History of a Pioneering Neuropeptide: Substance P

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1 Extraction, Biological Testing and Structural Elucidation

The initial discovery of an 'unidentified depressor substance in certain tissue extracts' by von Euler and Gaddum (1931) has been described in detail by von Euler (1977):

The discovery of substance P (SP) was unexpected but not wholly fortuitous. As a postgraduate student in 1930 I was allowed to work in H. H. Dale's laboratory in Hampstead, London. At that time acetylcholine (ACh) was of primary interest in the laboratory, and I was given the task of trying to demonstrate a release of ACh from the intestine on vagus stimulation. This led to some experiments in which an intestinal extract was tested for biological activity on the isolated jejunum of the rabbit. Since the contraction observed was not inhibited by atropine and since histamine—another of the favourite substances of the labo-

ratory—did not contract the rabbit's jejunum, it appeared that the effect observed was due to a new or at least unknown principle. Dale suggested that the work should be continued jointly with J. H. Gaddum, senior assistant in the laboratory. As a result the effect observed was systematically investigated, and after some time we were satisfied that the active agent was not identical with any of the compounds then known that had a stimulating action on the gut. A study of extracts from various organs also showed that this substance was present in appreciable quantities in the brain. We also observed that all extracts that contracted the gut also lowered the blood pressure, especially in the rabbit. In order to make quantitative estimations we used a purified standard preparation simply referred to as 'P' on the tracings and in the protocols. Some 30 years later, Gaddum wrote "We concentrated the active substance in the form of a stable dry powder known as preparation P (which probably contained about 15 units/mg). It is impossible to justify the widespread custom, started by Gaddum and Schild, of calling the active principle itself substance P, but it is probably too late to change that now." The active principle is now generally called substance P or SP, somewhat in analogy to PG for prostaglandins, which has the advantage of allowing convenient addition of qualificatory suffixes.

The substance could be distinguished from acetylcholine as it was not inhibited by atropine, and also from histamine because this amine did not contract the rabbit jejunum, which was used as the test organ. The substance was found only in extracts from gut and brain. All the extracts also lowered the blood pressure, especially in the atropinized rabbit.

Gaddum and Schild (1934) excluded adenosine derivatives in this extract, found its acid stability, its solubility in acetone and ethyl alcohol, and described it as a basic compound. They also called the stable dry precipitate, a powder, the first time 'substance P'. Von Euler (1936) removed large proteins from the tissue extracts by boiling at pH 4, followed by salting out the peptides by ammonium sulfate. This stable dry powder, which probably contained 15 units/mg substance P, was used in all the earlier experiments. The peptide nature of the active principle was indicated by inactivation with trypsin.

All the earlier papers measured the amount of substance P in biological units, although an International Standard has never been established. After the synthesis of substance P, an activity of about 200,000 biological units/mg was established, thus 1 unit is roughly equal to 5 ng substance P.

It is worth remembering what Dale (1933) said. In 1933, nearly at the end of his third Dohme lecture, Dale made a statement which is as topical now as it was then and may serve as a kind of warning. He said:

The discovery, in artificial extract from an organ or tissue, of a substance which on artificial injection produces a pharmacodynamic effect provides only a first item of presumptive evidence in support of a theory that the action of this substance plays a part in normal physiology. Much more evidence is required before we can attribute clearly defined functions to such a substance, as we can now do in the cases of histamine and acetylcholine. But even where this is already possible, we have still no evidence to justify the assumption that the

substance comes naturally into action in the body in the free condition in which we isolate and identify it in the laboratory after various unnatural chemical procedures.

Not much attention was paid to substance P for the next 20 years. At that time a number of peptides such as the hormones insulin, vasopressin and oxytocin, and other agents like bradykinin or angiotensin were known. But no methods were yet available for their isolation and establishing their structure.

Lembeck's interest in substance P emerged from other findings. Hellauer, the youngest coworker of Otto Loewi in Graz, published two papers with Loewi in 1940, in which it was shown that the dorsal roots of the spinal cord, in contrast to the ventral roots, do not contain acetylcholine. Long ago, Stricker (1876) had made an unusual observation: when he stimulated the peripheral endings of the cut dorsal roots in a dog, he observed a peripheral vasodilatation, measured by a mercury thermometer between the toes. This was a contradiction to the Law of Bell and Magendie which says that the dorsal roots contain only afferent nerve fibers. Stricker's observation was fully confirmed by Bayliss (1901) and was thereafter mentioned as an unexplained curiosity by the name 'antidromic vasodilatation' in physiology textbooks.

In his Walter Dixon Memorial Lecture, Dale (1935) commented:

When we are dealing with two different endings of the same sensory neuron, the one peripheral and concerned with vasodilatation and the other at a central synapse, can we suppose that the discovery and identification of a chemical transmitter of axon-reflex vasodilatation would furnish a hint as to the nature of the transmission process at a central synapse? The possibility has at least some value as a stimulus to further experiment.

Hellauer and Umrath (1948) injected subcutaneously crude extracts of ventral and dorsal roots into the ears of rabbits in order to find the transmitter substance of sensory fibers. The differences, observed only visually—as no suitable equipment to measure vasodilatation was available in these post-war years—seemed to Lembeck, having been trained in bioassay in Gaddum's laboratory, not at all convincing. But the approach raised his interest. Lembeck's laboratory facilities were also fairly primitive. The isolated organ bath to suspend a guinea pig ileum was home made, the guinea pigs were home bred and the kymograph was a heirloom of Otto Loewi's time. Lembeck prepared substance P containing ammonium precipitate and found that it caused contractions in the presence of atropine and an antihistamine. Therefore he interpreted it as the effect of substance P. He went to the slaughterhouse to collect ventral and dorsal roots and made simple extracts. And indeed there was at least ten times more activity in the dorsal as compared to the ventral roots (Lembeck 1953a). The dorsal roots had the activity of 4 units substance P/mg wet tissue, thus equaling roughly 20 ng substance P. Lembeck had not been aware before that the guinea pig ileum, used for the first time as a substance P bioassay, reacted to such a small amount. A proteolytic destruction of the activity of the dorsal and ventral roots could also be shown, which was a strong argument for substance P. As a second

bioassay he used the decrease in blood pressure of the atropinized rabbit, but there was no difference in the activity.

