The Dopaminergic Innervation Of The Brain Stem And Spinal Cord

An Anatomical Study On The Distribution Of The Neurotransmitter Dopamine And Its D_2 Receptor.

This study was carried out at the Department of Anatomy of the Erasmus University of Rotterdam, The Netherlands (Head Prof. Dr. J. Voogd).

Financial support by NEN-Dupont for the publication of this thesis is gratefully acknowledged.

The Dopaminergic Innervation Of The Brain Stem And Spinal Cord

An Anatomical Study On The Distribution Of The Neurotransmitter Dopamine And Its D_2 Receptor

De dopaminerge innervatie van de hersenstam en ruggenmerg
Een anatomische studie naar de verdeling van de neurotransmitter dopamine en zijn D_2 receptor

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr. P.W.C. Akkermans MA
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 8 juni 2000 om 13.30 uur.

door Hendrik van Dijken
geboren te Emmen

Promotiecommissie

Promotor	Prof. Dr. J. Voogd
Overige leden	Dr. J.C. Holstege (tevens co-promotor)
	Prof. Dr. P.G.M. Luiten
	Dr. P. Voorn
	Prof. Dr. C.I. de Zeeuw

Contents

Chapter I

General Introduction

General Introduction

Until the first half of the 19th century, the anatomy of the nervous system was studied mainly by means of macroscopic methods, like dissection, while microscopy was hampered by the lack of adequate staining techniques. However, when reliable fixation and staining techniques (like the Weigert and Golgi stains and the silver impregnation technique for degenerating fibers) became available, the knowledge of the nervous system rapidly increased. Since then a multitude of new research methods employed by an ever increasing number of scientists involved in studying the nervous system, have led to a rapid progress in our knowledge and - to some extent - our understanding of the nervous system.

The classical anatomical studies and especially the findings of Ramon y Cajal (1909), the great advocate of the neuron theory, have laid the foundation of our present knowledge of the nervous system. Cytoarchitectonic studies of this period, made a big impact on neuroscience and served as the basis of the nomenclature of the central nervous system as it is still used today. Data on the chemical identity and the specific function of (groups of) nerve cells became available more recently. Often the data complemented, but sometimes it conflicted with, the cytoarchitecture based subdivisions of the brain. This induced the introduction of alternative nomenclatures, not based on cytoarchitecture, but on pharmacological or functional characteristics. This thesis, which describes an anatomical study of the dopamine innervation of the brain stem and spinal cord and one of the receptors involved, i.e. of a chemically identified system characterized by containing the transmitter dopamine, reflects some of these great changes, which transformed classical neuroanatomy into a dynamic, functional science. In this chapter the subject will be introduced by a short description of the anatomy of the spinal cord and the brainstem, followed by a description of the chemical anatomy of the nervous system, with the main emphasis on the dopaminergic system and its receptors. Subsequently, we will provide insight into the scope of this thesis.

Anatomy of the spinal cord and brain stem

The central nervous system is composed of the spinal cord, the brain stem (which consists of the myelencephalon, the metencephalon and the mesencephalon) and the forebrain (cerebrum, which consists of the telencephalon and diencephalon). The spinal cord, the most caudal part of the central nervous system, receives sensory information from the entire body below the face, by way of the dorsal divisions (the dorsal roots) of the spinal nerves, and activates muscle fibers as part of a motor command for reflex or voluntary movement, by way of the ventral divisions (the ventral roots) of the spinal nerves. The brain stem is a complex rostral continuation of the spinal cord and contains several collections of cell bodies, among which the cranial nerve nuclei. Similar to the spinal cord, the brain stem receives sensory information from the face, and contains the motor nuclei innervating the muscles of the face, eyes, and the cranial parasympathetic system. In addition, much of the specialized sensory information originating from the cochlea and vestibular labyrinth, the eyes, the taste buds, the cardiovascular, respiratory, and digestive systems directly reaches the brain stem, where it is further processed. Both the brain stem and spinal cord have a dual function: on the one hand they distribute afferent sensory information to higher centers, thereby determining levels of arousal, and execute the motor commands from these higher centers, while on the other hand they are capable of independently organizing and executing a basic motor response on the basis of afferent sensory information.

Anatomy of the spinal cord

The spinal cord represents the most caudal part of the central nervous system. It is located within the canal of the vertebral column surrounded by cerebrospinal fluid. During its embryonic development there is surprisingly little change in the internal anatomical organization of the spinal cord. Its peripheral nerve fibers are grouped into segmental spinal nerves that emerge from the vertebral canal between the individual vertebrae. As a result, the rat spinal cord is subdivided into 8 cervical (C1-C8), 13 thoracic (Th1-Th13), 6 lumbar (L1-L6), 4 sacral (S1-S4) and 3 coccygeal segments. In the center of the spinal cord lies the central canal, which is a remnant of the space within the primitive neural tube. The spinal cord is bilaterally symmetrical. It consists of a centrally located gray matter, which is characterized by a large number of neuronal cell bodies, and is surrounded by white matter, which is characterized by a large number of nerve fibers. Sensory information from the skin, joints, muscles and, to some extent, the viscera of the trunk and limbs enters the spinal cord by way of the dorsal roots. The motoneurons in the spinal cord constitute the final common pathway for issuing commands for muscle contraction by way of the ventral roots. In the spinal cord there is an orderly arrangement of the sensory and motor nuclei controlling the limbs and trunk. In addition, the spinal cord contains various ascending pathways, that convey sensory information towards the brain, and descending pathways which originate in higher parts of the brain and terminate within the spinal cord.

The spinal gray matter

The spinal gray matter, in cross section, is more or less butterfly-shaped. The dorsal protrusions on each side are known as dorsal horns and the ventral protusions as the ventral horns. The area in between the ventral and dorsal horns, where the left and right side of the spinal cord are interconnected, is termed the intermediate zone. Based on cytoarchitectonic criteria, first presented by Rexed in cat (1952, 1954), the gray matter can be further subdivided into ten different cell layers or laminae, numbered I to IX from dorsal to ventral, with lamina X representing the area that surrounds the central canal (fig. 1).

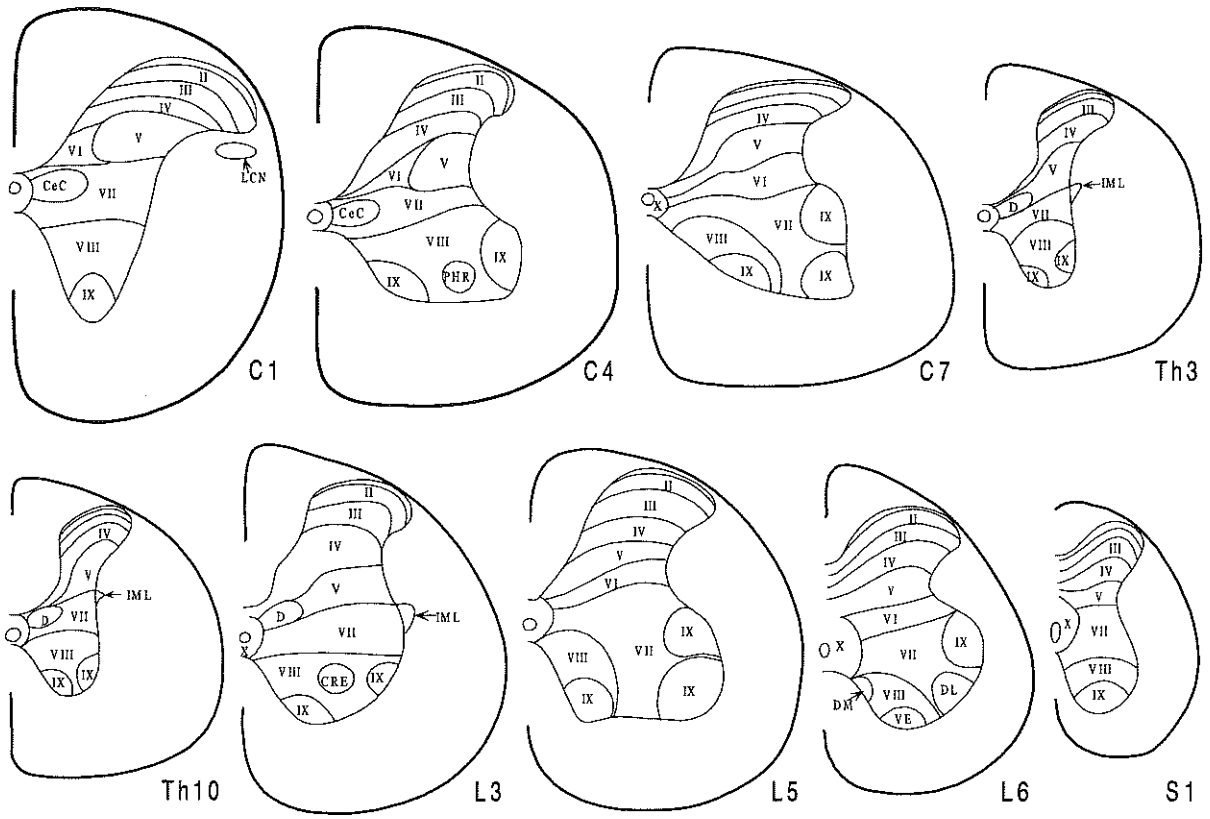


Figure 1: Diagrams of cross-sections of the spinal cord at cervical (C), thoracic (Th), lumbar (L) and sacral (S) levels. CeC, central cervical nucleus; LCN, lateral cervical nucleus; PHR, phrenic nucleus; D, dorsal nucleus (Clarke); IML, intermediolateral cell column; CRE, cremaster nucleus; DM, dorsomedial motor nucleus (SNB, spinal nucleus of the bulbocavernosus); VE, ventral motor nucleus; DL, dorsolateral nucleus; numerals I-X show the layers of Rexed.

