but these suffer from ambiguity and awkwardness. I propose that a male with false female traits be called a pseudogynous male and be symbolized ♂♀, and that a female with false male attributes be termed a pseudandric female and be symbolized ♀♂.

The pseudohermaphroditism of *I. obsoleta* appears to be the first demonstrated instance in the molluscs of this type of sexuality, which is known primarily from vertebrates (*19*). The kind of pseudohermaphroditism displayed by *I. obsoleta* is very unusual. The marked differences between populations, the correlation between the degree of pseudandry in adult females and the prevalence of penial organs in immatures, and the maintenance of population character despite apparent genetic exchange, together form a pattern that has not been reported in any other group of pseudohermaphrodites. Furthermore, this pattern suggests that in *I. obsoleta* pseudohermaphroditism is environmentally controlled. This hypothesis is supported by the results of a transfer experiment I conducted between a pseudandric and nonpseudandric population, using 400 *I. obsoleta*. Normal males transferred between the sites showed no change. But every pseudandric female moved to the nonpseudandric location either lost all male traits or kept only a small penial bump, while all their controls at the home site retained large penes. Similarly, some nonpseudandric females moved to the pseudandric location developed male attributes, while all their controls remained free of any male characteristics (*20*).

I also examined other neogastropods to determine how common anomalous sex traits are within the order. I found such traits in almost every species I collected, thereby increasing from 18 to 34 the number of species and subspecies in which they are known; these include members of all four superfamilies (*21*). Also, although they are not as extensive, my field data on *Urosalpinx cinerea, Nucella emarginata, Eupleura caudata, Nassarius vibex, Fasciolaria lilium hunteria, Terebra dislocata*, and others bear a striking resemblance to those on *I. obsoleta*. Populations differ in the prevalence of penial structures in adult females; the occurrence of these traits is related to the proportion of immatures bearing a penial organ; and populations maintain their character even when near different populations of the same species (*20*). These strong parallels indicate that environmentally controlled pseudohermaphroditism may be a major sexual phenomenon in the Neogastropoda and, indeed, may be the basic sexual pattern in the order.

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15. For example, at those sites in Table 1 where females lacked male traits, the total percentage of females found in the samples ranged from 43 percent at Radio Island to 69 percent at Bird Shoals; at those sites where the females had a penial organ, the total percentage of females in the samples ranged from 46 percent at Bogue Sound to 63 percent at Banks Channel. These figures do not suggest that the occurrence of male traits in females is related to a scarcity of males in a population. However, determining the true sex ratio of an *I. obsoleta* population is difficult. These are social animals with complex behavior that varies with the season and depends on the sex of the individual and its state of maturity, so samples taken from the same population within a short time may yield widely different sex ratios. Therefore, the question of whether there is any relation between sex ratio and the prevalence of anomalous male traits in females deserves more investigation.
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21. Prior to this study, anomalous male characters had been reported in females of 18 neogastropod species or subspecies (*1–12*). I have found such traits in the females of 12 other kinds of neogastropods. I have also discovered the ventral pedal gland, a female structure, in males of six neogastropod species and have seen penial organs in immatures of 12 neogastropod species. Anomalous sexual attributes of at least one sort are now known in *Murex florifer dilectus, M. pomum, Urosalpinx cinerea, U. cinerea follyensis, U. perrugata, U. tampaensis, Thais haemastoma floridana, T. haemastoma canaliculata, Nucella lapillus, N. emarginata, N. lima, Ocenebra erinacea, Eupleura caudata, E. caudata etterae, E. sulcidentata, Calotrophon ostrearum, Anachis avara, Mitrella lunata, Pisania tinctus, Melongena corona, Buscycon carica, B. contrarium, Nassarius vibex, N. trivittatus, Ilyanassa obsoleta, Fasciolaria lilium hunteria, Pleuroploca gigantea, Olivella biplicata, Marginella apicina, Conus mediterraneus, Terebra dislocata, Terebra protexta, Kurtziella cerina*, and *Propebela turricula*.
22. This work was done in partial fulfillment of Ph.D. requirements in Zoology at the University of North Carolina at Chapel Hill. I thank the faculty of the UNC Department of Zoology and the UNC Institute of Marine Sciences, Morehead City, N.C., for their assistance with this research, and give special thanks to C. E. Jenner for his guidance and support.