Holton and Holton (1952) found that the antidromic stimulation of the n. auricularis induced the release of ATP from the rabbit ear: the decrease in blood pressure caused by ventral and dorsal root extracts was about equal. But ATP contracted the gut only in amounts above 10 μg and could therefore not be involved in contraction of the guinea pig ileum induced by the released material. The old findings of Holton and Holton (1952) and Holton and Perry (1951) found confirmation in recent years, as ATP is a co-transmitter in many synapses (see Hökfelt et al. 1980).

When Lembeck showed his results to Gaddum, Gaddum critically asked "Could you exclude serotonin?" and Lembeck's reply was also a question "What is serotonin?" Gaddum thereafter informed Lembeck about the recent isolation of serotonin by Rapport, Green and Page (1948) and the previous work of Erspamer. Fisher of the Upjohn Company had just synthesized serotonin and sent Lembeck a 10-mg sample. Desensitizing the guinea pig ileum to serotonin by the large dose of 50 μg/ml did not influence the effects of the dorsal root extracts; therefore serotonin could be excluded.

In parallel with these investigations, Kopera and Lazarini (1953) studied the distribution of substance P in the bovine and feline brain. Simple extracts were prepared and tested on guinea pig ileum in the presence of atropine and an antihistamine. A high amount of substance P in the substantia nigra was noted. These results were confirmed and extended by investigations of Pernow (1953), Zetler and Schlosser (1955), and others (for a review see Pernow 1983).

All of these findings evoked a certain amount of interest in substance P. Changes in different functional stages of the brain were investigated by Zetler and Ohnesorge (1957), and investigations of the functions on the gut and on the central nervous system (CNS), even a search of antagonists under arbitrarily selected compounds (Stern and Huković 1961), led to a first Symposium on Substance P, held on 9 and 10 June 1961 in Sarajevo (Fig. 1 and Fig. 2). At the end of the symposium, all participants agreed that further progress would depend on the expected isolation of substance P. Pernow (1953) had already found that substance P could be absorbed on alumina and eluted with decreasing concentrations of aqueous methanol; in this way the activity was increased from a few biological units per milligram to about 3,000 units per milligram. Franz, Boissonas and Stürmer (1961) of Basel reported at the symposium that they had reached an activity of 30,000–35,000 units per milligram by chromatography and electrophoresis.

At a symposium of the New York Academy of Sciences under the title 'Structure and Function of Biologically Active Peptides: Bradykinin, Kallidin and Congeners' in 1963, 30 papers were devoted to bradykinin, for which the greatest progress including isolation and synthesis was achieved, and only eight to substance P (Whipple et al. 1963). The contributions from Basel on the isolation of substance P were the most remarkable ones, by Boissonas and Stürmer of

Fig. 1 Participants of the Symposium on Substance P, organized by P. Stern and held on 9–10 June 1961 in Sarajevo. The photograph was taken by U.S. von Euler (see Fig. 2). *Front row seated*, *from right*: K. Umrath, J.H. Gaddum, G. Zetler, V. Varagic, H. Caspers, E. Stürmer. *Second row seated*: B. Pernow (*third from right*), F. Lembeck (*second from left*). *Standing, from right*: S. Hukovic (*second*), W.A. Krivoy (*third*), K. Lissak (*fourth*), P. Stern (*fifth*), Marthe Vogt (*ninth, half-covered*)

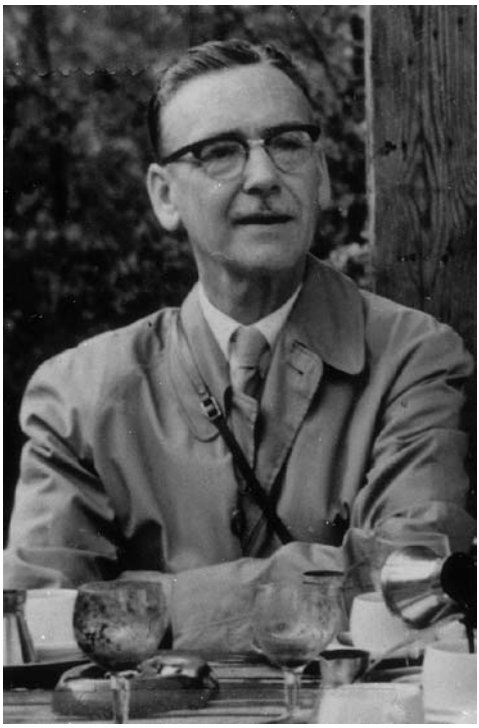

Fig. 2 U.S. von Euler. The photograph was taken by F. Lembeck at the occasion of the Symposium on Substance P in Sarajevo, 9–10 June 1961

Sandoz, by Vogler and coworkers of Roche, and by Zuber of Ciba-Geigy—they all had almost reached the goal, but the essential hit came from somewhere else.