In the classical nomenclature, lamina I is known as the marginal zone, lamina II as the substantia gelatinosa, laminae III and IV as the nucleus proprius, laminae V-VI as the base of the dorsal horn, laminae VII and VIII as the intermediate zone and lamina IX as the motoneuronal cell groups. Rexed's original description was based on the spinal cord of cat, but the lamination scheme has subsequently been adapted and applied to the spinal cords of other mammals, including the rat (Molander *et al.*, 1984; Molander *et al.*, 1989), and is now generally accepted as the standard subdivision of the vertebrate spinal cord. A potential problem with the laminar subdivision are the boundaries between laminae, which are zones of transition rather than exact lines of separation, since the laminar characteristics change sometimes gradually, sometimes more abruptly. The delineation is, therefore, open to considerable subjective interpretation as evidenced by the many minor differences in the various spinal lamination schemes proposed for the rat (see Molander *et al.*, 1984; Brichta and Grant, 1985; Molander *et al.*, 1989; Verburgh, 1990).

Lamina I, or marginal zone, covers the dorsal surface of the dorsal horn, of which it forms the upper most part, and curves smoothly around its apex, appearing largely as a thin layer of gray matter between the overlying zone of white matter (named the tract of Lissauer, containing many small myelinated axons), and the underlying gray matter of the dorsal horn. Lamina I contains many relatively small neurons of various types as well as large horizontal neurons (the marginal cells of Waldeyer), which are relatively sparse and directly project to the thalamus (Ribeiro-da-Silva, 1995). Functionally, lamina I serves as an important relay for nociception and temperature sense, but cells also respond to non-nociceptive primary afferent input. Lamina I cells project, mainly contralaterally, to the thalamus, hypothalamus and brainstem, apart from projections within the spinal cord (Willis *et al.*, 1995). Dorsal root fibers constitute the large majority of the afferent input to lamina I, consisting of both thinly myelinated and unmyelinated fibers, but there are also projections to lamina I that are derived from supraspinal levels, including the cortex, the hypothalamus (among which dopaminergic fibers, see chapter II), noradrenergic fibers from the locus coeruleus and serotonergic fibers from the raphe nuclei (Tracey, 1995; Willis *et al.*, 1995).

Lamina II, or substantia gelatinosa, is situated parallel to lamina I and is covered by that layer dorsally and laterally, but not medially. It is characterized by a scarcity of myelinated fibers, giving it a translucent (gelatinous) appearance in fresh tissue, staining darkly in cresyl violet preparations. Lamina II can be subdivided into an intensely stained outer zone (II_o) with densely packed cells and a less compact inner zone

(II_i) (Ribeiro-da-Silva, 1995). This subdivision is relevant, since subdivisions of the unmyelinated dorsal root afferents, as well as some supraspinal projections, appear to terminate preferentially in lamina II_i or II_o (see also chapter II), which is also reflected in the chemical heterogeneity of this area (Todd and Spike, 1993). The functional meaning of the subdivisions in lamina II is at present unclear. Lamina II neurons respond to nociceptive and non-nociceptive inputs and project primarily within the spinal cord (Willis *et al.*, 1995).

Lamina III is the widest layer and runs parallel to both lamina I and II. In contrast to lamina II, it receives a substantial number of myelinated dorsal root axons, which carry proprioceptive and cutaneous sensory information, but are not involved in the relay of pain and temperature information. Projections of lamina III are mainly within the spinal cord but some neurons project to the dorsal column nuclei or the thalamus (Molander and Grant, 1995).

Lamina IV forms a band beneath laminae II and III, but does not posses their characteristic lateral curvature. Lamina IV is broader than lamina III and its neurons are heterogeneous in size, including several large cells, which are not found in lamina III. Some of these cells may receive nociceptive input and contribute to the spinothalamic tract (Burstein *et al.*, 1990). Similar to lamina III, lamina IV receives many myelinated dorsal root afferents (Grant, 1995).

Lamina V forms the neck of the dorsal horn. It is characterized by the presence of many myelinated fibers in its lateral part, which gives this part a reticulated appearance. The general structure of the neuropil is not greatly different from lamina IV, hence the border between lamina IV and lamina V is unclear, especially medially. In comparison with lamina IV, lamina V contains a more heterogeneous population of cells (Molander and Grant, 1995). A relatively large number of neurons in lamina V contribute to the spinothalamic tract (Burstein *et al.*, 1990), in line with the input of, mainly thinly myelinated, nociceptive afferents to this lamina. Similar to lamina III and IV, lamina V receives proprioceptive and cutaneous sensory afferents and contains several cells projecting to the dorsal column nuclei and spinocervical tract. Medially in laminae IV and V, adjacent to the dorsal columns, there is longitudinal column of cells in the segments Th1-L2, known as the dorsal nucleus of Clarke, which relays information about limb position and movement to the cerebellum via the dorsal spinocerebellar tract (Matsushita and Hosoya, 1979)

Lamina VI exists only within the cervical and lumbar enlargements and forms the base of the dorsal horn (Verburgh, 1990). The input and projections of the cells in this lamina appear similar as those in lamina V. The border between lamina VI and lamina VII is demarcated by the transition from an area of cellular diversity to one of relative homogeneity.

Lamina VII constitutes most of the intermediate zone of the gray matter throughout the entire spinal cord, and regularly extends into the ventral horn. It contains a large variety of cells that receive a complex mixture of information from both primary afferent, spinal and supraspinal sources. Neurons in the intermediate zone may project to motoneurons, to many supraspinal nuclei or to other neurons throughout the spinal cord. In many cases they project to several different target areas through collaterals. Neurons in the intermediate zone have been subdivided based on specific criteria (like inhibitory versus excitatory or projecting preferentially to medial or lateral motoneurons or receiving a specific type of primary afferent input or on the basis of their chemical identity), which subcategories all overlap to a large extent. Therefore a comprehensive subdivision of the intermediate zone has not been identified (for review see Jankowska, 1992). An exception to this rule are autonomic preganglionic neurons, located laterally in lamina VII at the border of the gray and white matter in the segments Th1-L2, which form a longitudinal column also known as the lateral horn or the intermediolateral nucleus (Strack *et al.*, 1988). These neurons project to sympathetic ganglion cells in the sympathetic chain alongside the vertebral column, from where the sympathetic innervation of the entire body arises (Gabella, 1995). Some of these sympathetic preganglionic neurons are not located in the lateral horn, but more medially up to the central canal area in the intermediomedial nucleus (Strack *et al.*, 1988). At sacral levels (S1-S3), also laterally in the intermediate zone, preganglionic parasympathetic neurons are located that innervate the lower part of the trunk and the lower extremities . The parasympathetic innervation of the upper part of the body is derived from the brainstem (Haines, 1997).

Lamina VIII can be distinguished from lamina VII by the darker staining and more variable size of its cells. It is located in the ventromedial part of the ventral horn and contains commissural neurons, which project to the contralateral side.

Lamina IX is not a true layer, but consist of several groups of large motoneurons innervating the striated muscles of the limbs and the trunk. It can be subdivided into medial and lateral groups (for review see Kuypers, 1981). The medial motoneuronal groups innervate axial muscles, whereas lateral cell groups innervate the muscles of the body wall and the extremities. The spinal motoneurons of the lateral cell group are somatotopically organized. Thus, in the lateral motoneuronal cell group the motoneurons to flexor muscles are located dorsally, the motoneurons to extensor muscles are located more ventrally and laterally, and those supplying the muscles of the girdle and the proximal part of the upper limbs are located ventromedially. Motoneurons innervating proximal limb muscles are located more rostrally than those of distal muscles. Motor commands for voluntary movement reach the ventral horn via descending pathways from supraspinal levels, and connect to motoneurons primarily by way of interneurons, except for the projections to the medial motoneurons and, mainly in primates, the cortical projections to the lateral motoneuronal cell groups, which are especially important for steering distal extremity muscles involved

in fine manipulative movements. Reflex motor activity is also effectuated mainly by relay of sensory information via interneurons within the intermediate zone, with the exception of the Ia afferents derived from muscle spindles, that project directly onto motoneurons of the same (or functionally similar) muscles, from which they are derived.