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Substance P: Evidence for Diverse Roles in Neuronal Function from Cultured Mouse Spinal Neurons

Abstract. *Mouse spinal neurons grown in tissue culture were used to examine the membrane mechanisms of action of the peptide substance P. Two functionally distinct actions were observed, one being a rapidly desensitizing excitation, and the other being a dose-dependent, reversible depression of excitatory responses to the putative amino acid neurotransmitter glutamate. These effects on excitability suggest that substance P may play more than one role in intercellular communication in the nervous system.*

The presence of the peptide called substance P (SP) in specific neurons and pathways in the central nervous system (CNS) (*1*) has prompted many investigations into its role in neuronal function (*2*). Discrete pharmacological applications of SP onto central neurons in vivo and in situ alters their excitability (*3–10*), suggesting that the peptide mediates specific synaptic transmissions in the same way as a neurotransmitter (*3, 7–9*). The relatively slow time course of some SP responses (*4, 6, 7, 10*) and the finding that SP depresses responses to other putative neurotransmitters (*11, 12*) have led to the suggestion that the peptide may not function as a neurotransmitter (*4, 6, 7, 10, 11, 13*). We have used dissociated mouse spinal neurons grown in cell cultures as a model system to study the membrane mechanisms of action of SP, and we report here that the peptide clearly has at least two operationally distinct actions on membrane properties, including a rapid excitatory effect.

Spinal neurons were dissociated from 13-day-old mouse embryos and grown in tissue culture for 2 to 3 months according to methods described (*14, 15*). The neurons, growing as a monolayer in a plastic petri dish, were then brought to the modified stage of an inverted phase microscope and viewed at ×250 magnification

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with phase contrast optics. Intracellular recordings were made with conventional techniques by means of micropipettes filled with 3*M* KCl or 4*M* potassium acetate (resistance: 30 to 50 mohm). Substance P (Beckman) was either iontophoresed from a 4 m*M* solution brought to *p*H 4 with HCl or perfused from a 10 μM solution in a micropipette with a 5 to 10 μm tip. Glutamic acid (Sigma) was iontophoresed from a 1*M* solution brought to *p*H 8 with NaOH. We added 10 m*M* $MgCl_2$ to the bathing solution (minimum essential medium containing 10 percent horse serum) to block synaptic activity and allow clearer examination of the membrane events.

Brief (50 msec) iontophoretic applications of SP delivered within several micrometers of the surface of the cell evoked rapidly depolarizing excitatory responses in 31 of 78 cells tested in a dose-dependent, reversible manner (Fig. 1A). A similar excitatory response was observed on 9 of 29 cells tested by pressure application of the peptide. The depolarizing response decayed rapidly, disappearing within 100 msec for a 10-mV response (Fig. 1C). Sustained application of the peptide resulted in a depolarizing response associated with an increase in membrane conductance, both of which desensitized completely within 1 second (Fig. 1B). The excitatory effects of SP were directly compared to those of the putative amino acid neurotransmitter glutamate on 17 cells. Glutamate uniformly excited all cultured spinal cord cells (Fig. 1A). The time-to-peak and the decay of glutamate responses were both slower than those observed with SP-induced depolarizing responses of similar amplitude (Fig. 1C). Similar differences in the time courses of the membrane currents activated by the peptide and amino acid were observed under voltage clamp, indicating that the kinetics of the underlying conductance responses are different (*16*). Closely repeated applications of SP evoked short-lived depolarizations of constant amplitude when delivered at a frequency of 1 Hz. At 2 Hz the depolarizations decreased slightly in amplitude during a train of ten applications (Fig. $1D_1$). At 5 Hz a transient, slight increase in response amplitude and several millivolts of depolarization was rapidly followed by a complete loss of the response which was maintained over a 2-minute period of continued, repeated application (Fig. $1D_2$). A similar desensitization of the excitatory response was observed with pressure-applied peptide. Full recovery after complete desensitization was usually obtained within 5 seconds of termination of the iontophoretic applications (not shown). In contrast, closely repeated applications of glutamate began to summate at a frequency of 2 Hz, and at 5 Hz summated sufficiently to generate spikes (Fig. 1E). The depolarizing response to the amino acid has an inversion potential near 0 mV (*17*), whereas the peptide response has an inversion potential close to the peak of the spike (*16*). Thus the excitatory responses have underlying driving forces that are depolarized relative to resting potential but are not identical. The results show that there are clear differences in the pharmacology of excitatory responses to amino acid and peptide substances.