Erspamer had found that the salivary gland of a mollusc, *Eledone moschata*, contains besides serotonin the peptide eledoisin, and the skin of a South American frog, *Physalemus fuscomaculata,* contains the peptide physalaemin. Both peptides occur in large amounts and therefore their isolation and elaboration of their chemical structure was no problem. Lembeck once asked Erspamer about the motivation behind these experiments. His reply was short and precise: "It was just serendipity, nothing else". The strategy of his work was to test the activity of eledoisin, physalaemin and substance P on many bioassay preparations in vitro and in vivo. Ten of the amino acids in physalaemin were present in the not yet completed analysis of substance P as shown by the researchers in Basel. In a paper of Bertaccini et al. (1965) we find the following:

But the door still remained closed for several years. I think the next important step emerged from the Mediterranean Sea, like Aphrodite, but in the shape of a mollusk, *Eledone moschata*. Erspamer discovered eledoisin in its salivary glands and among so many other interesting peptides, shortly thereafter, physalaemin in the skin of a South American frog. The isolation and careful pharmacological analysis of these peptides led to a statement by Erspamer in 1965 (Bertaccini et al. 1965) which is worth quoting: It may be seen that as many as ten of the amino acids found in substance P are also present in eledoisin, and eight in physalaemin. The only amino acid lacking in the molecule of substance P is apparently the tremendously important methioninamide. The suspicion seems to be justified that the labile methioninamide residue has escaped the attention of research workers, who have isolated and studied substance P. We would suggest that this possibility be checked.

At this time, Lembeck compared eledoisin and physalaemin with substance P in terms of their effects on salivary secretion. This led to a profound speculation: could substance P, when released from the gut into the circulation, induce increased salivary secretion like secretin released from the gut induces increased fluid secretion from the pancreas? Lembeck and Starke measured salivary secretion after intravenous injection of the substance P preparation into chicken, rat and dog, the species used by Bertaccini et al. (1965) and observed a pronounced increase in salivary secretion (Lembeck and Starke 1968). In addition, they simultaneously recorded the blood pressure in the dog: substance P, at a dose that induced only a weak salivary secretion, evoked a profound decrease in blood pressure. From common experience it is known that the smell of a delicious meal can induce salivation, however, fortunately no fainting—so this romantic speculation had to be abandoned.

A few days later, on 4 May 1967, Lembeck made an important discovery in an unusual place: sitting in a convenient Intercity train, reading the *Frankfurter Allgemeine Zeitung*, he found a short note about the isolation of a 'salivary secretion stimulating factor' called 'sialogen' in peptide extracts from hypothalamus by Leeman and Hammerschlag (1967), presented at a Federation Meeting. Lembeck had the feeling that he was the only really fascinated reader of this

message, written by an unknown reporter. After his return to Tübingen, he sent Leeman all the results on salivation by air mail. Samples were exchanged and there was soon enough evidence that sialogen and substance P were identical.

Susan Leeman's work was a spin-off product of a rather complicated endocrinological test. Glucocorticoids are synthesized and released, but not stored in the adrenal cortex. When their synthesis is increased, the high amount of ascorbic acid in the rat adrenal cortex decreases. This reduction in ascorbic acid, which can easily be measured, was therefore a parameter for the assay of glucocorticoid production such that the effect of adrenocorticotrophic hormone (ACTH)—released from the pituitary and stimulating the synthesis of glucocorticoids—could be quantified. The neurohormonal control of the ACTH secretion is based on the release of the corticotropin-releasing factor (CRF). Leeman's project was to purify this factor (Leeman et al. 1962). The presence of CRF in a crude hypothalamic extract was verified and the extract was subjected to gel exclusion chromatography on Sephadex G-75 as the next step in a purification procedure. Each fraction from the column had to be injected into an anesthetized rat to measure the biological activity. Material from certain fractions rapidly caused a copious secretion of saliva. The sharp cut-off of sialogogic activity in the elution profile was shown to be caused most probably by the presence of vasopressin, which produced a marked blanching in the test rats and effectively inhibited the sialogogic response. Michael Chang joined the project and they turned from CRF to the isolation of the newly discovered hypothalamic peptide. The initial large-scale tissue extraction (the extract from 200 bovine hypothalami was applied to one column!) outgrew the facilities of the laboratory. Further steps were ion exchange chromatography and paper electrophoresis (Chang and Leeman 1970). The amino acid sequence of substance P was published in 1971 (Chang et al. 1971). Tregear et al. (1971) were able to synthesize the peptide and to produce Tyr^8-substance P which enabled the labeling with ^{125}I and thus permitted the development of a radioimmunoassay (Powell et al. 1973). Studer et al. (1973) showed that the substance P in gut was identical with that in brain.

This story is strongly reminiscent of what Gaddum wrote in 1954, and which will, hopefully, remain true in the future:

The most interesting discoveries (in pharmacology) are the unexpected ones, and in spite of the fact that research is much more organized today than it was once, unexpected discoveries are still made.

The availability of synthetic substance P dramatically changed the scenery in this field of research, and the number of publications on substance P increased steeply between 1970 and 1980 (see Pernow 1983). Previous material, compiled by Lembeck and Zetler in reviews 1962 and 1971 needed to be confirmed and extended. New methods allowed unexpected guidelines to a much better insight into the distribution of substance P in tissues and conclusions about its function. In the following only the keys to these new methods are described, while the full gain of the new results is described in the other chapters.

2
Masanori Otsuka and the Action of Substance P in the Spinal Cord

At the International Congress of Pharmacology 1972 in San Francisco Lembeck was elected Secretary General of the IUPHAR. His involvement in administration had considerably diluted his attention to the scientific program. When Lembeck met Marthe Vogt she asked whether he knew Masanori Otsuka, what Lembeck denied. "But he has confirmed your results on substance P from 1953", she said, and brought Otsuka and Lembeck together.

Otsuka had found that dorsal roots contain 20 times more substance P than ventral roots, and he first observed—by the use of a bioassay—that the substance P content of the dorsal horn declined after section of the dorsal roots; this means that substance P is produced in the dorsal root ganglion und transported by the central fibers of afferent neurons into the dorsal horn (Otsuka et al. 1972). Lembeck returned from California with a feeling like that of the gold digging people. Another confirmation came from B. Pernow: While the substance P content of the rat ventral horn is 134±33 pmol/g substance P, that of the dorsal horn is 1070±160 pmol/g wet tissue (see Pernow 1983).