Lamina X forms the area around the central canal and comprises most of the dorsal and ventral gray commissures. It seems to contact the ventral white commissure at all levels of the spinal cord. However, the borders of lamina X are ambiguous. Cells in lamina X receive primary afferent fibers, possibly with the emphasis on nociceptive and visceral input (Molander and Grant, 1995). In addition there are also supraspinal projections terminating within lamina X, including a prominent projection from the monoaminergic cell groups. The role of lamina X in the spinal cord is unclear. In view of its inputs (visceral, nociceptive and monoaminergic) and its chemical heterogeneity with many different neuropeptides, it may be regarded as the internal autonomic or limbic core region of the spinal cord.

Outside the gray matter, just lateral to the apex of the dorsal horn, a group of small cells is present, throughout the length of the spinal cord. In the first three cervical levels, this nucleus is known as the lateral cervical nucleus (Molander and Grant, 1995), which is also seen in other species, including human, and projects to the midbrain and the thalamus (Burstein *et al.*, 1990). Below the high cervical segments, the nucleus is known as lateral spinal nucleus, which is not normally seen in other vertebrates. It consist of neurons of varying size and form, projects to the midbrain tegmentum, hypothalamus and thalamus and receives both nociceptive and non-nociceptive sensory input.

The spinal white matter

The white matter surrounding the spinal gray matter is subdivided into different fiber zones or funiculi (see Haines, 1997 for review). The dorsal funiculi are located dorsal to the dorsal root entry zone. The left and right dorsal funiculi are separated by the dorsal septum. The ventral funiculi are located ventrally and largely separated by the anterior median fissure. However, fibers traversing these funiculi may cross to the contralateral side through the ventral commissure located just dorsal of the anterior median fissure. The lateral funiculi, subdivided into dorsolateral and ventrolateral funiculi, are located in the lateral parts of the spinal white matter. The various funiculi not only contain the ascending and descending fibers connecting the spinal cord with supraspinal levels, but also contain many propriospinal fibers that interconnect different spinal levels. The area occupied by these fibers is termed the fasciculus proprius and is located close to the spinal gray matter in all the funiculi throughout the length of the spinal cord (Kuypers, 1981). These proprio-spinal fibers are important for intersegmental communication as part of a large number of spinal reflexes.

Ascending systems in the spinal cord

There are several ascending fiber tracts for conveying information to supraspinal levels, which are located in different areas of the spinal white matter. These systems relay afferent information to the brain for three purposes: perception, arousal, and motor control. Three groups of fiber tracts are usually distinguished: (1) the dorsal column system, (2) the anterolateral system and (3) the spino-cerebellar system (Haines, 1997).

The dorsal column system contains myelinated and unmyelinated fibers derived from the ipsilateral spinal ganglia, although fibers originating from neurons in the ipsilateral spinal cord (mainly laminae III-V) also contribute significantly to this system. The dorsal column fibers convey sensory information, including proprioception, discriminative touch and vibration sense, and are strongly somatotopically organized (Grant, 1995). Some of the fibers, originating from spinal neurons, also contribute nociceptive information. Two main subdivisions of the dorsal column nuclei can be distinguished, especially at higher levels of the spinal cord: the gracile and cuneate fasciculi. The cuneate fasciculus contains information from sacral, lumbar and lower thoracic ganglia and spinal levels and terminates in the cuneate nucleus in the lower brainstem, while the gracile fasciculus contains information from high thoracic and cervical levels and terminates in the gracile nucleus. From these dorsal column nuclei, the medial lemniscus arises, which terminates in the contra-lateral thalamus, where they synapse mainly on neurons in the ventral posterior lateral nucleus, which, in turn, send their axons to synapse mainly in the somatic sensory cortex. Because somatotopic relationships are preserved throughout the system, the somatic sensory cortex contains an orderly representation of the body surface.

The anterolateral system is the second major ascending system that mediates somatic sensation (Haines, 1997; Willis *et al.*, 1995). It is actually composed of various ascending pathways, including spinoreticular, spinomesencephalic, spinothalamic and spinohypothalamic fibers (see Tracey, 1995 for review) and is located in the ventral and ventrolateral funiculus, i.e. the anterolateral portions of the spinal cord white matter. The fibers in the antero-lateral system are derived from neurons from both sides of the spinal cord, but with a contralateral predominance, and from several different laminae (mainly lamina I, and V-VII), although the relative contributions of the various laminae depend on the exact site of termination in the various brainstem areas. In addition it seems likely that individual fibers project to different brainstem areas through collateral projections. Fibers involved in contralateral projections cross in the ventral white commissure to reach the anterolateral system. There is a rough somatotopic organization of the anterolateral system with fibers from progressively more rostral spinal cord segments assuming a more ventral and medial position. The anterolateral system is the main pathway for conveying nociceptive and temperature information to the brainstem and thalamus (Willis *et al.*, 1995). Projections to the medulla are focussed on the reticular formation, especially its medial part. A similar

projection exists to the medial pontine reticular formation, in addition to a prominent projection to the parabrachial nuclei in the dorsolateral pons. The mesencephalic projections terminate in the superior colliculus, the periaqueductal gray and the reticular formation. The spinothalamic projection is the main pathway by which nociceptive and temperature information reaches the thalamus. It terminates in the ventropostero-lateral nucleus, the intralaminar nuclei and the posterior nuclear group of the thalamus. Interestingly in the rat, an important part from the spino-thalamic projections are derived from the upper cervical levels (Willis *et al.*, 1995). There are also spinal projections to the hypothalamus and more rostrally located areas of the telencephalon, like the septum, the nucleus accumbens, the amygdala, the bed nucleus of the stria terminalis and other areas generally considered as a part of the limbic system (Willis *et al.*, 1995). The number of these projections are of similar magnitude as those projecting to the thalamus and they may provide a direct input of nociceptive information into (mainly limbic) telencephalic areas, and thus may play a role in the affective components of pain sensation.

There are two major fiber tracts in the spinal cord that project to the cerebellum: the dorsal spinocerebellar tract and the ventral spinocerebellar tract, which are both located on the ventrolateral surface of the spinal cord (Tracey, 1995; Voogd, 1995). Fibers in the dorsal spinocerebellar tract are mainly derived from neurons of the dorsal nucleus of Clarke, which is located slightly dorsolaterally of the central canal bilaterally at spinal segments Th1 - L2 and receives proprioceptive (and some cutaneous) information from the lower extremities and the trunk. Most fibers in the dorsal spinocerebellar tract reach the cerebellum via the restiform body. Fibers in the ventral spinocerebellar tract are derived from neurons in the intermediate zone, including some cells at the ventrolateral border of the ventral horn, called spinal border cells, which all receive proprioceptive information especially from the lower extremities. These fibers project mainly via the brachium conjunctivum. Information from the forepaw and neck regions reaches the cerebellum via dorsal column projections to the external cuneate nucleus via the rostral spinocerebellar tract from the cervical enlargement or from neurons at the C1-C3 segments, which are located in the central cervical nucleus, which occupies the same position in the cervical spinal cord as the column of Clarke neurons in the Th1-L2 segments. In addition there are neurons in the cervical intermediate zone projecting to the cerebellum via the "rostral spinocerebellar tract" of Oscarsson. Spinocerebellar cells terminate as mossy fibers in the cerebellum and their projections are usually bilateral, with an ipsilateral predominance. In this respect, the spino-olivary projections may also be mentioned, since these projections may influence climbing fiber activity in the cerebellum.

Descending systems in the spinal cord

The descending fiber tracts in the spinal cord may be subdivided in two groups: those originating from the cortex and those originating from the brainstem (for review see Kuypers, 1981).

The corticospinal axons originate from pyramidal cells in layer V of the somatosensory cortex, which includes not only the primary motor and sensory cortex, but also various areas in the prefrontal cortex in the rat (corresponding to premotor and supplementary motor areas) and in the parietal cortex (corresponding to sensory and visual association cortex). The corticospinal fibers run through the corona radiata, the internal capsule and the pedunculus cerebri to form the pyramidal tract in the medulla. At the transition of the medulla oblongata and the spinal cord the pyramidal tract crosses to the contralateral side. In the rat spinal cord the cortico-spinal fibers are located in the ventral part of the dorsal funiculus, in contrast to its localization in the dorsolateral funiculus like in most higher mammals like the human. Fibers originating from the sensory areas of the cortex terminate in all laminae of the dorsal horn, while fibers originating from motor areas of the cortex terminate in the intermediate zone and the ventral horn, mostly on interneurons. However, direct projections to motoneurons also exist in the rat, especially at low cervical levels, from where the distal forepaw muscles, involved in fine manipulative movements, are innervated. A few fibers do not cross at the pyramidal decussation but stay on the ipsilateral side to run as the anterior corticospinal tract in the ventral funiculus. Fibers from this tract terminate bilaterally in the medial part of the ventral horn, both on interneurons and motoneurons, which are primarily involved in axial movements.

