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Differential Sensitivity of Tetrodotoxin of Nociceptive Neurons in 4 Species of Leeches

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We have recently shown that the nociceptive neurons (N cells) in the leech can be segregated into a medial type (N_{med}) and a lateral type (N_{lat}) according to their physiological and pharmacological properties. We now report that the Na-dependent action potential (AP) of the 2 cell types in *Macrobdella* have differential sensitivities to tetrodotoxin and that the APs of the N-cell homologs from different leech species also vary considerably in their response to TTX.

The normal AP of the N cells in the 4 leech species was exclusively Na-dependent; its overshoot varied logarithmically with $[Na]_o$, was unaffected by the presence of Mn and absence of Ca, and could not be elicited in Na-free Ringer's solutions. The decrease in the maximal rate of depolarization ($\dot{V}_{max}$) of the Na-dependent AP of the N_{med} cell in *Macrobdella* produced by TTX followed a reverse Langmuir curve for bimolecular reaction with an ED_{50} of 9 μM. Almost complete blockage was obtained at 50 μM. In contrast, TTX inhibition of $\dot{V}_{max}$ in the N_{lat} cell reached saturation at a level of only 55% reduction in $\dot{V}_{max}$ even at doses in excess of 200 μM TTX. The simplest interpretation of these data is that there are 2 types of Na channels in the N_{lat} cell. This is further supported by the fact that the dose-response data could be well fitted by a Langmuir curve, assuming that the N_{lat} cell possesses 2 populations of Na conductances—one insensitive to TTX and one (like the N_{med} cell) with an ED_{50} to TTX of 9 μM, the 2 populations being present in a 0.45:0.55 ratio, respectively. Similar results were obtained for the 2 pairs of N cells in *Haemopis*. In the N cells of *Hirudo*, 50 μM TTX reduced $\dot{V}_{max}$ by only 25%, with no difference between N_{med} and N_{lat}. In the phylogenetically less advanced glossiphoniid leech *Haementeria*, which possesses only 1 N-cell homolog, the AP was completely blocked by 1 μM TTX, with an approximate ED_{50} of 15 nM.

These results provide evidence that the N_{lat} cell of *Macrobdella* has 2 types of Na channels that can be segregated by their different sensitivities to TTX. The N_{med} cell possesses only 1 type with an ED_{50} in the low-micromolar range. In contrast, the N cell of *Haementeria* is 1000 times more sensitive to the drug with an ED_{50} in the low-nanomolar range, as is the case for most mammalian Na channels. This diversity in sensitivity to TTX may reflect structural and functional differences in the Na channels of leech N cells at the molecular level both between species and within a single cell type. These results may provide a framework with which to gain insight into the functional diversity and evolution of ion channels among phylogenetically closely related neurons.

The specific affinity of Na channels for tetrodotoxin has made this toxin an important tool for the study of the structure and properties of the Na channel (Catterall, 1980; Ritchie and Rogart, 1977). Previously, we have shown that the Na-dependent action potential of various functionally identified leech neurons respond differentially to TTX and procaine in a way specific for each cell type within a species (Johansen et al., 1984b; Kleinhaus and Prichard, 1983; Yang et al., 1984). In this study we have used TTX as a probe to investigate the phylogenetic variation in sensitivity to TTX among Na channels within a well-defined population of homologous nociceptive sensory neurons (Nicholls and Baylor, 1968) from 4 species of leeches.

Typically, in hirudinid leeches, each segmental ganglion of the CNS possesses 4 N cells that, apart from sharing a sensory modality, are characterized by their similar morphology (Johansen et al., 1984a, b; Muller and McMahan, 1976) and action potential waveforms (Keyser and Lent, 1977; Nicholls and Baylor, 1968). However, the 4 cells are not identical, and can be classified into a medial (N_{med}) and a lateral (N_{lat}) type according to several of their physiological and pharmacological properties (Johansen et al., 1984a; Sargent et al., 1977). The 2 populations differ from one another within a particular leech species in their receptive fields (Blackshaw et al., 1982; Johansen et al., 1984b), their synaptic connections (Johansen and Kleinhaus, 1985a), their pharmacological responses (Johansen et al., 1984b, 1986a; Sargent et al., 1977), and the presence of antigenically distinct molecules that can be recognized by monoclonal antibodies (Johansen et al., 1984a; McKay et al., 1983).