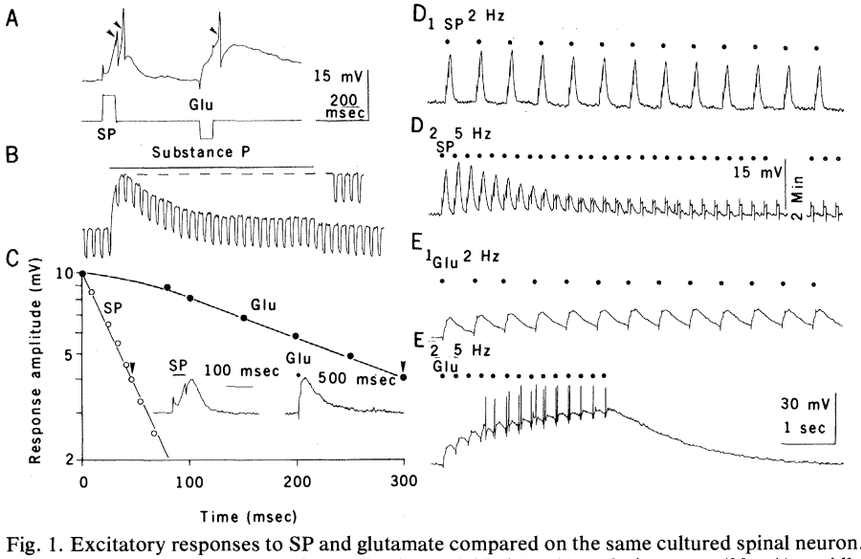

Fig. 1. Excitatory responses to SP and glutamate compared on the same cultured spinal neuron. (A) Brief (50 msec) iontophoretic applications of SP (25 nA) and glutamate (20 nA) rapidly depolarize the cell above threshold for generation of spikes (marked by arrowheads), which are attenuated by the frequency response of the pen-recorder. The iontophoretic current trace is below the membrane potential trace. Resting potential: −52 mV. (B) Sustained application of a 40-nA current delivered through the SP pipette (marked by bar above trace) leads to depolarization and associated increase in membrane conductance which desensitizes completely. Downward deflections are voltage responses to −0.2-nA current stimuli. The inset shows voltage responses at same potential as peak of depolarizing response. The abrupt change in potential at the end of the SP iontophoresis is due to the coupling artifact of several millivolts between the iontophoretic current and the voltage recording. Membrane potential: −60 mV. (C) Voltage decays of equal-sized SP and glutamate responses are plotted semilogarithmically. Downward arrowheads show time constants of decay; that for the glutamate response is about sixfold greater than that for the SP response. (D_1) Depolarizing responses to 50-msec applications of SP (25 nA) diminish slightly when applied at a frequency of 2 Hz. (D_2) At 5 Hz the responses desensitize completely and remain desensitized for 2 minutes. (E_1) Depolarizing responses to 50-msec applications of glutamate (20 nA) summate at the 2-Hz delivery rate and produce spikes at 5 Hz (E_2). Membrane potential (C) to (E): −80 mV.