The descending pathways from the brain stem to the spinal gray matter may be divided in two main groups: diffuse projections to all laminae of the spinal cord originating from monoaminergic cell groups in the brainstem and projections involved primarily in motor coordination and focussed mainly on neurons in the intermediate zone (Holstege, 1991). The diffuse monoaminergic projections to the spinal cord may be divided in three groups: serotonergic projections originating from the caudal raphe nuclei and adjacent ventromedial reticular formation, noradrenergic projections from the nucleus coeruleus and subcoeruleus and the A5 cell group and dopaminergic projections from the posterior hypothalamus. These diffuse projections are characterized by a high degree of collateralization, such that neurons innervating cervical segments may, through collaterals, also innervate lumbar segments. In spite of the diffuse character of the descending monoaminergic projections, some topical relations have been identified in these systems (Holstege and Kuypers, 1987), such that the rostral part of the caudal raphe nuclei (i.e. the raphe magnus) seems to project predominantly to the dorsal horn, while more caudal parts (i.e. the raphe pallidus and obscurus) projects predominantly to the ventral horn, including the motoneuronal cell groups. Similarly, the locus coeruleus may project to the dorsal horn, while the subcoeruleus may project predominantly to the ventral horn and the A5 nucleus projects specifically to the preganglionic neurons in the lateral horn. For the

dopaminergic projection such a topical organization has not been identified as yet. Although the various monoaminergic cell groups in the brainstem project to virtually all the layers throughout the spinal cord, their projections are not completely identical and some differences exist (for details see Chapter II)

The brainstem areas that project mainly to the intermediate zone and show a very low degree of collateralization can be subdivided in a medial and a lateral group, based on their termination site in the spinal cord (Kuypers, 1981). The medial group terminates in the ventromedial part of the spinal gray matter, both on interneurons and motoneurons, thus controlling the muscles of the trunk and proximal extremities. These projections are important for maintaining balance and in postural fixation. The main components of the medial group are the reticulospinal tract (originating in the dorsal part of the caudal pons and medullary reticular formation), the medial vestibulospinal tract (originating in the medial and caudal vestibular nuclei), the lateral vestibulospinal tract (originating in the lateral vestibular nucleus), and the tectospinal tract (originating from the superior colliculus), which all descend in the ventral funiculus. The lateral group projects to the dorsolateral part of the intermediate zone where the interneurons are located that project to motoneurons of muscles of the distal extremities. The main component of this group is the rubrospinal tract, which originates in the caudal, magnocellular portion of the red nucleus. The rubrospinal tract descends in the contralateral dorsolateral funiculus. Some neurons in the dorsolateral pontine reticular formation also contribute to the lateral group of descending brainstem pathways.

Anatomy of the brain stem

The brain stem (truncus cerebri) is located between the spinal cord and the diencephalon (see Haines, 1997). This part of the brain can be divided into three major regions: the medulla oblongata (myelencephalon), the pons with the cerebellum (metencephalon) and the midbrain (mesencephalon). Although the pons and cerebellum together constitute the metencephalon, only the pons and not the cerebellum is considered a segment of the brainstem. The transition zone between the medulla and spinal cord is located approximately at the level of the foramen magnum. This is also the level of the pyramidal decussation. The cranial nerves that emerge from the medulla are the hypoglossal nerve (XII), leaving the medulla ventromedially, and the laterally emerging accessory (XI), vagus (X) and the glossopharyngeal (IX) nerves. The abducens (VI), facial (VII) and vestibulo-cochlear (VIII) nerves exit the brainstem at the junction of the pons and medulla. The medulla oblongata is the rostral extension of the spinal cord. The pons lies rostral to the medulla and appears as a protuberance on the ventral surface of the brain stem. It contains a massive set of neurons that relay information from the cerebral hemispheres to the cerebellum. The trigeminal nerve (V) enters the pons laterally at the middle level. The trochlear nerve (IV) leaves the brainstem at the dorsal side, at the transition between pons and mesencephalon. The midbrain is located rostral to the pons and is the smallest part of the brain stem. The oculomotor nerve (III) leaves the mesencephalon ventromedially.

As the rostral continuation of the spinal cord, the brain stem follows a developmental plan that is much like that of the spinal cord. The sensory input and motor output of the brain stem is carried by cranial nerves. Most of the cranial nerves arise from, or terminate on, nuclear groups in the brain stem and innervate structures of the head and neck, except for the vagus nerve, which innervates viscera in the rostral half of the body. The motor nuclei of the brainstem are located medially and ventrally and are derived from the embryonic basal plate, while the sensory nuclei of the brain stem are located mostly dorsally and laterally and are derived from the alar plate.

The motor nuclei of the brain stem can be subdivided into three sets of nuclei. One set of motor nuclei is located dorsomedially and innervates striated muscles of the head, that are derived from occipital myotomes (the tongue muscles) or derived from the orbit (the eye muscles). These nuclei include the hypoglossal nucleus (located in the medulla), innervating the tongue muscles and the abducens and trochlear nuclei (located in the pons) and the oculomotor nuclei (located in the mesencephalon), innervating the eye muscles. A second set of motor nuclei develops to form the preganglionic parasympathetic nuclei. These are the dorsal motor nucleus of the vagus nerve and the inferior salivatory nucleus in the medulla, the superior salivatory nucleus in the pons and the Edinger-Westphal nucleus in the mesencephalon. More ventrally and laterally, a third group of motor nuclei develops to innervate striate muscles that originate from the pharyngeal arches. These nuclei comprise the ambiguus nucleus of the medulla, and the facial nucleus and the motor trigeminal nucleus in the pons.

The sensory nuclei of the medulla, which are derived from the alar plate, are located dorsolaterally. They comprise the trigeminal nuclei, the nucleus of the solitary tract, the cochlear nuclei, and the vestibular nuclei. The trigeminal nuclei, which mainly receive general sensory input from the skin, mucosa, muscles and joints of the head and neck, consists of several subnuclei, which include the spinal trigeminal nucleus, located in the medulla and caudal part of the pons, the nucleus princeps located in the rostral pons and the mesencephalic nucleus of the trigeminal nerve. The most caudal part of the spinal trigeminal nucleus, which is a rostral continuation of the spinal dorsal horn, receives primarily pain and temperature information from the head and neck. Proprioceptive and exteroceptive sensory information is conveyed to more rostral parts of this nucleus and the nucleus princeps. The mesencephalic nucleus contains ganglion cells, with their peripheral projections terminating as muscle spindles in the mastication muscles and their central terminations on motor neurons of the trigeminal motor neurons. Other sensory nuclei in the medulla are the gracile and cuneate nuclei, which receive general

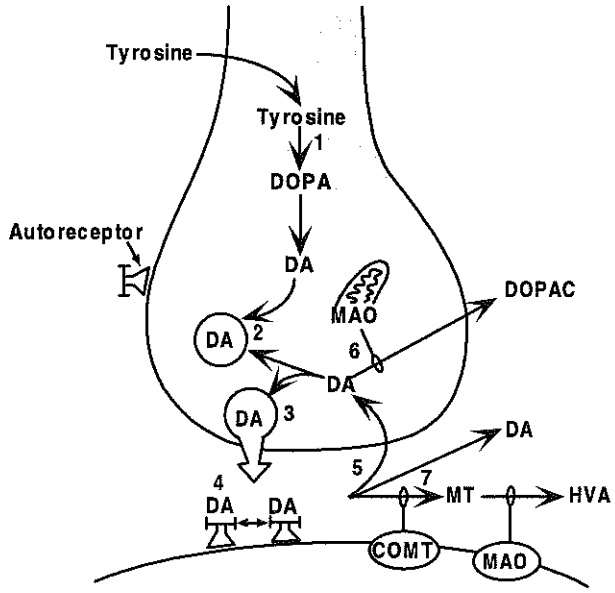


Figure 2: The key steps in the biosynthesis and degradation of dopamine (DA) are shown in this dopaminergic synapse (Adapted from Sachar, 1985). (1) Enzymatic synthesis (see also fig. 3). (2) Uptake and storage of dopamine by storage granules. (3) Release of dopamine. (4) Receptor interaction. (5) Reuptake. (6) Degradation. (7) Inactivation. COMT, catechol-*O*-methyltransferase; DA, dopamine; DOPA, dihydroxyphenylalanine; DOPAC, dihydrophenylacetic; HVA, homovanillic acid; MAO, monoamine oxydase.

sensory information from the entire body except the head. However, these nuclei do not receive pain and temperature information, which, instead, is processed in the dorsal horn of the spinal cord. The nucleus of the solitary tract is a sensory nucleus receiving visceral information from the cardiovascular system (blood pressure and heart rate), the digestive system (e.g. gastric extension), the respiratory system (e.g. oxygen levels, etc.) as well as taste information from the tongue and pharynx. The cochlear nuclei are involved in processing auditory information. The vestibular nuclei receive information from the vestibular labyrinth and are involved in the (reflex) control of eye movements, head movements and movements of the body as a whole (balance and posture).