This study represents an extension of these observations, comparing TTX sensitivity of lateral and medial N cells from 4 species of leeches. In addition to the 3 common hirudinid species, *Hirudo, Haemopis,* and *Macrobdella,* are included the phylogenetically less advanced glossiphoniid leech *Haementeria,* which possesses only 1 pair of N cells (Kramer and Goldman, 1981). The results provide evidence that the Na channels of the different N-cell homologs, based on their TTX sensitivity, are surprisingly heterogeneous and can be segregated into at least 3 different types: 1 insensitive to TTX, 1 with an ED_{50} in the micromolar range, and one with an ED_{50} in the nanomolar range. Furthermore, we show that 2 types of TTX-sensitive Na channels are present in roughly equal proportions in the lateral N cell of *Macrobdella,* whereas the medial N cell possesses only 1 type.

Materials and Methods

Leeches of the species *Macrobdella decora, Hirudo medicinalis,* and *Haemopis marmorata* were obtained from commercial suppliers and kept in springwater at 15°C. Specimens of the glossiphoniid leech *Haementeria ghilianii* were the generous gift of Drs. K. J. Muller, E. McGlade-

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McCulloh, and Dr. E. M. Burreson and were maintained at room temperature (22–24°C).

The current clamp techniques for intracellular recording from leech neurons have been described in detail elsewhere (Kleinhaus, 1976, 1980; Nicholls and Baylor, 1968). Electrodes were filled with 4 M potassium acetate and had resistances of 15–20 MΩ. Segmental ganglia were dissected and pinned out in normal Ringer's solution (110 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Tris-HCl, pH 7.4) and the nociceptive neurons in the different leech species identified by their size, position, and electrical parameters, as described by Nicholls and Baylor (1968), Keyser and Lent (1977), and Kramer and Goldman (1981). To facilitate electrode penetration and to remove possible diffusion barriers to the toxins, the connective capsules overlaying the ganglia were completely removed with specially sharpened forceps. This procedure leaves the membrane of the neuronal somata in the ganglia directly exposed to the bath solution with only a few remnants of glia attached.

TTX (Sanyo) was dissolved in Ringer's solution from the powder. Saxitoxin (STX) was the generous gift of Dr. C. Y. Kao and experimental Ringer's solutions were made up from an aqueous 2 mM STX stock solution. In some experiments, Ca was substituted for by either Mn or Mg and outward K currents were blocked by adding 25 mM tetraethylammonium chloride (TEA) and 3 mM of 4-aminopyridine (4-AP) to the Ringer's solution (Johansen and Kleinhaus, 1986b).

Permanent recordings were made with a PDP-8 computer system that digitized and stored the traces at 23 kHz for later reproduction on an incremental plotter. The maximal rates of depolarization ($\dot{V}_{max}$) of the Na-dependent action potential of the N cells were analyzed by a computer subroutine written for this purpose (Kleinhaus and Prichard, 1979; Yang et al., 1984). The ED_{50} for TTX inhibition of $\dot{V}_{max}$ of the Na-dependent action potentials was estimated from the data points. From this value the theoretical Langmuir curve for bimolecular reaction (Cuervo and Adelman, 1970; Kao and Walker, 1982) was computed using the equation:

$$\frac{\dot{V}'_{max}}{\dot{V}_{max}} = \frac{1}{1 + ([TTX]/ED_{50})}$$

where $\dot{V}'_{max}$ is the maximal rate of depolarization in the presence of toxin.

Experimental procedure

To determine the inhibition by TTX of the Na-dependent action potential, each ganglion was first equilibrated in the control Ringer's solution for 5 min. Then the N cells in the ganglia were identified and the average $\dot{V}_{max}$ of 3–5 elicited action potentials was determined for each individual cell. The recording electrode was withdrawn and the cells incubated in the experimental TTX containing Ringer's solution. After 5–10 min, the cells were reimpaled and the average $\dot{V}'_{max}$ of 3–5 action potentials in the presence of the toxin was again determined. The ratio, $\dot{V}'_{max}/\dot{V}_{max}$, for each cell was then used as a measure of the level of TTX inhibition. After each experiment, the toxin was washed out by perfusion with normal Ringer's solution, and it was ascertained that at least 80% recovery of $\dot{V}_{max}$ of the AP could be obtained, to exclude data from damaged cells. With this procedure, data from medial and lateral N cells from the same ganglia were collected simultaneously under identical conditions.