Konishi and Otsuka (1974a) investigated the effects of substance P and other peptides on the isolated spinal cord of the frog. Thereafter they used the spinal cord of newborn rats (Konishi and Otsuka 1974b). The dorsal root was placed in a suction electrode that was stimulated and the ventral root was placed in a recording suction electrode. Depolarization in the ventral root was measured after the addition of substance P and L-glutamate, and the activity of substance P was estimated to be about 200 times higher than that of glutamate which was regarded as the leading candidate for the excitatory transmitter in the spinal cord (Curtis and Johnston 1974). It was not before 1994 (Ueda et al. 1994) that the co-release of substance P and glutamate was shown. Yanagisawa et al. (1984) developed the isolated spinal cord tail preparation of the newborn rat, in which they could demonstrate capsaicin-induced nociceptive responses. (For the neurotransmitter functions of mammalian tachykinins, see also the review by Otsuka and Yoshioka 1993.)

3
Radioimmunoassay and Immunohistochemistry of Substance P

At Nobel Symposium 37 (1976, Stockholm; organized by U.S. von Euler and B. Pernow) under the title 'Substance P', (Fig. 3), the dominant role of the immunological methods based on the radioimmunoassay of Powell et al. (1973) was evident. Susan Leeman agreed to accept Rainer Gamse as coworker at the Harvard Medical School in order to learn the radioimmunoassay. When he returned to Graz after a year, he opened the way to many new projects. Thomas Hökfelt presented, based on 23 impressive slides, the distribution of substance P in the central and peripheral nervous system and made comments on the results which are worth reading even today.

Fig. 3 Participants of the Nobel Symposium 37, entitled 'Substance P', organized by U.S. von Euler and B. Pernow and held in Stockholm in June 1976. *Lowest step, at right*: E.G. Erdös. *Second step, from right*: U.S. von Euler (*second*), G.F. Erspamer (*third*), S.E. Leeman (*fourth*), K. Krnjevic (*fifth*). *Third step, from right*: B. Pernow (*second*), W.A. Krivoy (*third*), G. Zetler (*fourth*), P. Oehme (*second from left*), F. Lembeck (*first from left*). *Fourth step, from right*: S. Rosell (*second*), J.L. Henry (*second from left*), R. Gamse (*first from left*). *Fifth step, from righ*t: M. Otsuka (*third from left*), T. Hökfelt (*second from left*). *Last step, from right*: K. Folkers (*first*), A. Carlsson (*second*)

The word 'tachykinin' was a creation of Erspamer. On isolated smooth muscle preparations eledoisin, physalaemin and substance P caused faster contractions than bradykinin, and to designate this difference at a time when none of these peptides had been isolated he created these descriptive names which are still used today. Two peptides closely related to substance P, neurokinin A and neurokinin B, were discovered in mammals, and many further tachykinins were isolated from lower animals.

The immunohistochemical technique, including the immunofluorescence technique, the peroxidase technique, the very sensitive peroxidase-antiperoxidase technique and the 'double staining techniques' allowed the identification of more than 20 peptides in neurons of the brain, spinal cord and in the periphery. In several cases peptides occur together with a 'classical' transmitter in the same neuron (Hökfelt et al. 1980). Calcitonin gene-related peptide (CGRP) was an unexpected finding and remarkable, because its distribution and functions are closely related to those of substance P (for a review see Wimalawansa 1996). Somatostatin was also found concomitantly with substance P (Gamse et al. 1981). The concentration of these and many other peptides in the CNS is about 1,000 times lower than that of monoamines and 100,000 times lower than that of amino acids. But peptides may activate their receptors at a much lower concentration than classical transmitters. Classical transmitters are synthesized not only

in the cell body, but also at the nerve endings, and a reuptake mechanism exists. Peptides are produced only in the cell body and are carried by the axonal transport to the nerve terminals where no reuptake mechanism seems to exist. Classical transmitters usually cause a rapid response of short duration, whereas the peptides are responsible for a long-lasting effect. It has to be emphasized that the new findings of Hökfelt and many others provided a completely new view of the afferent, autonomic and central nervous systems. The findings also include new aspects of the organization of neuronal pathways within the CNS. Finally the coexistence of peptides with classical transmitters expanded the original principle of Dale (i.e., one neuron–one transmitter) to a system which seems to be adequate for the great variety of neuronal functions, especially within the CNS (for a review see Hökfelt et al. 1980).

4
Nicolas Jancsó and Capsaicin

It was in 1967 when Lembeck attended the Annual Meeting of the Hungarian Pharmacological Society in Budapest. He extended the journey to Szeged where he met Nicolas Jancsó in his laboratory, which was full of all kind of experimental equipment and had hundreds of bottles on the shelves. Lembeck knew Jancsó's work on the extinction of pain by capsaicin (Jancsó et al. 1959). Lembeck invited Jancsó to Tübingen for a seminar, and Jancsó arrived a few months later with his wife and Thuranszky, the owner of a pre-war Mercedes car. Jancsó gave his seminar on a very hot summer day, at two o'clock in the afternoon. He carefully explained his simple, but conclusive experiments, and Lembeck was probably the only one who was convinced that he was right. After the availability of synthetic substance P there were so many projects to deal with, that only with some delay we started to unravel the functional implications of substance P-containing afferent fibers by the action of capsaicin. The initial stimulus for this work was the work of Nicolas Jancsó, Aurelia Jancsó-Gábor and János Szolcsányi (1967) who reported:

1. By antidromic electrical stimulation of the sensory nerves (saphenous or trigeminal) of rats, the following signs of an inflammatory response could be elicited: arteriolar vasodilatation, enhancement of vascular permeability, protein exudation, fixation of injected colloidal silver onto the walls of venules and, later, their storage in histiocytes.
2. The inflammatory response induced by electrical stimulation could not be altered by parenterally administered atropine, physostigmine, hexamethonium, phentolamine, dibenamine, propranolol, promethazine, chloropyramine, or methysergide.
3. After the degeneration of the sensory nerve, capsaicin, xylene, mustard oil and ω-chloroacetophenone did not evoke inflammation. Hence, these substances induce inflammation purely or dominantly through the involvement of sensory nerves.