Except for the motor and sensory nuclei that innervate the peripheral nerves, there are several nuclei in the brainstem, which project within the CNS. These nuclei include the inferior olive, which is located ventrally in the medulla rather than dorsolaterally and is a relay center for sensory and motor information to the cerebellum, and the lateral reticular nucleus, located ventrolaterally in the lower medulla, which projects to the cerebellum and receives afferents from a large number of brain structures including the cortex and the spinal cord. In this respect it is similar to the more medially located inferior olive, although their mode of termination in the cerebellum (respectively as mossy fibers and as climbing fibers) is very different. Another conspicuous nucleus is the superior olive, which is located ventrolaterally in the pons and is involved in processing auditory information. The parabrachial nucleus, which is situated around the brachium conjunctivum in the dorsolateral pons, is involved in processing autonomic (mainly from the nucleus of the solitary tract) and nociceptive (derived from the spinal and trigeminal dorsal horn) information, with strong reciprocal connections with the hypothalamus. A dorsal protrusion of the mesencephalon is the inferior colliculus, which is specifically involved in processing auditory information. Directly rostral to the inferior colliculus a similar, albeit more flattened, protrusion is formed by the superior colliculus. Together the inferior and superior colliculi can be seen macroscopically as four small elevations on top of the rostral brainstem, hence the name corpora quadrigemina. Located immediately below the corpora quadrigemina in the pons and mesencephalon around the aqueduct is the periaqueductal gray (PAG), which plays an important role in autonomic control. Ventrolaterally of the PAG and dorsolaterally from the substantia nigra in the mesencephalon, a large rounded cell group is present, termed the red nucleus, which is usually subdivided in caudal magnocellular and rostral parvocellular parts. Its main inputs arise from the cerebral cortex and the cerebellum, while its efferent projections are to the inferior olive (through the central tegmental tract) and contralaterally to the spinal intermediate zone, through the rubrospinal tract.

In addition to the various cell groups described above, the core of the brain stem is formed by a large number of neurons collectively known as the reticular formation and comparable to the intermediate zone of the spinal cord. The medullary, pontine and mesencephalic reticular formation consists of a heterogeneous population of cells, traversed by fiber bundles of some of the major tracts, giving it the reticular appearance. Although the reticular formation can be subdivided in different areas on the basis of cytoarchitecture (parvocellular and gigantocellular in

the medulla) or position (medial, lateral etc), this subdivision (and its delineation) is often confusing and an increasing number of nuclei in the reticular formation are now being defined on functional or chemical criteria, rather than on anatomical characteristics only (see e.g. Holstege, 1996). Thus specific areas in the brainstem reticular formation have been described that are involved in producing or organizing specific stereotyped behavior (like locomotion, balance and posture, respiration, vocalization, micturition, vomiting, swallowing etc.), or controlling autonomic functions (like cardio-vascular control and digestive functioning) or monitoring and controlling different types of afferent sensory information. Other areas, usually located more rostrally in the brainstem, are specifically involved in coordinating these basic functions in a context of relaxation, fight and flight, sexual arousal or sleep. Obviously, a particular brainstem area may be involved in several of these functions. These functional brainstem areas often coincide with anatomically defined areas, but may also be smaller or partly located outside such an area. In spite of these developments, the anatomical delineations are still most widely used. For the description in this thesis of the dopaminergic fibers and terminals and the D_2 dopamine receptors in the brain stem of the rat, the nomenclature used in the atlas of the rat brain by Paxinos and Watson has been adopted.

Chemical anatomy of the central nervous system

The great majority of the neurons in the central nervous system communicate with one another through chemical substances, called neurotransmitters, which are stored within neurons and released upon depolarization of a neuron generally with the purpose to act upon one or more other neurons (the target). Most neurotransmitters are released at specialized sites known as synapses (fig. 2). A synapse consists of a pre- and postsynaptic membrane, belonging to two different neurons, with an intercellular space, termed the synaptic cleft, in between. A neurotransmitter is generally released in the synaptic cleft by exocytosis from synaptic vesicles at the presynaptic membrane. It then traverses the synaptic cleft to bind at receptors at the postsynaptic membrane. In the majority of the cases the postsynaptic membrane of a synapse belongs to a dendrite or a cell soma, but part of a terminal or the axolemma also may serve as the postsynaptic membrane. Most transmitters are released by the axon terminals of a presynaptic neuron, but release from dendrites or somata also occurs. In addition, there may be release of neurotransmitter from non-synaptic sites of a neuron, which is known as non-synaptic or volume transmission. Neurotransmitters thus released may diffuse relatively large distances through the intercellular space to act on distal receptor targets. These target receptors are usually located on the neuronal membranes, but glial cells also display receptors on their membrane. The binding of a transmitter to a receptor may cause alterations in electrical or biochemical properties or gene expression of the cell, depending on the specific properties of the transmitter, the receptor and their interaction. A particular neurotransmitter may act at different (sub)types of receptors, but one receptor (sub)type usually interacts with only one transmitter. Substances that act on a receptor are termed ligands and neurotransmitters are the natural ligands of their cognate receptors. However, ligands that do not normally occur in the brain (like synthetic drugs or substances from plants) may act on one or more types of receptors, if they pass through the blood-brain barrier. These non-natural ligands may thus be used as tools to influence brain functioning, e.g. as therapeutic or experimental pharmaca. If a ligand is labeled (and its receptor-binding properties have not been significantly compromised), it may be used to identify the location (and/or the functional characteristics) of the receptors that bind this particular ligand.

In the past 50 years, an increasing number of chemicals that act as a neurotransmitter have been identified (see e.g. Nieuwenhuys, 1985). In order to determine whether a chemical substance may be considered a neurotransmitter three basic conditions have to be fulfilled: (1) The transmitter should be present in a neuron at its release sites; (2) it should be released after stimulation of the neuron and (3) after release it should produce a specific effect on another neuron, preferably through a specific receptor, which effect can be mimicked by exogenous application of the substance (see also Siegel, 1994; Haines, 1997). However, these conditions are not absolute and are still debated. The neurotransmitters that have been identified so far are usually divided in two groups: small molecules (with fewer than 10 carbon atoms) and neuropeptides (with more than 10 carbon atoms). The small molecules are further subdivided in four groups: (1) amino acids (Gamma-aminobutyric acid –GABA-, glycine and glutamate); (2) biogenic amines (acetyl-choline and the monoamines); (3) nucleosides and nucleotides (adenosine and ATP) and (4) the gaseous substance nitric oxide (NO). At present over 50 neuropeptides have been identified and there are several subdivisions in use to categorize these neuropeptides, mostly based on the area where they were first encountered or on the most well known member of a particular group e.g. opioid peptides (like enkephalin and dynorphin); posterior pituitary peptides (vasopressin and oxytocin); tachykinins (like substance P and neurokinins); glucagon related peptides (e.g. vasoactive intestinal peptide); pancreatic polypeptide-related peptides (e.g. neuropeptide Y) and many others. The receptors, associated with the various transmitters, also can be subdivided into two groups: those mediating fast synaptic transmission, in which case the receptor is itself an ion channel, and those mediating slow synaptic transmission, in which case the receptor is associated with a G-protein. Fast synaptic transmission is associated exclusively with small molecule transmitters, while G-protein coupled receptors are associated both with the group of small molecule transmitters and the group of neuropeptides.

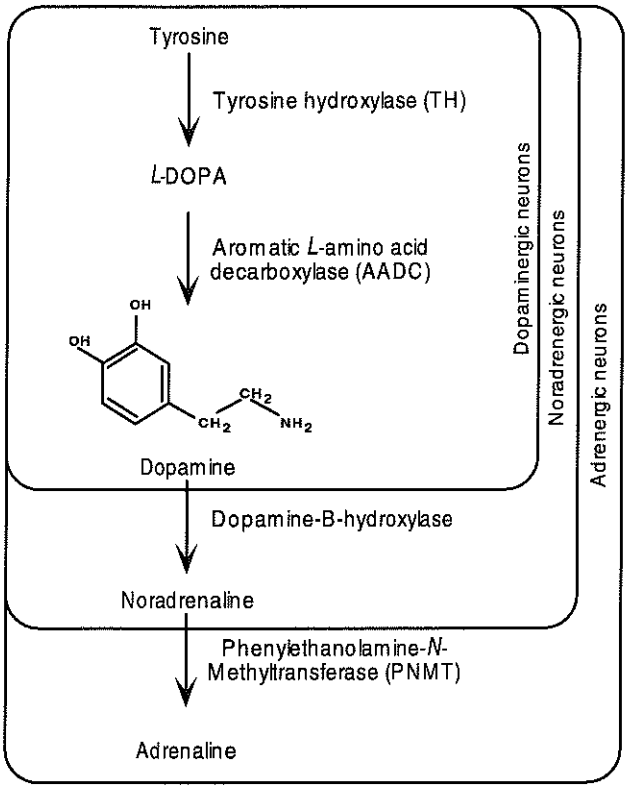


Figure 3: Key steps in the biosynthesis of monoamines. In dopaminergic neurons tyrosine is converted to the neurotransmitter dopamine. In noradrenergic neurons the precursor dopamine is converted to noradrenaline. In adrenergic neurons the precursor noradrenaline is converted to the neurotransmitter adrenaline.