Results

TTX sensitivity of the Na-dependent action potential of lateral and medial N cells in Macrobdella

We have previously reported that $\dot{V}_{max}$ of the action potential of the sensory neurons and Retzius cells in hirudinid leeches is approximately linearly dependent on the extracellular Na concentration (Kleinhaus and Prichard, 1976, 1983). Furthermore, both the medial and the lateral N cells of *Macrobdella* are unexcitable in Na-free Ringer's solutions, the overshoot of their action potentials (AP) varies logarithmically with $[Na]_0$ (Fig. 1), and the AP persists unattenuated when Mn is substituted for Ca. The normally occurring action potential of the nociceptive neurons is therefore almost exclusively Na-dependent, although a small Ca flux through Ca channels (Johansen and Kleinhaus, 1985b) may also be making a minor contribution to $\dot{V}_{max}$ (less than 2%). To eliminate contamination from this Ca current, most of the experiments on the effect of TTX on the action potential were done in Ringer's solutions in which Mn or Mg was substituted for Ca. The results from such experiments, from experiments performed in normal Ringer's solutions, and from experiments in which the outward current was additionally blocked with K-channel blockers, were indistinguishable from one another within the resolution of the experimental methods. However, it should be pointed out that even when the Na current dominates the total membrane current, $\dot{V}_{max}$ of the AP is not necessarily proportional to the Na conductance (Cohen et al., 1981). The ED_{50} data for toxin block in the present study are therefore to be considered only as estimates for comparative purposes, since they are derived from an indirect measurement of the Na conductance. On the basis of studies in which a comparison has been made, the deviation from the actual K_i is likely to be on the order of 2–5-fold (Pappone, 1980; Ritchie and Rogart, 1977), which is orders of magnitude less than the differences in toxin sensitivity reported here.

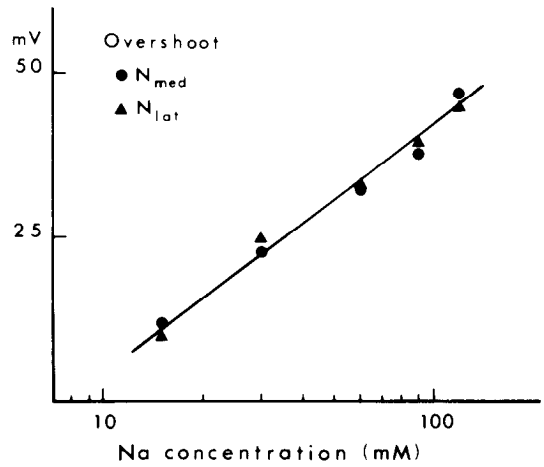


Figure 1. Na dependence of the action potential overshoot from lateral (N_{lat}) and medial (N_{med}) N cells of *Macrobdella*. The overshoot of both cell types varied logarithmically with $[Na]_0$. Since the determination was carried out in normal Ringer's solution, the slope deviated from the slope of 58 mV/decade expected for a purely Na-dependent event. Each *data point* represents the average of 3 determinations.

Figure 2 shows the dose-dependent reduction of $\dot{V}_{max}$ of the Na-dependent AP produced by TTX in medial and lateral N cells in *Macrobdella*. The data points for the medial N cell could be well fitted by a Langmuir curve for bimolecular reaction with an ED_{50} of 9 μM, which indicates a single population of binding sites reacting 1-to-1 with TTX. However, the TTX dose–response curve for the lateral N cell was strikingly different from that of the medial N cell. The TTX inhibition of $\dot{V}_{max}$ in this cell reached saturation at a level of only 55% reduction in $\dot{V}_{max}$ even when doses in excess of 200 μM TTX were applied. The simplest interpretation of this data is that there are 2 types of Na channels, with a different sensitivity to TTX in the N_{lat} cell. This is further supported by the fact that the dose–response data could be approximated by a Langmuir curve, assuming that the N_{lat} cell possessed 2 populations of Na conductances: 1 insensitive to TTX and one (like in the N_{med} cell) with an ED_{50} to TTX of 9 μM, the 2 populations being present in a 0.45:0.55 ratio, respectively.

Since the data from N_{lat} and N_{med} cells were recorded under identical conditions, after complete removal of the glial sheet, it is unlikely that the difference in their sensitivities could be accounted for by different diffusion barriers. In addition, the