4. Capsaicin desensitization inhibited the signs of inflammation induced both by antidromic stimulation of the sensory nerve and by orthodromic stimulation of pain sensitive nerve terminals with irritants.
5. The experiments suggest that a mediator substance, a neurohumor, is released by orthodromic or antidromic stimulation of pain sensitive nerve terminals and that this substance is responsible for the signs of inflammation produced by some substances.

The question was whether the proposed 'neurohumor' was indeed substance P. The paper by Gamse, Holzer and Lembeck (1980) supplied a clear answer: "Familiar with antidromic vasodilatation from the work of Hellauer and Umrath (1948), we checked whether antidromic vasodilatation, so far only shown by Bayliss (1901) in the dog, could be reproduced in the rat hind leg. By means of a hand-made drop-recorder, we found that the venous outflow from the hind leg was increased by a 1-min stimulation of the saphenous nerve for about 10 min, comparable to the result of Bayliss (1901) in the dog." In capsaicin-pretreated rats an almost complete inhibition was observed (Lembeck and Holzer 1979). The postocclusive vasodilatation was also inhibited after capsaicin pretreatment (Lembeck and Donnerer 1981). Janscó had demonstrated that the 'neurogenic inflammation' consisted of vasodilatation and plasma extravasation. Substance P also evoked a plasma extravasation by histamine release from nearby mast cells, whereas CGRP did not. These events seemed to be closely related to the 'nocifensor system', which Sir Thomas Lewis had found by studies on human skin (see Lembeck 1985). It consisted of a peripheral neurogenic vasodilatation and plasma extravasation (flare and wheal response), providing a defense mechanism which removes exogenous or endogenous toxic material by increase of blood flow and lymph drainage (see also Lembeck 1988). When selective substance P (NK_1) receptor antagonists became available, it was definitively proven that substance P is the mediator of neurogenic plasma extravasation (Lembeck et al. 1992).

Vasodilatation and plasma protein extravasation by substance P are, together with the contraction of various smooth muscles, non-neural effects. Could substance P, released in regions of the CNS, induce vasodilatation besides the stimulation of defined neurons? Lembeck and Starke (1963) tested purified substance P extracts, which were free of amines and ATP, containing different amounts of substance P. The extract of substantia nigra contained enough substance P to produce a pronounced increase in capillary permeability, whereas the other extracts were correspondingly less active. It was concluded that those parts of the brain which are rich in substance P might have a particularly high exchange rate between the capillaries and the surrounding tissue. Hypothalamic blood flow was measured in conscious rabbits by the ^{133}xenon washout technique. Intrahypothalamic injections of substance P at doses of 50 and 500 ng were performed; the results suggested that substance P may cause an increase in blood flow via the endogenous release of acetylcholine which in turn stimulates

an intracerebral noradrenergic pathway (Klugmann et al. 1980). Neither publication found an echo and no further experiments of this kind were carried out.

5
Peptide and Nonpeptide Transmitters in Primary Afferent Neurons

Of the primary afferent neurons, Aβ fibers detect innocuous stimuli applied to skin, muscle and joints. Stimulation of Aβ fibers can reduce pain, such as when these fibers are activated by rubbing one's hand. Thinly myelinated Aδ fibers and the most slowly conducting C fibers transmit the rapid, acute, sharp pain, and the delayed, more diffuse, dull pain, respectively. Aδ nociceptors respond to intense mechanical stimuli and to intense heat. Most C fibers are polymodal, responding to noxious thermal, chemical and mechanical stimuli, whereas others are mechanically insensitive, but respond to noxious heat; however, most C fiber nociceptors respond to chemical stimuli such as capsaicin or acid. Tissue injury might sensitize so-called 'silent' receptors. The peptidergic group of unmyelinated C fibers contains tachykinins (substance P and neurokinin A) and expresses trkA (high affinity tyrosine kinase) receptors for nerve growth factor. The second population expresses P2X receptors, specific ATP-gated ion channels. Further peptides found in C fibers include CGRP, released concomitantly with substance P (for a review see Holzer 1992), somatostatin, vasoactive intestinal polypeptide (VIP) and galanin, with much less well defined functions. Besides peptides, glutamate is most probably the predominant excitatory neurotransmitter in all primary afferent neurons.

In contrast to vision, olfaction or taste, sensory nerve endings that detect painful stimuli ('nociceptors') are not localized to a particular anatomical structure, but are dispersed over the body, innervating skin, muscle, joints and internal organs. The transmission of pain has raised the greatest interest in recent years. The vanilloid receptor VR1 was cloned and functionally characterized; it is activated by noxious heat, capsaicin and acid (for a review see Szallasi and Blumberg 1999). The VRL-1 receptor, expressed in a subset of medium- to large-diameter myelinated neurons, is activated by extreme noxious heat. VR1 and VRL-1 belong to a larger family of transient receptor potential channels (for review see Julius and Basbaum 2001).