Another type of peptide action, observed in 9 of 17 cells tested by means of microiontophoresis of SP and in 4 of 11 cells studied by pressure application of SP, consisted of a dose-dependent, reversible depression of glutamate responses by the peptide (Fig. 2). Both the onset of, and recovery from the antagonism were rapid (Fig. 2A). No desensitization of the SP-mediated depression of glutamate responses was detected during a 5-minute application period of the peptide. Prior activation of glutamate-receptor coupled conductance was not necessary since glutamate responses were equally well depressed when a 3-minute interval was interposed between glutamate responses obtained in the absence and presence of the peptide (Fig. 2B). The attenuation of glutamate responses was often seen after activation of the rapidly desensitizing excitatory response evoked by SP (Fig. 2, A and B) when membrane conductance and potential had returned to control values (Fig. 2C). When the peptide was applied slowly in small stepwise increments, the rapidly desensitizing property of the excitatory SP response prevented any detectable change in membrane potential or conductance, but produced a dose-dependent decrease in the amplitude of the glutamate response (Fig. 2D).

The resolution of the dissociated spinal neuron preparation has revealed two distinctly different functional effects of SP on cultured spinal cord neurons: (i) a rapidly depolarizing, rapidly desensitizing excitatory transmitter-like response, and (ii) a nondesensitizing de-

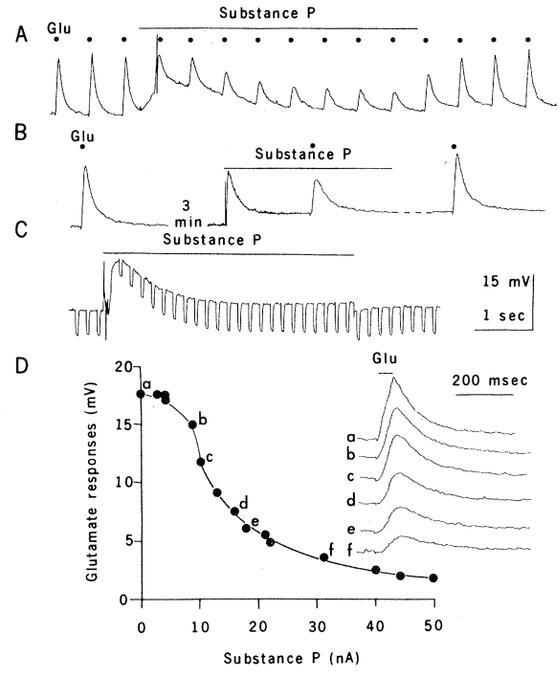

Fig. 2. Modulation of glutamate responses by SP. (A to D) Recordings from two cultured spinal neurons. (A and C) Application of a 30-nA current delivered through SP pipette (marked by bar above trace) leads to a depolarizing response associated with an increase in membrane conductance both of which desensitize completely. Downward deflections in (C) are voltage responses to −0.15-nA stimuli. The abrupt change in potential at the end of the iontophoresis in (C) is a coupling artifact between the iontophoretic current and the voltage recording. Depolarizing responses to 50-msec application pulses of glutamate (20 nA) are rapidly and reversibly attenuated by SP (A). (B) The antagonism of glutamate responses by SP does not necessitate activation of glutamate channels immediately preceding SP application because the peptide depresses glutamate responses as well when a 3-minute waiting period is interposed between a control glutamate response and SP iontophoresis (40 nA). The glutamate response on the right (with the interrupted line serving as an artificial baseline) is shown for visual comparison of control and depressed responses. Apparent residual depolarization during SP iontophoresis is a coupling artifact. The initial part of the trace during SP iontophoresis is at one-fifth of recording speed for the rest of the trace. Membrane potential (mV): (A) −55, (B, C) −65. (D) Responses to 20-nA, 50-msec current pipette delivered through the glutamate are depressed by increasing the SP iontophoretic current. Specimen records are on the right. A slowing of the glutamate response time course is evident at the higher SP currents. Membrane potential: −80 mV.