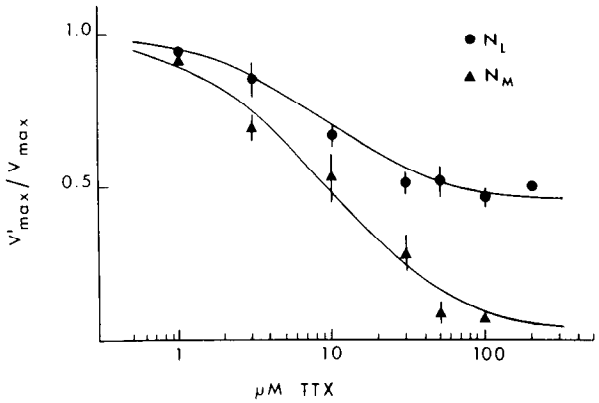


Figure 2. Dose-response curves for TTX inhibition of the Na-dependent action potential of lateral (N_L) and medial (N_M) N cells in *Macrobdella.* $\dot{V}'_{max}$ is $\dot{V}_{max}$ in the presence of drug. *Data points with error bars* represent the average of 10–15 determinations with SEM. *Data points without error bars* are the mean of 3–5 determinations. The *Langmuir curve for* N_M was computed as described in Materials and Methods using an ED_{50} of 9 μM. The *Langmuir curve for* N_L was computed from: $\dot{V}'_{max}/\dot{V}_{max} = 0.55/1 + ([TTX]/ED_{50}) + 0.45$. It was assumed that the lateral N cell possessed 2 populations of Na conductances: 1 insensitive to TTX and 1 with an ED_{50} of 9 μM, the 2 populations being present in a 0.45:0.55 ratio, respectively.

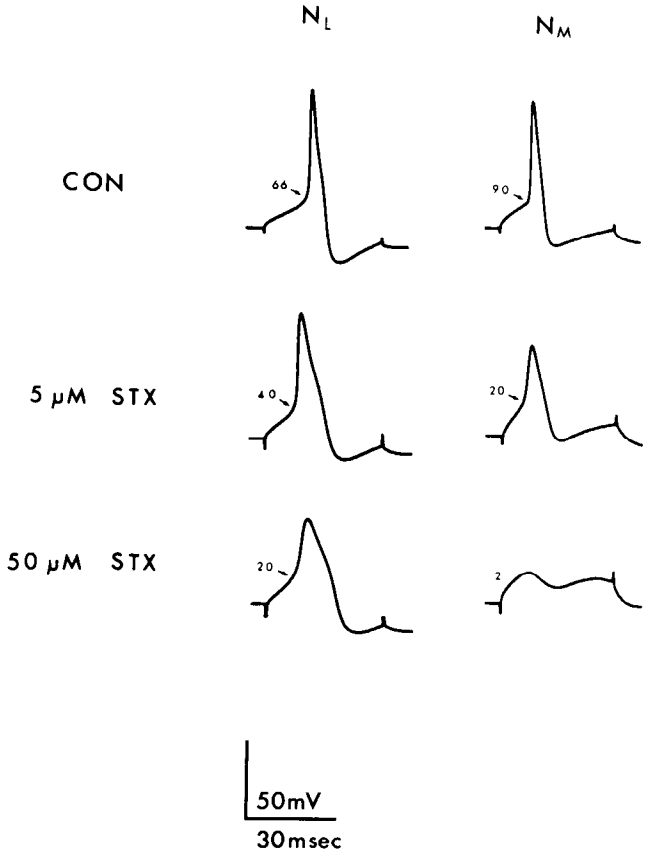


Figure 3. Dose-dependent inhibition by STX of action potentials in N cells from *Macrobdella.* The figure shows the action potentials of a lateral (N_L) and a medial (N_M) N cell before (*CON*) and after incubation for 10 min in 5 and 50 μM *STX. Numbers* indicate $\dot{V}_{max}$ of the action potential in V/sec. After washout of the toxin, the action potentials recovered completely (not shown).

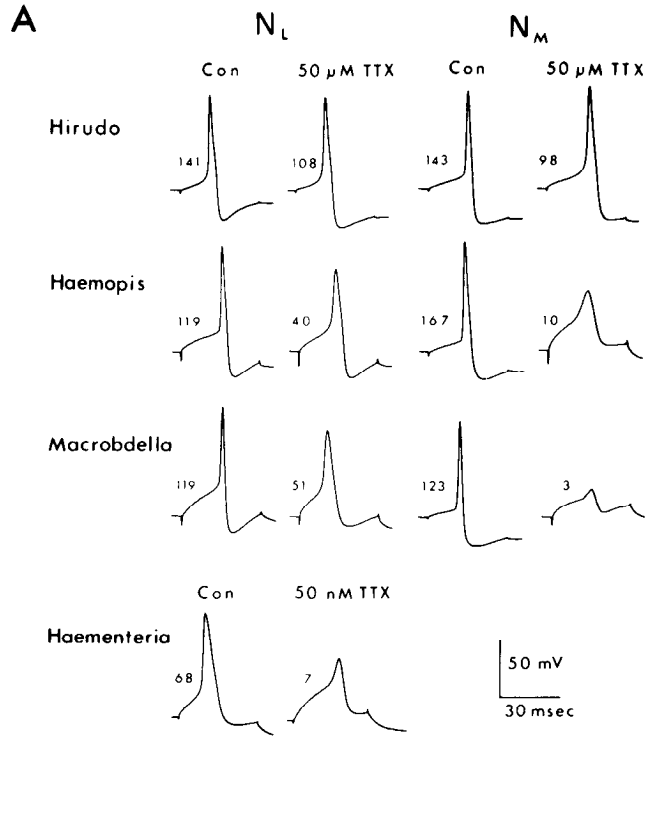
toxin STX, which is thought to compete for the same binding site as TTX on the Na channel (Colquhoun et al., 1972; Ritchie and Rogart, 1977) even though structurally different (Kao and Walker, 1982), also inhibited $\dot{V}_{max}$ of the Na-dependent AP of the 2 cell types in a way similar to that of TTX (Fig. 3). These results, therefore, strongly suggest that the N_{lat} cell in *Macrobdella* possesses 2 types of Na channels, which can be segregated by their different sensitivities to TTX, whereas the N_{med} cell has only 1 type, with an ED_{50} in the low-micromolar range. Questions concerning the relative distribution of the 2 types of Na channels in the N_{lat} cell were not investigated further—for example, whether 1 Na channel type could be restricted to the soma, whereas the other type would be specifically located in the axon and neuronal processes.