This thesis is focussed on one particular small molecule transmitter: the monoamine dopamine and one of its receptors: the G-protein coupled dopamine D_2 receptor (see end of this chapter: Scope of the present study). Below the current knowledge of dopamine and its receptors will be shortly reviewed.

Monoaminergic transmitters and their receptors

The term monoamines is used to collectively address a group of substances, which act as transmitters in the brain. They are the indolamines, i.e. serotonin and histamine, and the catecholamines i.e. adrenaline, noradrenaline and dopamine. Monoamine containing neurons and their pathways in the brain stem were visualized for the first time by Dahlström and Fuxe (1964) using the Falck-Hillarp formaldehyde histofluorescence technique. This technique is based on the finding that monoamines in the brain become fluorescent after formaldehyde treatment. Fluorescence of indolamines (i.e. serotonin) is yellow, while catecholamine fluorescence is green. Thus it became possible to localize the various serotonergic and catecholaminergic cell groups in the rat brain stem, which they designated B1-B9 and A1-A12, respectively. Based on previous pharmacological and biochemical data it was inferred that the rostrally located A9-A12 groups were dopaminergic, while the more caudally located cell groups A1-A8 were noradrenergic. This basic subdivision of the monoaminergic cell groups in the brain is still used today with some additions, specifically three adrenergic cell groups in the lower brainstem (C1, C2, and C3) and four small dopaminergic cell groups in the diencephalic area of the brain (A13-A16). Since the initial experiments by Dahlström and Fuxe, more sophisticated techniques like immunocytochemistry and *in situ* hybridization have revealed many additional details about the morphology of the monoamine-containing neurons. In addition many data have emerged about the function and localization of the monoaminergic receptors.

Indolamines

Two indoleamines act as transmitters in the brain: serotonin and histamine. Serotonin, also known as 5-hydroxytryptamine (5-HT), was first isolated from platelets as the factor in serum with a tonic effect (hence: serotonin) on blood vessels (Rapport *et al.*, 1948). Serotonin belongs to a group of aromatic compounds called indoles with a five-membered ring containing nitrogen joined to a benzene ring. In the brain, serotonin is synthesized from the aminoacid tryptophan in two steps. First, a hydroxyl group is inserted at the 5 position on the indole ring by means of the enzyme tryptophan hydroxylase, which is the rate-limiting enzyme. The resulting molecule is then decarboxylated by 5-hydroxytryptophan decarboxylase to form 5-HT or serotonin (Frazer and Hensler, 1994).

Interestingly, 5-hydroxytryptophan decarboxylase and aromatic *L*-amino acid decarboxylase (an important enzyme in the production of dopamine, see later) appear to be identical. Thus the same enzyme is involved in the production of dopamine as well as serotonin (Jaeger *et al.*, 1984).

Serotonin-containing neurons in the brain stem are located in the mesencephalon, pons and medulla oblongata (Dahlström and Fuxe, 1964). The serotonin cell bodies, located on or near the midline (raphe) of the brain stem, can be divided into a rostral (superior) and a caudal (inferior) group (reviewed by Jacobs and Azmitia, 1992). The superior group consists of four main nuclei: the caudal linear nucleus (CLi; B8), the median raphe (MnR; B8 and B5) and its laterally displaced cells, the B9 neurons lying just dorsal to the medial lemniscus (ml), and the dorsal raphe nucleus (DR; B7 and B6). The inferior group consists of five main nuclei: the nucleus raphe obscurus (ROb; B2), the nucleus raphe pallidus (RPa; B1 and B4), the nucleus raphe magnus (RMg; B3) and a group of neurons ventrolaterally adjoining B3. B3 neurons are also located in the medulla at the level of the nucleus raphe obscurus (B2) just dorsal to the inferior olive and, more laterally, in the LPGi and the RVL. The few serotonergic cells located dorsally in the medulla were designated B4.

Whereas the serotonergic cell bodies are restricted to clusters of cells in the brain stem, their fibers innervate virtually every area of the brain. However, there are significant regional differences in their distribution (for details see Steinbusch, 1981; Björklund and Skagerberg, 1982; Jacobs and Azmitia, 1992; Kolta *et al.*, 1993; Halliday *et al.*, 1995). The superior group mainly projects rostrally towards the forebrain, whereas the inferior group projects caudally to the brain stem and spinal cord.

Serotonergic receptors

Originally, two classes of serotonin receptors were distinguished, namely 5-HT_1 and 5-HT_2 receptors, based on their specific affinity for serotonin and spiperone respectively. At present, seven major groups of serotonin receptors are recognized (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7), with at least seven distinct subtypes, each with a unique pharmacological and behavioral profile, and a distinct anatomical distribution (Jacobs and Azmitia, 1992; Halliday *et al.*, 1995).

The 5-HT_1 receptor family consists of four subtypes (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, and 5-HT_{1D}). 5-HT_{1A} receptors are usually defined using 8-hydroxy-2(di-*n*-propylamino)tetralin (8-OH-DPAT) and are concentrated within the raphe nuclei, the neocortex, septum and hippocampus. They act as cell body autoreceptors, but are also responsible for some of the postsynaptic actions of serotonin, especially in the hippocampus and cortex. 5-HT_{1B} sites only have been described in rat brain, by serotonin binding in the presence of the 5-HT_{1A} ligand 8-OH-DPAT. They were found to be densely distributed in the substantia nigra and globus pallidus. The 5-HT_{1B} receptor is present in the membrane of serotonergic axon terminals in rats and primates, where it acts as an autoreceptor with an inhibitory effect on the release of serotonin. 5-HT_{1A} and 5-HT_{1B} receptors both act by inhibiting adenylate cyclase. The highest levels of the 5-HT_{1C} receptor are present in the choroid plexus, with much lower but detectable levels in the hippocampus and cortex. 5-HT_{1C} receptors act by stimulating phosphatidylinositol hydrolysis. 5-HT_{1D} receptors share many of the properties of the 5-HT_{1B} receptors and were identified by pharmacological criteria only in brains of species devoid of the 5-HT_{1B} receptor.

The 5-HT_2 receptor, characterized by its high affinity for spiperone, is located postsynaptically and is associated with the fine serotonergic fibers in the middle layers of the cerebral cortex, mostly in layer IV. It is responsible for many of the behavioral effects of serotonin. The most recently described serotonin receptors are the 5-HT_3 and 5-HT_4 receptors. Both receptor types were initially identified in peripheral neurons, but subsequently they were also observed in the brain. 5-HT_3 receptors accumulate in the substantia gelatinosa of the spinal cord (suggesting a possible role in nociception) and in the area postrema of the medulla, with lower amounts in hippocampus, septum and amygdala.

Histamine

Histamine is the other indolamine transmitter in the brain (Nieuwenhuys, 1985) and is the least studied of the monoamines, even though there is a widespread histaminergic innervation of nearly all regions of the brain, including the spinal cord (Steinbusch, 1991). Histaminergic neurons are located primarily in the tuberomammillary nucleus of the posterior hypothalamus.

Histamine is formed from the amino acid histidine by decarboxylation, a reaction that is catalyzed by the enzyme histidine decarboxylase (HDC) or by aromatic amino acid decarboxylase (AADC). The effects of histamine on neurons are relatively slow in the onset and of long duration, similar to the majority of the effects of noradrenaline and serotonin. At present, three major groups of histamine receptors (H_1, H_2, and H_3) are recognized (Green, 1994). Stimulation of the H_1-receptor is associated with increased formation of cAMP and cGMP, increased phosphoinositide turnover, and ion shifts. H_2-receptors, on the other hand, are directly linked to adenylyl cyclase through a G protein. Stimulating the H_3 presynaptic receptor reduces release of endogenous histamine, indicating a function as inhibitory autoreceptor.

The presence of histamine receptors has been described in several brain regions (Green, 1994). High densities of H_1-receptors were found in rat hippocampus, but the density of H_1-receptors in brain regions varied among species. Interestingly, there is no similarity between the regional distribution of the H_1-receptor and histamine. The striatum of the guinea pig and the basal ganglia of primates show especially dense concentrations of H_2-receptors as compared to hippocampus and cortex. The H_3 autoreceptor is especially prominent in the cortex and basal ganglia.