pression of excitatory responses to glutamate. The first effect falls within the definition of conventional neurotransmitter action in that it involves activation of membrane conductance independent of voltage (*18*). Such a rapid excitatory event induced by SP contrasts with various reports of slow excitatory responses on central (*4–7, 10, 13*) and peripheral neurons (*8*). The rapidly desensitizing nature of this peptide-induced excitation should act to limit the efficacy of synaptic excitation, in contrast to glutamate-mediated excitation which does not desensitize (*19*) and can easily summate (Fig. 1E). The desensitizing property might also make it difficult to observe the response in the intact CNS when pharmacologic applications SP last longer than 0.1 second. Thus this response is unlikely to be any of those previously reported.

The transmitter-like action observed in this system suggests that SP may mediate excitatory synaptic events which are both rapid in onset and brief in duration, cannot summate, and exhibit refractoriness. Physiologically elaborated, rapidly desensitizing excitatory events mediated by acetylcholine (*20*) and dopamine (*21*) have been reported in several invertebrate ganglia. We have observed similar rapidly desensitizing excitatory responses to iontophoresis and pressure application of the opioid peptides leucine- and methionine-enkephalin (*22, 23*), to the purine nucleoside inosine (*24*), and the benzodiazepine flurazepam (*24*). Whether these substances can cross-desensitize pharmacologically or interact physiologically remains to be investigated. The excitatory signals generated by the endogenous peptide substances may function as evanescent excitatory neurotransmitters in the vertebrate CNS whereas glutamate appears to be a neurotransmitter that can mediate summating forms of synaptic excitation.

The second action of the peptide, involving depression of glutamate-activated events independent of any other effects on membrane properties, falls outside the definition of conventional neurotransmitter action and is similar to the previously reported depression of glutamate responses on cultured neurons by leucine-enkephalin (*25, 26*). Previous investigators have (*6, 11, 12*) and have not (*27*) reported an interaction of SP with glutamate or other putative neurotransmitters. Modulation of the efficacy of transmitter action on postsynaptic membranes by peptides should serve to alter specific synaptic events in the CNS.

The data presented here suggest that SP may serve diverse functions in interneuronal communication (*28*). The results also indicate that the functional effects of the peptide are dependent on the pattern of peptide release. Rapidly repeated or sustained release should initially excite the postsynaptic cell and then depress its excitability by attenuating glutamate-mediated synaptic excitation. Exactly where and when SP functions and in what manner will require recording from SP-containing neurons and studying their interactions with target cells. It might be possible to use such cells grown in culture to examine the physiology of peptide-mediated communication in the mammalian CNS.

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Receptor-Mediated Internalization of Fluorescent Chemotactic Peptide by Human Neutrophils

Abstract. *Tetramethylrhodamine labeled* N-*formyl-Nle-Leu-Phe-Nle-Tyr-Lys is a potent chemoattractant for human neutrophils. Binding of this peptide to living neutrophils was observed by means of video intensification microscopy. At 37°C, diffuse membrane fluorescence was seen initially, followed by rapid aggregation and internalization of the fluorescent peptide. These processes are dependent on specific binding to the formyl peptide chemotactic receptor.*

Neutrophil chemotaxis is a complex behavioral response in which the cell exhibits directed migration in response to a chemical gradient. This implies that the cell is able to sense not only the concentration and chemical structure of the chemoattractant, but its directionality as well. Because of the vectorial nature of this signal, the molecular mechanisms of signal recognition or transduction may be more complex than that defined for other hormonally responsive cells, which must sense magnitude only.

Initial characterizations of the interaction of several chemotactic factors with neutrophils have demonstrated specific cell surface receptors for the factors, including formyl peptides, C5a (a complement-derived anaphylatoxin), and a urate crystal induced factor (*1*). In each case, the potency of a factor to induce chemotaxis correlated closely with its binding affinity for the specific receptor.