We have previously shown that there are segmental differences in the distribution of lateral and medial N cells based on their antigenicity (Johansen et al., 1984a) and their response to procaine (Johansen et al., 1984b). In the sex ganglia (ganglia 5 and 6), the 1 pair of N cells found per ganglion is of the medial type, whereas the single pair of N cells present in each of the terminal ganglia 20 and 21 is of the lateral type. Application of TTX to the N cells from these ganglia showed that their response was consistent both with the previous observations and those of the present study, and confirmed the N-cell distribution pattern deduced from our earlier work.

TTX sensitivity of the Na-dependent action potential of lateral and medial N cells from 4 species of leeches

The difference we found between Na channels of N cells within 1 leech species induced us to conduct a comparative study of TTX sensitivities of N cells from different leech species. In addition to *Macrobdella,* we examined 2 other hirudinid species, *Hirudo* and *Haemopis,* from the order Gnathobdellidae—leeches with jaws. From the phylogenetically less specialized order Rhynchobdellidae, comprised of leeches characterized by the presence of a proboscis, we investigated the glossiphoniid species *Haementeria.* Although the organization and many elements of the CNS of *Haementeria* are similar to those of the hirudinid species (Kramer, 1981; Kramer and Goldman, 1981) the CNS of *Haementeria* differs from these in several respects; for example, there are fewer neurons per ganglion (Macagno, 1980) and each ganglion has only 1 pair of N-cell homologs (Kramer and Goldman, 1981). This pair of N cells probably corresponds most closely to the lateral N cell type of hirudinid leeches, since a pair of N-cell homologs could be found in ganglion 20 but not in the sex ganglia in spite of an extensive search. However, we cannot rigorously rule out the presence of N cells in the sex ganglia of *Haementeria,* since we were unable to examine all the cells in the anterolateral package of any one ganglion because of the small size of the cells.

The $\dot{V}_{max}$ of the action potential of the N-cell homologs from all the leech species examined was, as in the case of *Macrobdella,* almost exclusively Na-dependent (data not shown). Figure 4*A* presents representative records of the TTX inhibition of the action potential of the N-cell homologs in normal Ringer's solution in the 4 leech species. The action potential is shown before and after application of TTX. The number beside each trace indicates the value of $\dot{V}_{max}$ in *V*/sec. In all cases the effect of TTX was completely reversible, and full recovery of the height and $\dot{V}_{max}$ of the AP were obtained. Figure 4*B* gives a comparison of the effects of 50 μM TTX in the hirudinid species and 100 nM TTX in the case of *Haementeria* on $\dot{V}_{max}$ of lateral and medial N cells. The data represent the mean of 15–20 determinations on each cell type with SEM. In *Hirudo,* 50 μM TTX decreased $\dot{V}_{max}$ only by about 20–25%, and there was no statistically significant difference between the responses of the lateral and medial N cells. The decrease of $\dot{V}_{max}$ of 20–25% was the maximal inhibition that could be obtained even when 100–200 μM TTX