Catecholamines

Catecholamines are substances that are characterized by a catechol ring, i.e. a 3,4-dihydroxylated benzene ring. The catecholamine neurotransmitters - dopamine, noradrenaline, and adrenaline - are all synthesized from the *L*-amino acid tyrosine in a common biosynthetic pathway (see fig. 3). The first enzyme, tyrosine hydroxylase (TH), converts the amino acid tyrosine to 3,4-dihydroxyphenylalanine or *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and is rate limiting for both dopamine and noradrenaline. *L*-DOPA is next decarboxylated by aromatic *L*-amino acid decarboxylase (AADC) to yield dopamine. In noradrenergic and adrenergic neurons, dopamine-β-hydroxylase (DBH) converts dopamine to noradrenaline. Lastly, in adrenergic neurons phenylethanolamine-N-methyltransferase (PNMT) methylates noradrenaline to form adrenaline, using *S*-adenosylmethionine as a methyl donor. Interestingly, there is a considerable homology in the nucleic acid sequences encoding for TH, DBH and PNMT, which appear to be linked on the same chromosome. It has therefore been suggested that the genes for these enzymes have evolved from a common ancestral gene.

Within neurons (fig. 2), high concentrations of catecholamines are stored in vesicles by means of amine transporters. After the release of a catecholamine from vesicles into the synaptic cleft or another part of the extraneuronal space, it will be inactivated by (re)-uptake into the catecholaminergic neuron by means of a cellular transporter, which is highly specific for a particular catecholamine. Catecholamines may also be inactivated enzymatically. Two main pathways are known: methylation of the 3-hydroxyl group of the catechol ring, which reaction is catalyzed by catechol-*O*-methyltransferase, or oxidative removal of their amino group by monoamine oxidase (MAO). Since MAO is present within neurons, it will only inactivate catecholamines that are not present within vesicles or otherwise protected.

Adrenaline

In the initial studies of Dahlström and Fuxe (1964), the amount of adrenaline in the brain was found to be too low to detect by spectrofluorometric analysis. Subsequent biochemical studies, however, demonstrated that the brain stem did contain adrenaline (Versteeg, 1976), a finding that was confirmed by immunohistochemical evidence showing the existence of three adrenaline-containing cell groups in the caudal medulla, which have been designated C1, C2, and C3 (Armstrong *et al.*, 1982; Hökfelt *et al.*, 1984; Kalia *et al.*, 1984). The rostral cell groups C1 and C2 are identical to the rostral parts of the catecholaminergic cell groups A1 and A2, respectively. The cell group C3 is located in the rostral medulla in the region of the medial longitudinal fasciculus.

Adrenaline-containing terminals have been observed in many areas of the central nervous system, including the brain stem and spinal cord (Fleetwood-Walker and Coote, 1981; Hökfelt *et al.*, 1984). The most prominent terminal fields in the brain stem and spinal cord include the ventral part of the locus coeruleus, the nucleus solitarius, the dorsal nucleus of the vagus, and the intermediolateral nucleus of the spinal cord, of which the latter three nuclei contain especially high levels of adrenaline. Experimental evidence suggests that the adrenergic projection to the spinal cord originates mainly from cell group C1 (Ross *et al.*, 1981; Armstrong *et al.*, 1982; Hökfelt *et al.*, 1984).

Noradrenaline

Neurons that synthesize noradrenaline are restricted to the pontine and medullary tegmental regions. Seven noradrenergic cell groups, designated as A1-A7, have been described in rat brain (Dahlström and Fuxe, 1964). The functional significance of the existence of several discrete subgroups of noradrenergic neurons in the central nervous system, however, has remained a controversial issue for a long time. Early anatomical studies of the projections of central noradrenergic neurons led to the widely accepted view of noradrenergic cells as a class of diffusely projecting neurons (Moore and Bloom, 1979). Two noradrenaline systems were distinguished: the locus coeruleus (LC) and the lateral tegmental system. Since then, it became increasingly evident that subgroups of central noradrenergic neurons have different and mostly non-overlapping projections and thus should be considered separate anatomical entities (Grzanna *et al.*, 1987; Grzanna and Fritchy, 1991; Aston-Jones *et al.*, 1995). In the rat CNS, the differential distribution of noradrenergic fibers derived from neurons in the locus coeruleus and those derived from non-coerulean noradrenergic neurons is particularly striking in the spinal cord and brain stem (Björklund and Skagerberg, 1982; Westlund *et al.*, 1983; Grzanna *et al.*, 1987; Lyons and Grzanna, 1988; Grzanna and Fritchy, 1991). In these regions, noradrenergic fibers from the locus coeruleus are primarily distributed to sensory nuclei while noradrenergic fibers of non-coerulean neurons are distributed to motor nuclei and the intermedio-lateral cell column (Grzanna and Fritchy, 1991).

Adrenaline and Noradrenaline receptors

Effects of both adrenaline and noradrenaline are mediated through adrenergic receptors. Two families of α-adrenergic receptors (α_1 and α_2) and one family of β-adrenergic receptors exist. At the present time, three subtypes of α_1- (α_{1A}, α_{1B}, and α_{1C}), three subtypes of α_2- (α_{2A}, α_{2B}, and α_{2C}), and three subtypes of β- (β_1, β_2, and β_3) adrenergic receptors have been identified.

Each subtype of the α-adrenergic receptor displays a unique distribution pattern, the α_1 adrenergic receptors being more restricted in their distribution than the α_2 adrenergic receptors. Of the α_1-receptor family, the α_{1A}-receptor is primarily localized in regions of the olfactory system, several hypothalamic nuclei, and regions of the brainstem and spinal cord, particularly in areas related to motor function. Additional regions include the intermediate layers of the cerebral cortex, cerebellum, and the hippocampal formation (McCune *et al.*, 1993; Day *et al.*, 1997). The α_{1B}-receptor is localized in most thalamic nuclei, dorsal and median raphe nuclei, and intermediate and deep layers of the

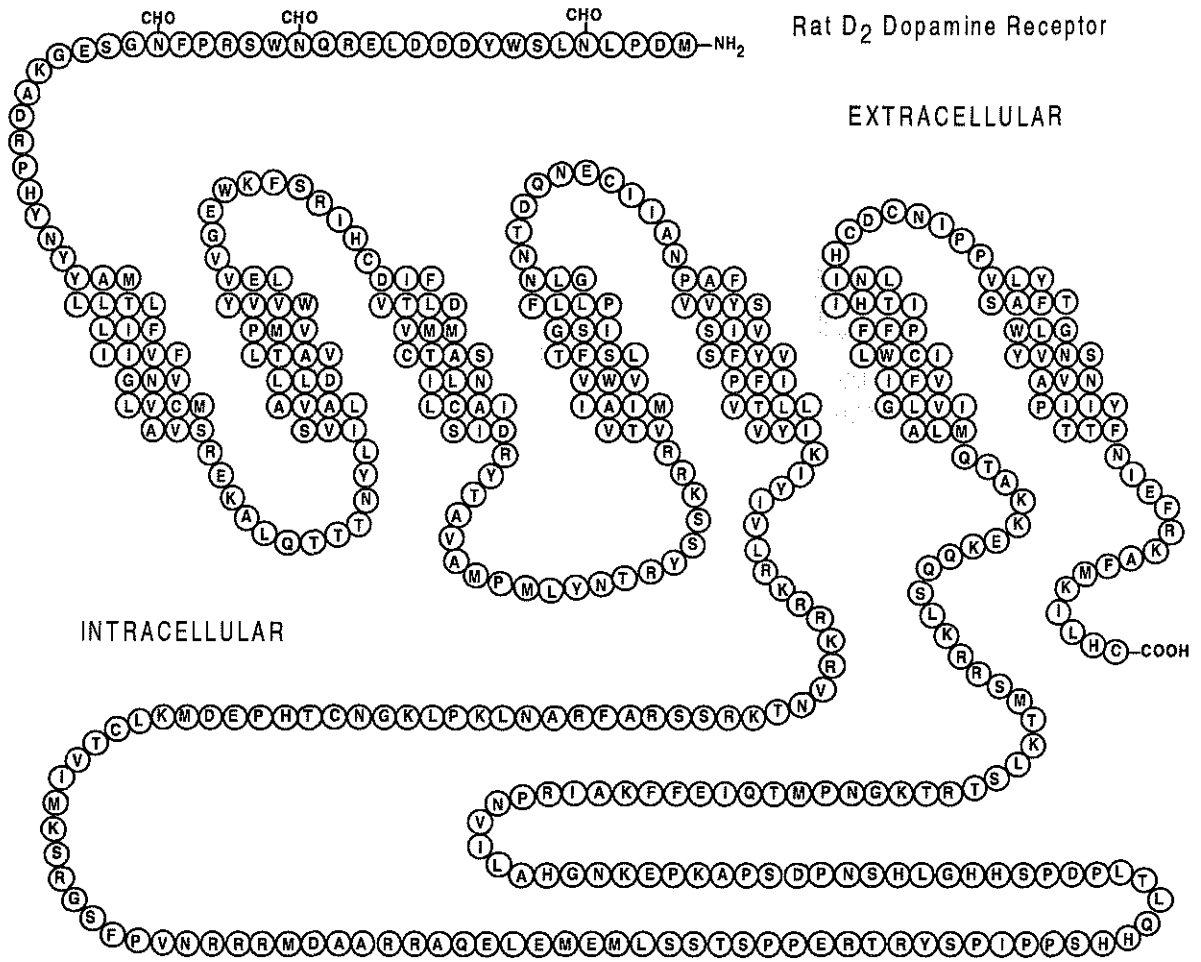


Figure 4: Proposed amino acid topography of the rat D_2-dopamine receptor, deduced from its cDNA sequence (Sibley, 1992). Adapted from Ariano, 1993.