Neutrophils are believed to sense the directionality of a chemical gradient by a spatial, rather than a temporal, mechanism (*2*). The cell continuously determines the difference in concentration across its own dimensions. Because the chemotactic factors interact with cell surface receptors, it has been postulated that differential receptor occupancy across the cell membrane may account for recognition of directionality. Receptors nearest the source of the attractant, and therefore exposed to the highest relative concentration, would be occupied to a greater extent than those receptors on the same cell which are furthest from the source of attractant. In order to observe directly the binding and distribution of a chemotactic factor, we have prepared a fluorescent chemotactic peptide that retains biological activity. This peptide binds specifically to the plasma membrane of human neutrophils, rapidly aggregates, and is subsequently internalized.

Synthetic *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys [formyl peptide (*3*)], believed to be an analog of naturally occurring bacterial products that induce leukocyte chemotaxis, is a potent chemoattractant for human neutrophils, exhibiting an EC_{50} (concentration producing a response 50 percent of maximal) for chemotaxis of 0.4 n*M* and EC_{50} for binding of 1.3 n*M*. There are approximately 120,000 binding sites per neutrophil (*4*). Compared to the nonfluorescent formyl peptide, the potency of the tetramethylrhodamine conjugate of this peptide (TMR-peptide) (*5*) in inducing chemotaxis is only slightly decreased, the EC_{50} being approximately 1.0 n*M*. The TMR-peptide also competes effectively for the formyl peptide receptor, exhibiting an EC_{50} of 3.0 n*M* in a competitive binding assay.

Binding of the TMR-peptide to human neutrophils was observed by means of an RCA silicon intensifier target TV camera (TC1030H) attached to a Zeiss Photomicroscope III equipped with epifluorescence. Video output was recorded on a Panasonic recorder (NV-8030) and displayed on a Hitachi 8-inch TV monitor (VM-905AU), from which Polaroid photographs were taken (*6*).

Neutrophils that were exposed to TMR-peptide for 1 minute at 37°C displayed a diffuse and relatively homogeneous membrane fluorescence (Fig. 1, a and b). By 2 minutes, 20 to 50 aggregates of intensely fluorescent material could be seen superimposed upon the diffuse background fluorescence (Fig. 1, c and d). These aggregates were seen only in the focal plane of the plasma membrane, suggesting that the aggregates were confined to the membrane at this time point. By 3 minutes, these aggregates had increased in size and intensity on the membrane, and fluorescent endocytic vesicles, which displayed the saltatory motion characteristic of cytoplasmic organelles, were seen within the cell (Fig. 1, e and f). When cytoplasmic streaming was observed by phase-contrast, the fluorescent vesicles moved with the same velocity and direction as the cytoplasmic organelles. During the next 2 minutes, most of the fluorescent material remaining on the membrane was internalized and the numerous small endocytic vesicles coalesced into 5 to 10 larger vesicles (Fig. 1, g and h). The fluorescent vesicles correlated with easily definable, cytoplasmic lucent vesicles on the corresponding phase micrograph, suggesting that phase dense lysosomes had not, as yet, fused with the vesicles. In most cells, the coalescence of fluorescent vesicles continued, so that at 10 minutes, many cells displayed only one or two intensely fluorescent vesicles (Fig. 1, i and j). This pattern remained stable for 30 to 60 minutes. All of the neutrophils bound and internalized TMR-peptide to the same extent, providing direct evidence that the cells are homogeneous with regard to the presence of the formyl peptide receptor.

When the binding was carried out at 4°C for 120 minutes, the diffuse, homogeneous membrane fluorescent pattern was seen (similar to that in Fig. 1b). If the cells that had bound TMR-peptide at 4°C were then warmed on the microscope stage to 37°C, aggregation and internalization by individual cells could be followed. The time course and events were identical to those shown in Fig. 1.

To demonstrate that the rhodamine label being observed in Fig. 1 was attached to the original formyl peptide rather than to a hydrolytic product, we prepared a formyl peptide containing both ^{125}I and rhodamine in the same molecule

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