Primary afferent C fibers are stimulated at their peripheral endings by many exogenous and endogenous substances such as capsaicin, mustard oil, xylol and other compounds which easily penetrate the skin. Histamine, acetylcholine and serotonin, jointly occurring in the stings of nettle, belong to this group of compounds. When the sting tip breaks off, the mixture is released into the dermis. Following stimulation, substance P is released from the peripheral terminals of sensory neurons, as shown by the vasodilatation and plasma extravasation due to antidromic electrical nerve stimulation. Plasma extravasation may be augmented by the release of histamine from mast cells, specifically by basically charged peptides, like substance P, but not by CGRP. This recalls the 'nocifensor system' claimed by Sir Thomas Lewis. He showed the cutaneous 'wheel and flare

response' to be of neurogenic origin, absent after chronic denervation, in clinical studies on the human skin. As antidromic vasodilation can only be evoked by nerve stimulation, it cannot be regarded as physiological. But under physiological conditions two actions occur: firstly transmitter release at the peripheral terminals, and secondly neuronal conduction to the central terminals of the dorsal root fibers within the CNS. At this site, the release of substance P takes place, and the amount of substance P is increased under inflammatory conditions in the periphery. This is therefore a nociceptive pathway. But the capsaicin-sensitive C fiber afferents serve not only the signaling of pain—they are much more the afferent part of the autonomic nervous system (which we erroneously regard as being only efferent).

Alarming messages recruiting one or the other defense reaction usually arise from higher brain centers, but the initiating information is signaled to the brain from the periphery. The signal might activate a single, unconscious response or recruit more than one of the possible defense reactions. The peripheral pathways which signal the alarming event to the CNS seem to be mainly afferent neurons which share certain common properties:

1. They are small diameter, unmyelinated C-fibers.
2. They synthesize substance P and other peptides, and transport them to the terminals where they are released by nervous impulses.
3. These neurons are capsaicin-sensitive, i.e., a small dose of capsaicin causes the release of the peptide, and systemic treatment with a very large dose of capsaicin causes these neurons to loose their function or to degenerate.
   The loss of function after systemic capsaicin pretreatment can therefore be regarded as evidence for the involvement of these afferent fibers in the response. It does not allow neurochemical definition of the type of neuron involved. Therefore it has been agreed to speak of 'capsaicin-sensitive neurons'. This limitation is essential because other C-fibers exist which are not capsaicin-sensitive: e.g., cold fibers (Petsche et al. 1983) and osmosensitive fibers in the hepatic portal vein (Stoppini et al. 1984).

6
Involvement of Substance P in Autonomic and Neuroendocrine Reflexes

Orthodromic conduction and transmitter release from capsaicin-sensitive neurons can evoke several reflex responses:

1. At the spinal level it induces the scratching syndrome in mice (Piercey et al. 1985).
2. Cardiovascular reflexes mediated by capsaicin-sensitive afferent fiber stimulation are set in action either by a stimulation or a withdrawal of the noradrenergic vasoconstrictor tone (Juan and Lembeck 1974; Donnerer et al. 1988).

3. Stimulation by heat induces a heat loss reaction in rats (Cormarèche-Leyden et al. 1985; Donnerer and Lembeck 1983).
4. The micturition reflex in rats caused by distension of the urinary bladder involves capsaicin-sensitive afferents (Holzer-Petsche and Lembeck 1984; Maggi and Meli 1988) whose activation might depend on ATP.

Endocrine regulations influenced by capsaicin-sensitive afferents and possibly comediated by tachykinins have also been revealed:

1. The hepatic portal vein of rats contains osmoreceptors and glucoreceptors (Stoppini et al. 1984) which are sensitive to hypoglycemia and signal this information to hypothalamic centers via capsaicin-sensitive afferents, which in turn leads to release of adrenaline from the adrenal medulla to upregulate the blood glucose concentration (Amann and Lembeck 1986; Donnerer 1988).
2. The release of ACTH from the pituitary can be evoked either by emotional or various peripheral 'stressors'. Capsaicin-sensitive neurons seem to convey such peripheral signals to the CNS. Cold exposure of rats is known to cause the release of ACTH; in capsaicin-treated rats, this response is abolished. Other peripheral stimuli like intraperitoneal administration of formalin, which is painful, or intravenous injection of isoprenaline induce the release of ACTH; the effect of these treatments is abolished in capsaicin-treated rats. Emotional stress, induced by restraint conditions, which is not conveyed by capsaicin-sensitive afferents but by other central pathways, remains unchanged in capsaicin-treated rats (Amann and Lembeck 1986; Lembeck and Amann 1986; Donnerer and Lembeck 1988). Capsaicin-pretreated rats have normal levels of plasma ACTH, which means a normal function of the endocrine regulation of glucocorticoids. Only the stimulation of the immediate release of ACTH, emerging from afferent neuronal signals, is under the influence of capsaicin-sensitive afferents.
3. In the rat the fertilized egg cell develops to a blastocyte within 3 days—during transport into the rat uterine cavity . In the meantime endocrine influences transform the uterine mucosa to the decidua, an essential requirement for nidation. These endocrine adaptations via hypothalamus and pituitary are set into action by afferent messages originating from the stimulation of vagina and cervix during copulation . These messages run via capsaicin-sensitive neurons to the CNS (Traurig et al. 1984a, 1984b). The existence of this neural pathway had already been shown by Marthe Vogt in 1933.
4. Lactation in the rat is regulated by prolactin and oxytocin. The stimulation of oxytocin release is induced by ultrasonic vocalization of the pups, by pheromones and by suction on the nipples which are richly innervated by substance P-containing neurons. When normal pups were fed by capsaicin-treated dams, the amount of milk they drank and their gain in weight were

both reduced by 20% (Traurig et al. 1984c). This is another example of the involvement of capsaicin-sensitive afferents in an endocrine regulation.

These few examples of autonomic reflexes and endocrine regulations present evidence that the afferent part is mediated by capsaicin-sensitive neurons. It is known that the n. vagus or the n. splanchicus, described in textbooks as 'efferent', contain a high amount of afferent fibers. Immunohistochemistry showed that substance P fibers account for about 10% of all fibers in the cat vagus nerve (Gamse et al. 1979).

