicillamine. The intravenous injection of pentobarbital in the

Table 2. The comparative results of the effects of nereistoxin with a variety of drugs on the spinal cord.

| Drug       | Dose, i.v. (mg/kg) | Ventral root Reflex potentials | Spontaneous discharges | Dorsal root Reflex potentials | Spontaneous discharges | Afferent action potentials |
|------------|-------------------|--------------------------------|------------------------|-------------------------------|------------------------|--------------------------|
| Nereistoxin| 10                | ↓                               |                        | ↓                             |                        |                          |
| Picrotoxin | 1                 | or ↑                            |                        | ↓                             |                        |                          |
| Strychnine | 0.2               | ↑                               | ↑                      | -                             | ↑                      |                          |
| Nicotine   | 0.5               | ↑*                              |                        | -                             |                        |                          |
| Tromorine  | 20                | or ↑                            | ↑                      | -                             |                        |                          |
| Cysteamine | 100               | ↑                               |                        | -                             | ↑                      |                          |
| Guanidine  | 100               | -                               | ↑                      | -                             | -                      |                          |
| Tetrodotoxin| 0.01             | ↓                               |                        | ↓                             |                        |                          |

* Monosynaptic spinal reflex potentials are not included.

↑: Increase, ↓: Decrease, −−: No change.
doses of 15 to 30 mg/kg potentiated the depression of the spinal reflex potentials caused by nereistoxin but remarkably inhibited the increased spontaneous ventral discharges. Although atropine (0.5 to 1.0 mg/kg, i.v.) inhibited salivation and markedly potentiated mydriasis, it had no effect on the spinal effect of nereistoxin. Other drugs including D-penicillamine and physostigmine, which were complete or partial antagonists against the neuromuscular blocking effect of nereistoxin, did not modify the spinal effects.

Table 2 indicates the comparative results of the effects of nereistoxin with a variety of drugs on the spinal cord. The effects of nereistoxin was qualitatively different from those of the drugs tested in the present experiments.

2. **Effects on nerve terminals**

In the control records, the antidromic nerve action potentials of the ventral filament evoked by orthodromic stimulation of the gastrocnemius nerve consisted of a synchronous response with short latency of 3 to 4 msec (short latency response) and mostly an asynchronous discharges with longer latency (long latency response). The injection of 20 to 30 μg of nereistoxin into the popliteal artery augmented both of the short and long latency responses 1 to 2 minutes later. Fifty to 60 μg facilitated both antidromic responses at the early phase but gradually depressed only the short latency response, leaving aside the facilitated long latency response, which was still observed after the recovery of the

![Fig. 4. Muscle action potentials recorded from belly-tendon electrodes (upper trace) and nerve action potentials recorded antidromically from filament of L7 ventral root (lower trace) following efferent stimulation of gastrocnemius nerve.](image-url)
short latency response. Slight but no significant change of the evoked muscle potentials of the gastrocnemius muscle was observed during this blocking phase. Higher dose, 120 μg progressively inhibited both antidromic responses in association with moderate depression of the muscle potentials (Fig. 4).

The effect of nereistoxin was compared with those of 5 μg of d-tubocurarine and succinylcholine. The intraarterial injection of 5 μg of d-tubocurarine completely inhibited both antidromic responses, leaving the muscle potential unchanged. While, the same dose of succinylcholine augmented the short latency and the long latency response in a characteristic of the repetitive firings at the early stage, as previously reported by Blaber and Goode (5). These facilitatory effects were followed by the inhibition of both antidromic responses. The effects of either drug recovered within 10 minutes and were of much shorter duration than that of nereistoxin.

Furthermore, the intraarterial injection of 5 μg of a cholinesterase inhibitor, neostigmine, which has been reported to be a partial antagonist against nereistoxin in the neuromuscular junction, remarkably reversed the inhibition of both antidromic responses induced by nereistoxin for 10 to 15 minutes. However, additional injection of the same or higher dose of neostigmine never produced the reversal effect.

In order to study the relationship between the time course of the spinal effects and of the effect on antidromic responses of nereistoxin, the spinal reflex potentials, spontaneous ventral discharges and antidromic responses were recorded simultaneously in the same spinal preparations. The intravenous injection of 10 mg/kg of nereistoxin produced facilitation of both antidromic responses for about 15 minutes at the early stage. Following this early phase, the depression of both antidromic responses with moderate decrease of the muscle potential and the depression of the spinal reflex potentials appeared. The increase in the spontaneous discharge of the ventral root also began to appear.