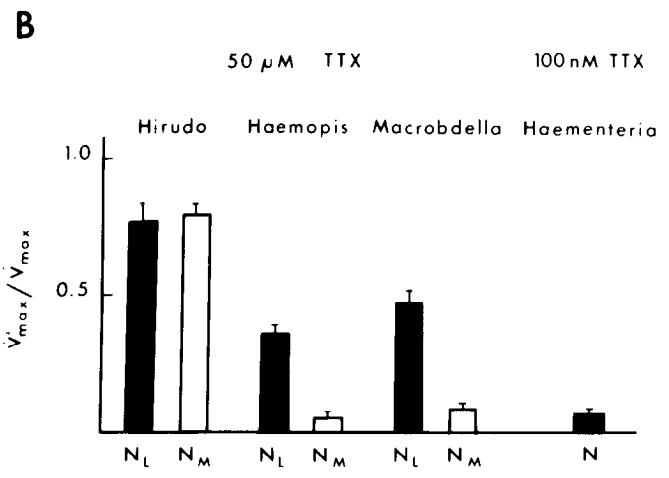


Figure 4. Comparison of the effect of TTX on the Na-dependent action potential of medial (N_M) and lateral (N_L) N cells from 4 species of leeches. *A*, Action potential of N_M and N_L before (*Con*) and after incubation in 50 μM TTX for the hirudinid leeches and in 50 nM TTX in the case of *Haementeria*. *Numbers* besides each trace indicate $\dot{V}_{max}$ of the action potential in V/sec. In all cases, full recovery of the action potential was obtained after washout of the toxin (not shown). *B*, Bar graph comparing the inhibition by 50 μM TTX of $\dot{V}_{max}$ of N_M and N_L from the 3 hirudinid species. Data from each cell type represent the mean with SEM of 10–15 determinations. In the case of the *Haementeria* N-cell homolog, only 100 nM TTX was applied.

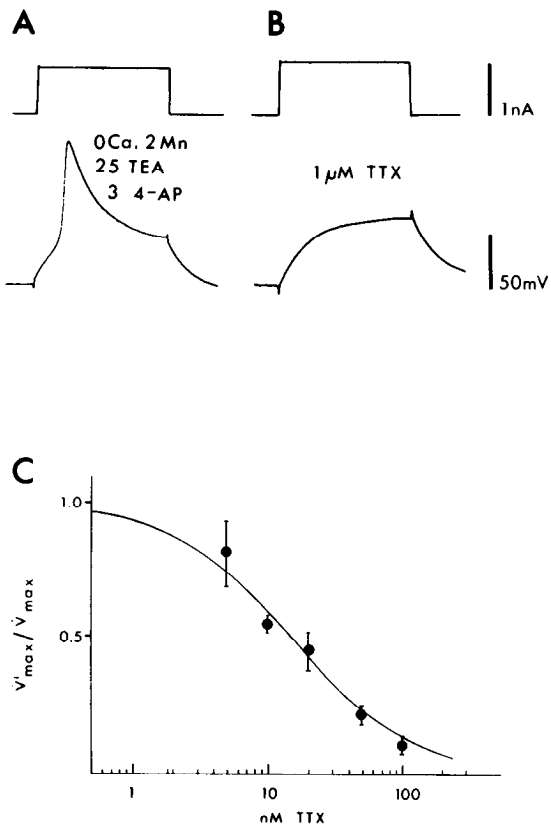


Figure 5. Dose-dependent inhibition by TTX of the action potential in the N-cell homolog of *Haementeria*. *A* and *B*, In the *lower traces*, the action potential (*A*) was completely blocked after the application of 1 μM TTX (*B*). In this experiment, outward current was blocked by 25 mM TEA and 3 mM 4-AP, and the Ca current was eliminated by substitution of Mn for Ca. *Upper traces* show the applied current of 40 msec duration. *C*, Langmuir dose–response *curve* of TTX inhibition of $\dot{V}_{max}$ of the action potential, calculated as described in Materials and Methods, using an ED_{50} value of 15 nM. *Data points* represent the mean of 5–8 determinations with SEM.

was applied. In contrast, 50 μM TTX reduced $\dot{V}_{max}$ of the medial N cells in *Haemopis* and *Macrobdella* by 90–95%, whereas $\dot{V}_{max}$ of the lateral N cells from the 2 species were reduced by only 50–65%. We did not investigate the dose–response relationship of the N_{lat} cell in *Haemopis* in any further detail, but, as was the case for the N_{lat} cell of *Macrobdella* (Fig. 2), the inhibition of $\dot{V}_{max}$ reached a plateau, which suggests the coexistence of a TTX-sensitive and a TTX-insensitive population of Na channels in this neuronal cell type. In *Haementeria*, 90–95% inhibition of $\dot{V}_{max}$ was obtained by 100 nM TTX, which indicates that the Na channels of the N cells in this species are 1000-fold more sensitive to TTX than the Na channels in the N_{med} cells of *Macrobdella* and *Haemopis*. Complete blockage of the Na-dependent action potential could be obtained with 1 μM TTX, as shown in Figure 5, *A* and *B*. In this case, all outward current was blocked by TEA and 4-AP and the Ca current was eliminated by the substitution of Mn for Ca. The reduction in $\dot{V}_{max}$ with increased TTX concentration followed a Langmuir curve for bimolecular reaction with an ED_{50} of 15 nM (Fig. 5*C*). Since this value of ED_{50} is derived from an indirect measurement of Na conductance, the actual K_i value is likely to be somewhat lower as discussed by Ritchie and Rogart (1977) and Pappone (1980). This makes the TTX sensitivity of the Na channel in the N cell of *Haementeria* comparable to the TTX sensitivity of most mammalian Na channels (Hille, 1984; Stallcup, 1977).