7
Tachykinin Genes, Precursors and Receptors

A new era in tachykinin research began in the 1980s through application of recombinant DNA technology. Nakanishi and his coworkers (1983–1986) opened the way to investigate the biosynthesis of substance P and first isolated its gene and precursor protein containing 384 amino acid residues (Nawa et al. 1983; Kawaguchi et al. 1986). Currently the list of mammalian tachykinins is extended by the discovery of hemokinins and endokinins. In addition, there is a large number of nonmammalian tachykinins including eledoisin in mollusca, scyllorinin I and II in fishes, physalaemin and others in amphibia. They all share the same carboxyl terminal with the mammalian tachykinins.

The *Xenopus* oocyte expression system combined with electrophysiogical measurements was the key to the molecular structure and function of the three tachykinin receptors. Intranuclear injection of oocytes with cDNA encoding the receptor, or injection of cRNA into the cytoplasm of an oocyte produces a functional, foreign receptor–channel complex in the membrane of the oocyte. After application of its specific ligand, an electrophysiological response can be recorded. It was shown that the preferred receptors for substance P, neurokinin A and neurokinin B are encoded by different mRNAs. The investigation of the relative activities on various test preparations showed that the functional site of the NK_1 receptor is activated predominantly by substance P, that of the NK_2 receptor by neurokinin A, and that of the NK_3 receptor by neurokinin B (see Henry et al. 1987). The membrane topology of all three receptor molecules is that of rhodopsin-type receptors, consisting of seven hydrophobic membrane-spanning domains with an extracellular amino terminus and a cytoplasmic carboxyl terminus (Nakanishi 1991).

8
Colocalization of Substance P with Peptides and Amino Acids

Like other neurotransmitters, substance P is stored mainly in the synaptosomal fraction of nerves and in microsomal fractions of brain homogenates. The binding sites of substance P in synaptic vesicles are extractable with ether and chloroform. These and other observations suggest that the specific substance P

binding is phosphatidyl serine, which is of interest since this lipid is a constituent of synaptic membranes and vesicles.

Until the 1970s it had been a widespread belief that a neuron contains and releases only one transmitter according to Dale's concept of one neuron–one transmitter. Hökfelt et al. (1978) showed the co-existence of substance P and serotonin in the same neuron, a finding later confirmed by Cuello et al. (1982). Numerous examples of the coexistence of substance P and serotonin exist not only within the CNS, but also in the periphery. Thus, substance P, neurokinin A and serotonin coexist in carcinoid tumors (Bergström et al. 1995). When Lembeck (1953b) first found serotonin in a carcinoid tumor, he noted another gut-stimulating compound with a different Rf-value. By desensitization with large doses of substance P and serotonin, respectively, he found an indication for the presence of both compounds. But the methods available at that time were not sufficient for a clear proof and he mentioned this finding only in the discussion but not in the summary of the paper.

Numerous examples of the coexistence of peptides with other peptides, amines and amino acid neurotransmitters are now established. The functional significance of more than one transmitter is still not fully understood. If a neuron stores and releases a fast-acting transmitter (e.g., an amino acid) and a slow-acting transmitter (e.g., a peptide), then upon excitation it will produce a fast excitatory or inhibitory postsynaptic potential (EPSP or IPSP) and in addition slow EPSPs and IPSPs in postsynaptic cells (Otsuka and Yanagisawa 1987). For example, if γ-aminobutyric acid and substance P are co-released, a short-lasting inhibition and a prolonged excitation will be produced (Otsuka and Yoshioka 1993). In some circumstances, multiple transmitters released from a nerve terminal may diffuse across some distance and selectively act on cells equipped with the respective receptors, called 'chemically addressed transfer of information' (Iversen 1986).

An interesting issue is the co-release of substance P and glutamate. De Biasi and Rustioni (1988) first showed the coexistence of glutamate and substance P in dorsal root ganglion neurons and at their central terminals. Donnerer and Amann (1994) demonstrated that blockade of ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) type inhibits afferent nerve-mediated autonomic reflexes. Ueda et al. (1994) found with a continuous on-line monitoring method that glutamate is released from capsaicin-sensitive primary afferent fibers in the rat. The inhibition—by the NMDA receptor antagonist MK-801—of the central transmission of reflexes mediated by capsaicin-sensitive afferents points to an essential role of glutamate in these reflex responses, whereas substance P antagonists had no effect (Juranek and Lembeck 1996). A decrease of the capsaicin-induced release of glutamate from afferent nerve terminals by clonidine (Ueda et al. 1995a) and by morphine (Ueda et al. 1995b) has also been demonstrated, confirming and explaining earlier findings of Donnerer et al. (1988) on the depressor effect. A facilitation of the glutamate release by co-released substance P was assumed (Juranek and Lembeck 1997).

The life cycle of substance P ends by internalization of the receptor-bound peptide and enzymatic breakdown. The degradation of substance P is difficult to define, as several organs (kidney, spleen, liver and intestine homogenates) have a high capacity to inactivate it. One has to differentiate between elimination of substance P released into the circulation, and substance P destroyed locally such as in the brain. By using the rat salivary response as a bioassay, Lembeck et al. (1978) showed that the highest degree of elimination of substance P occurs in the liver and hind limbs, followed by the kidney. More than 80% of substance P infused into the portal vein is degraded by the liver (Lembeck et al. 1978). Several cytosolic and membrane-bound peptidases capable of degrading substance P have been extracted from the brain. A partly purified neutral endopeptidase was first described by Benuck and Marks (1975). Degradation of substance P by a neutral metallopeptidase system of synaptosomal fractions of brain also exists (Berger et al. 1979). Neutral endopeptidase subsequently turned out to significantly inhibit the transmitter action of substance P.