DISCUSSION

In the present experiments, nereistoxin as well as its derivatives produced tremor and twitching in the limbs and body trunk of the spinal preparations. These components of convulsion, tremor and twitching were associated only with increase in the spontaneous repetitive discharges of the ventral root. Pentobarbital at the dose sufficient to depress the spinal activity abolished both the discharges and the EMG activated by nereistoxin. Whereas gallamine inhibited only tremor and twitching without any change of the ventral discharges. On the other hand, nereistoxin facilitated the antidromic discharges of the motor nerve in response to efferent stimulation which originate in the first node and the nerve terminal (6-10). A similar but more remarkable facilitation was also obtained by a depolarizing agent, succinylcholine. Kato and Fujimori (11), and Standaert and Adams (12) reported that succinylcholine elicited twitching in association with the increased repetitive antidromic discharges, and suggested that the twitching is attributable to the effect of succinylcholine on the motor nerve terminals or muscle fibers. The tremor and twitching by succinylcholine, however, did not synchronized with the increase in
the antidromic motor nerve impulses from the ventral filaments. Thus, this effect of nereistoxin does not seem to be the contributing factor to produce tremor and twitching.

In contrast to the increase in the spontaneous discharges of the ventral root, the reflex potentials at the ventral as well as dorsal roots were depressed or blocked. The action potentials at the dorsal root in response to afferent stimulation, under the condition of the depression of reflex potentials, were never impaired. Furthermore, the inhibitory effects of nereistoxin on the reflex potentials were not modified by the acute sectioning of both ventral and dorsal roots. These results indicate the presence of the site of inhibitory effect within the spinal cord.

The monosynaptic reflex potentials are depressed 1) by increase in presynaptic inhibition, 2) by increase in postsynaptic inhibition and 3) by functional depression of spinal motoneuron (13). Pretreatment of the animals with either picrotoxin known to block presynaptic inhibition (14, 15) or strychnine known to block postsynaptic inhibition (16–20) was found ineffective in reversing the blocking action of nereistoxin on the monosynaptic reflex potentials. The pre- or postsynaptic inhibition, therefore, could be excluded from the mechanism for the effect of nereistoxin on the spinal cord. The depolarization of the spinal motoneuron was likely to be the action mechanism, since the mode of action of nereistoxin in the antidromic discharges of the motor nerve was similar to that of succinylcholine. However, in contrast to the latter agent, nereistoxin increased the spontaneous discharges of the ventral root which reflected the activity of spinal motoneuron. Thus, it appears unlikely that the spinal motoneuron is functionally depressed by nereistoxin. No information on the inhibitory mechanism of the monosynaptic reflex potentials was available in the present experiments.

The polysynaptic reflex potentials are affected by interneuronal activity in addition to the above-mentioned three factors for the monosynaptic reflex mechanism. The exclusion of the spinal cord segments by transection significantly restored the depression of the polysynaptic reflex potentials following administration of nereistoxin but not that of the monosynaptic reflex potentials. This finding suggests that some unknown interneurons of the widespread, longitudinal neuronal chain in a number of cord segments, which play similar role to that of D cell in presynaptic inhibition (21), relate with the inhibitory mechanism of nereistoxin on the polysynaptic reflex potentials.

As pointed out above, nereistoxin showed the increase in the spontaneous repetitive discharges at the ventral root in synchronization with tremor and twitching. This increase was not affected by spinally acting drugs such as picrotoxin (14, 15), strychnine (16–20), mephenesin (22–24), nitrazepam (25, 26) and physostigmine (27–29), or a cholinolytic drug, atropine. Pentobarbital at the dose sufficient to depress spinal activity exhibited nonspecific antagonism against the increased ventral discharges induced by nereistoxin. On the other hand, the reduction of the connected spinal cord segments by transection of spinal cord resulted in the significant recovery from the increased discharges, indicating again that some unknown interneurons within spinal cord play an important role in this ventral discharges. Therefore, it is assumed that nereistoxin activates at least two
kinds of interneurons which are excitatory to initiation of ventral discharges and inhibitory to polysynaptic reflex potentials. This assumption is also supported by the fact that the depression of polysynaptic reflex potentials continued consistently during the relatively periodic appearance of the ventral discharges and even after the ventral discharges almost disappeared.

**SUMMARY**

Nereistoxin and its derivatives produced a marked inhibition or a block of the spinal mono- and polysynaptic reflex potentials and the dorsal root reflex potentials, and increased the spontaneous discharges of the ventral root in association with the manifestation of tremor and twitching of the skeletal muscles in the spinal cat with Cl transection. These effects of nereistoxin were also demonstrated in the anesthetized or gallamine-immobilized intact cat. The acute section of the ventral and dorsal roots in the spinal cat did not modify the effects. On the other hand, the reduction of the number of connected cord segments by transection of the spinal cord at L1 and S2 levels resulted in the significant recovery from the increased spontaneous discharges as well as the depressed polysynaptic reflex potentials produced by nereistoxin. In addition, the increase in the ventral discharges was almost completely prevented by the treatment of pentobarbital.

The antidromic discharges in the motor nerve evoked by the efferent stimulation were activated and then depressed after the intravenous injection of nereistoxin. Synchronized with the depressing effect, the activity of the EMG was also moderately depressed.

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