The results show that there can be a surprising heterogeneity in the TTX sensitivity of Na channels within a well-defined group of homologous neurons, even from closely related species. In N cells from the 4 leech species investigated in the present study, at least 3 types of Na channels seem to be distinguishable on the basis of their differential TTX sensitivities—1 type in-

sensitive to TTX, 1 type with an ED_{50} in the micromolar range, and 1 type with an ED_{50} in the nanomolar range. Furthermore, in some of the N cells 2 types of TTX-sensitive Na channels are present in different proportions within the same cell. In the N_{lat} cell of *Macrobdella* and *Haemopis,* the ratio of TTX-sensitive to insensitive Na channels is roughly 1:1, whereas in *Hirudo* N_{med} and N_{lat} cells, the ratio is approximately 1:3. Only 1 type of TTX-sensitive Na channel appears to be present in *Haementeria* N-cell homologs.

Discussion

The Na channels in nerve and muscle cells are functionally similar and are thought to be highly conserved structurally across wide phylogenetic boundaries (Hagiwara, 1983; Hille, 1984). Most Na channels are characterized by being sensitive to TTX in nanomolar concentrations (Hille, 1984; Kao, 1966); however, variation in TTX sensitivity exists. Several instances of TTX-insensitive Na conductances have been reported (Fukuda and Kameyama, 1980; Hagiwara and Takahashi, 1967; Kleinhaus and Prichard, 1976), and in many invertebrate preparations, Na channels are sensitive only to micromolar concentrations of the toxin (Keenan and Koopowitz, 1981; Kleinhaus and Prichard, 1983; Lee et al., 1977).

In this study, we have investigated variations in TTX sensitivity among Na-dependent action potentials within a well-defined population of nociceptive sensory neurons from 4 species of leeches. So many properties are shared by the neurons (sensory modality, morphology, position in the ganglia, action potential waveform, ion dependence of the AP) that it seems reasonable to assume that they are true homologs, derived from a common ancestor. However, in spite of their many common traits, the 4 N cells of the hirudinid species can be segregated into 2 pairs—medial and lateral—that differ antigenically and pharmacologically and that are differently distributed in the CNS (Johansen et al., 1984a, b). It is not known whether these differences are correlated with any functional specialization of the 2 types of N cells. Interestingly, the less advanced glossiphoniid leech *Haementeria* has only 1 pair of N cells. This pair probably corresponds most closely to the lateral N cell type of the hirudinid species, given its distribution in the CNS, which is comparable to that of the lateral N cell in *Macrobdella.* Thus it is feasible, although other possibilities exist, that, in the hirudinid leeches, medial-type N cells with additional specialized properties have evolved from the original single pair of lateral N cells by duplication (Johansen et al., 1984a). Instances of supernumerary sensory neurons have been reported in the leech (Kuffler and Muller, 1974), and some lineage mutations in the nematode are known to lead to reiterations of particular neurons (Chalfie et al., 1981). Another piece of evidence supporting this scenario is that medial and lateral N cells have completely overlapping sensory fields in the skin, both of which cover a whole hemisegment (Blackshaw et al., 1982). By contrast, each of the three touch (T) and the two pressure (P) sensory neurons have discrete, nonoverlapping sensory fields (Nicholls and Baylor, 1968), and no other distinguishing properties of these cells within each specific sensory modality have yet been reported.