9
Substance P and Neurokinin A in the Gut

Although substance P was discovered in 1931 in the gut and brain, by a fall in the blood pressure and by the contraction of the rabbit ileum, no-one asked what its physiological role might be in the latter. Acetylcholine and noradrenaline were accepted as gastrointestinal neurotransmitters, but about a dozen other compounds were known to contract intestinal smooth muscle, such as histamine, oxytocin, later on serotonin, bradykinin and other compounds. The first hint that substance P is involved in neuronal functions was the finding of Ehrenpreis and Pernow (1953) that the aganglionic, inactive part of the rectosigmoid in Hirschsprung's disease contained significantly less substance P than control pieces, whereas the proximal, hyperactive part showed normal levels of substance P. Substance P was found in all parts of the intestine of animal species and man. The guinea pig ileum was later used as a bioassay for substance P, as it is particularly sensitive to this peptide. The method of Holzer and Lembeck (1979) to record peristalsis in the isolated guinea pig ileum offered experimental possibilities to investigate the action of substance P on propulsive motility.

The essential breakthrough came from immunohistochemistry. Two fundamental types of neurons supply the digestive tract: an extensive population of neurons contained within the gut wall (intrinsic neurons) and an extrinsic innervation by cholinergic and noradrenergic autonomic fibers as well as by substance P-containing sensory neurons from dorsal root ganglia and the vagus nerve. The substance P-containing nerve cells in the myenteric ganglia issue a very dense network of varicose nerve fibers to other myenteric ganglia, as well as fibers to the longitudinal and circular muscle layers, to the submucous ganglia and to the mucosa. The number of substance P cell bodies in the myenteric plexus has been underestimated. Only 2.8%–3.5% of the myenteric neurons contained substance P, but in segments of the intestine in a culture medium with

colchicine for 24 h, about 20% of the total population are substance P-containing neurons. The proportion of substance P neurons in the submucous plexus is about 11%. In the human intestine, the substance P neurons have a similar distribution as those in the guinea pig gut (Holzer and Holzer-Petsche 1997a).

The extrinsic sensory substance P neurons are fewer than the intrinsic neurons. The extrinsic neurons are capsaicin-sensitive whereas the intrinsic substance P neurons are not. They sense intestinal pain (colic) or they induce reflexes (vomiting) or diarrhea to expel toxic material (for a review see Holzer and Holzer-Petsche 1997b).

The enteric nervous system, already separated from the autonomic nervous system by Langley (1932), consists of a large number of neurons, in the range of the number of neurons within the spinal cord. Immunohistochemistry led to the detection of many other neuropeptides in the gut, besides those mentioned above, such as neuropeptide Y, somatostatin, cholecystokinin, VIP, and others (for review see Holzer and Holzer-Petsche 1997a, 1997b).

10
Tachykinin Receptor Antagonists: The Role of Substance P Revisited

Antagonists of substance P, acting specifically like atropine on muscarinic acetylcholine receptors, were an old dream of pharmacologists. The attempts to find such a compound on a simple preparation like the guinea pig ileum were not successful. A new approach was chosen by Snider et al. (1991) and led to the discovery of the first specific nonpeptide antagonist CP96345. It was found by a robot and Lembeck was keen to see this assembly. So he went to the Pfizer Company in Groton, CT, and saw how 96-hole plates with cells containing substance P receptors were taken from the deep-freeze and brought to room temperature. Radioactively-labeled substance P was injected into the samples, 96 different compounds were added and left for a suitable time in the wells. Thereafter, the fluid of the wells was removed by suction, the probes dried by microwave and transferred to a scanner.

The scanner measured the amount of labeled substance P, and the result was a print-out in gray shades on a piece of paper. Binding of substance P to the receptor was 'black', full inhibition of the binding by the antagonist was 'white'. The most cumbersome work was the selection of the many substances and the preparation of suitable dilutions. The man who had set up this robot left the company after the screening of some 20,000 compounds, being unaware that among them the antagonist CP96345 had already been detected. The antagonist was specifically active on the fall in blood pressure induced by injection of substance P and neurokinin A, but not on that by VIP or CGRP. It inhibited the plasma protein extravasation induced by mustard oil in rats when injected intravenously or given orally. On intestinal smooth muscles specific effects of the antagonist were shown at concentrations below 1 μM, whereas at higher concentrations nonspecific effects were seen also. No effect was observed on the peri-

staltic reflex. Cardiac effects indicated an effect of both enantiomers on calcium channels.

We probably made an error. The substance P antagonist completely inhibited the effects of substance P released by antidromic nerve stimulation or capsaicin from the peripheral terminals of primary afferent fibers. We knew that substance P is also released from the central terminals of these fibers in the substantia gelatinosa of the dorsal horn of the spinal cord, as shown in vitro on spinal cord slices of the rat (Gamse et al. 1979) and in vivo in the cat (Duggan et al. 1987). We assumed that several reflexes and endocrine regulations, which were blocked following capsaicin pretreatment, should also be blocked by the substance P antagonist. But we did not find an action of the substance P antagonist. At the same time the co-release of substance P and glutamate was shown (Ueda et al. 1994). All the centrally mediated effects evoked or blocked by capsaicin were shown to be inhibited by the NMDA receptor blocker MK-801, therefore being mediated by glutamate (Juranek and Lembeck 1997). The question remains what the physiological effect of substance P at these synapses might be. In the meantime many new and better antagonists blocking NK_1 receptors have been synthesized or isolated from plants, e.g., Compositae (Yamamoto et al. 2002).

As a summary it can be stated that decades of intense research in the field of substance P, tachykinins, and their receptors have pioneered significant advances in the field of physiology, pathophysiology and pharmacology, yet the great pharmacological opportunities are still to come.

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