Our data show a surprising diversity in the TTX sensitivity of the Na-dependent action potential among the different leech species in this group of phylogenetically closely related nociceptive cells. Three types of Na channels can be distinguished by their sensitivity to TTX. One type, insensitive to TTX, exists in varying proportions in lateral N cells from *Haemopis* and *Macrobdella* and in both N_{lat} and N_{med} cells from *Hirudo.* In addition, it is noteworthy that the Na-dependent action potentials of the Retzius cells from these species also are completely resistant to TTX (Kleinhaus and Prichard, 1976; J. Johansen and A. L. Kleinhaus, unpublished observations). A second type of Na channel sensitive to TTX, with an ED_{50} of approximately 9 μM, is present in medial N cells from *Macrobdella* and *Haemopis,* and it contributes a little more than half of the $\dot{V}_{max}$ of the lateral N cells from these 2 species. This degree of sensitivity to TTX also seems to be shared by the Na channels from the other sensory neurons (P and T cells) in *Macrobdella* (Kleinhaus and Prichard, 1983). A third type of Na channel, about 1000-fold more sensitive to TTX, with an ED_{50} of 15 nM, is present in the single N cell type of *Haementeria.* This difference in sensitivity to TTX among the nociceptive leech neurons is not likely to be the result of diffusion barriers or variable access of TTX to the Na-channel receptors. The soma membrane of the neurons was directly exposed to the toxins by completely removing the surrounding glial sheet, and the TTX binding site of the Na channel is located on the outer membrane surface (Hille, 1984).

The reason for the diversity in sensitivity to TTX among leech neurons and the explanation of its possible functional or biological relevance are not clear. Twarog et al. (1972) proposed, after investigating the TTX and STX sensitivity of 22 species of bivalve molluscs, that tolerance to high levels of TTX and STX in some of the species had evolved as a protection against ingestion of STX-producing dinoflagellates, which periodically are very abundant in these species' natural habitat. There is clearly also good reason for the nervous system of the TTX-synthesizing species of fish and amphibians to be resistant to TTX (Kao, 1966). However, resistance or low sensitivity to TTX does not seem to be of major adaptive value for leeches, although *Hirudo* and *Macrobdella* are bloodsucking leeches that occasionally prey on amphibians (Sawyer, 1981), among which a few species contain TTX. The mud leech *Haemopis* is carnivorous and preys on small invertebrates, whereas *Haementeria* feeds strictly on the blood of mammals and reptiles (Sawyer, 1981). These 2 species, representing low and high levels of TTX sensitivity, respectively, are therefore not exposed to TTX or STX in their natural habitat. Consequently, it seems unlikely that adaptive pressure from exposure to TTX- or STX-containing prey has played a role in determining the structure of the TTX-sensitive site on the leech Na channel. Furthermore, a general hypothesis of high TTX tolerance as an adaptation to toxin exposure cannot explain the wide differences in TTX sensitivity of mammalian Na channels reported from studies of various tissues and neurons in culture (Fukuda and Kameyama, 1980; Stallcup, 1977; Yoshida et al., 1978), the presence of 2 kinds of TTX-sensitive Na channels within a single cell, as is found in some of the nociceptive neurons (this study) and in squid giant axon (Sevcik, 1976), and the occurence of TTX-resistant Na channels as a consequence of denervation of mammalian muscle fibers (Harris and Thesleff, 1971; Pappone, 1980). Rather, the TTX and STX binding site is part of a structural feature of the Na channel against which animals and organisms at diverse stages of evolution (i.e., puffer fish, amphibians, octopuses, gobys, dinoflagellates, and cyanobacteria) have independently produced a toxin (Hille, 1984). This site on the Na channel is obviously of structural or functional importance, since it has been highly conserved through evolution; the majority of previously described Na channels are sensitive to TTX and STX (Hille, 1984), and TTX sensitivity of Na channels is known of phylogenetically as early as in the phylum platyhelminthes (Keenan and Koopowitz, 1981). Changes in the structure of the TTX binding site, as revealed by altered affinity to TTX (in the absence of toxin exposure), may therefore reflect important modifications of channel properties and function, the nature of which have yet to be identified. However, it should be noted that changes in TTX affinity of Na channels do not necessarily reflect major structural changes in the channel molecule. Methylation of 1 or more external carboxyl groups by trimethyloxonium ions suffices to make Na channels of frog nerve and muscle highly resistant to both TTX and STX (Spalding, 1980),

and light treatment with trypsin of some molluscan neurons also increases resistance to TTX without affecting other properties of the Na conductance (Lee et al., 1977).

Thus, the differences in sensitivity to TTX among the nociceptive leech neurons reported in the present study strongly suggest that there are structural differences in the Na channels of these neurons, both between species and within a single cell type. The reason for this variation in sensitivity to TTX is not clear. However, evolutionary evidence indicates that the TTX binding site of the Na channel consists of a highly conserved amino acid sequence which may be structurally and functionally important. Thus, changes in this sequence, as revealed by its altered affinity for TTX, may reflect functional changes in the Na channel. A more detailed analysis of the present findings using voltage- and patch-clamp techniques to investigate the kinetic properties of the different N-cell Na conductances may provide a framework for studying the functional diversity and evolution of ion channels within phylogenetically closely related neurons.

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