cortex, hippocampus, cerebellum, and several other regions of the brain (McCune *et al.*, 1993; Day *et al.*, 1997). The α_{1C}-receptor has not yet been identified in tissues. The distribution of the α_{1D}-receptor is the most discrete of the α_1-receptors. It is localized in the olfactory bulb, cerebral cortex, hippocampus, reticular thalamic nucleus, regions of the amygdala, motor nuclei of the brainstem, inferior olivary complex and spinal cord (Day *et al.*, 1997). Of the α_2-receptor family, the α_{2A}-receptor is most abundant in the locus coeruleus, but is also found in many other brain areas including the brain stem, spinal cord, hippocampus, hypothalamus, olfactory bulb, cerebral cortex, pons, and cerebellum (McCune *et al.*, 1993; Nicholas *et al.*, 1993; MacDonald and Scheinin, 1995; Scheinin *et al.*, 1994). The α_{2B}-receptor is only found in the thalamus (McCune *et al.*, 1993; Nicholas *et al.*, 1993; Scheinin *et al.*, 1994; MacDonald and Scheinin, 1995) and the α_{2C}-receptor has been primarily observed in the basal ganglia and hippocampus, but it is also found in the olfactory bulb, cerebral cortex (particularly the cingulate cortex), pons and cerebellum (Nicholas *et al.*, 1993; Scheinin *et al.*, 1994; MacDonald and Scheinin, 1995).

The brain contains both β_1- and β_2-receptors, which cannot be differentiated in terms of their physiological functions. Adrenaline and noradrenaline are equally potent agonists for the β_1-receptor, but adrenaline is more potent than noradrenaline for β_2-receptors. β-receptors act through stimulation of adenylyl cyclase. β_1-adrenergic receptors predominate in the cerebral cortex, the hippocampus, the Islands of Calleja, and several nuclei of the thalamus. Approximately equal levels of β_1- and β_2-receptors occur in the substantia nigra, the olfactory tubercle, cerebral cortex, the medial preoptic nucleus, and all nuclei of the medulla. β_2-adrenergic receptors predominate in the molecular layer of the cerebellum, and in several thalamic nuclei (Rainbow *et al.*, 1984). The density of β_1-receptors varies in different brain areas to a greater extent than that of β_2-receptors. It has been suggested that this is due to the presence of β_2-receptors on glia or blood vessels.

Dopamine

Until the late 1950's dopamine (DA = 3,4-dihydroxyphenethylamine; also known as oxytyramine or hydroxytyramine) was exclusively thought of as a precursor in the biosynthesis of the catecholamines noradrenaline and adrenaline (Blaschko, 1939). Thereafter, it became clear that there existed an important and independent dopamine system, involved

in the regulation of many CNS functions.

The brain stem dopamine-containing neurons are confined to the mesencephalon and diencephalon as originally described by Dahlström and Fuxe (1964). Most dopaminergic neurons are found in the mesencephalic dopamine cell system, which includes the retrorubral nucleus (A8), the substantia nigra (A9) and the adjacent ventral tegmentum of the midbrain (ventral tegmental area of Tsai; A10). The neurons of the diencephalic dopamine cell system (groups A11-A14) are considerably fewer in number than the mesencephalic dopamine cells. These dopaminergic cell groups include (Dahlström and Fuxe, 1964; Fuxe, 1965; Fuxe and Hökfelt, 1966; Björklund and Nobin, 1973): (1) The rostral periventricular cell group (A14) with neurons scattered in the periventricular hypothalamic region, from the level of the anterior commissure back to the rostral border of the median eminence; (2) the dorsal hypothalamic cell group (A13) comprising neurons clustered in the medial zona incerta, just ventromedial to the mammillothalamic tract; (3) the tuberal cell group (A12) located in the arcuate nucleus and the adjacent pan of the periventricular nucleus; and (4) the caudal cell group (A11) distributed in the posterior and dorsal hypothalamic areas and in the periventricular gray matter of the caudal thalamus.

The best known projection from A11-A14 cell groups is the tuberohypophyseal dopamine system, which originates in the A12 cell group and innervates the median eminence and the neural lobe and pars intermedia of the pituitary (Fuxe and Hökfelt, 1966; Björklund and Nobin, 1973; Fuxe *et al.*, 1974). It seems likely that neurons in the other cell groups give rise to local diencephalic innervations, although these projections are less well established. The only extradiencephalic projection of the A11-A14 cell groups identified so far is the descending diencephalospinal dopamine system (Commissiong *et al.*, 1978; Björklund and Skagerberg, 1979; Blessing and Chalmers, 1979; Commissiong and Neff, 1979; Hökfelt *et al.*, 1979), which, in the rat, has been found to innervate various parts of the spinal cord (Skagerberg *et al.*, 1982). This A11 group is widely distributed along the rostrocaudal axis ranging from the posterior and dorsal hypothalamic areas, periventricular gray of the caudal thalamus, to the mesencephalic periaqueductal gray. In the frontal plane, neurons of the A11 group can be found along the dorsal and medial aspects of the fasciculus mammillothalamicus and, more caudally, in between the fasciculus retroflexus and the ventricle in the region of Darkschewitsch' nucleus.

Dopamine receptors

Dopamine exerts its diverse effects by specific, high-affinity binding to G protein-coupled receptors (for reviews see Civelli *et al.*, 1991; Sibley and Monsma, 1992; Gingrich and Caron, 1993; O'Dowd, 1993). In the late 1970's the neurotransmitter dopamine was thought to act through only two types of receptors, known as the D_1 and D_2 dopamine receptors (Kebabian and Calne, 1979). Pharmacological and biochemical data showed that, the D_1-receptor was responsible for the stimulation of adenylate cyclase, whereas the D_2-receptor mediated the inhibition of adenylate cyclase, or had no effect on it. These differential effects on the activity of adenylyl cyclase became a major distinguishing feature between D_1- and D_2-receptors. However, the results obtained with molecular biological techniques showed that the D_1- and D_2-receptors in fact represented two classes of dopamine receptors, which may be further subdivided on the basis of structural and functional similarities (Strange, 1991; Sibley and Monsma, 1992). The rat D_2-receptor was the first dopamine receptor to be cloned (Bunzow *et al.*, 1988). This receptor was isolated by low stringency screening of a rat brain cDNA library using the hamster β_2-adrenergic receptor cDNA as a probe. This cloning strategy, which is based on evolutionary conservation of a sequence, is commonly referred to as homology screening and has proven to be the most effective in identifying new members of the dopamine receptor gene family. The probes were subsequently used to clone the human D_2 dopamine receptor gene and a second form of the receptor ($D_{2(long)}$). These discoveries were quickly followed by the cloning of the D_1 dopamine receptor, called the D_1-receptor in rat and D_{1A}-receptor in human (Dearry *et al.*, 1990; Gerfen *et al.*, 1990; Sunahara *et al.*, 1990; Zhou *et al.*, 1990). Thereafter, probes based on the D_1 and D_2 dopamine receptors have led to the identification of other genes coding for the D_3 (Sokoloff *et al.*, 1990), D_4 (van Tol *et al.*, 1991; Todd *et al.*, 1992), D_5-receptors (Civelli *et al.*, 1991; Sunahara *et al.*, 1991; Tiberi *et al.*, 1991; Weinshank *et al.*, 1991), and two D_5-related pseudogenes (Civelli *et al.*, 1991). They are judged to be pseudogenes because of the presence of numerous in-frame stop codons that would produce truncated proteins and, presumably, nonfunctional receptors (Civelli *et al.*, 1991). Two additional members of the D_2 family, the rat D_3- and D_4-receptors have been cloned by low stringency hybridization using probes derived from the D_2-receptor. Thus, there are two known D_1-like receptors (D_1 and D_5) and three known D_2-like receptors (D_2, D_3, and D_4)

All the presently known dopamine receptor isoforms are members of the supergene family of receptors that couple to their effectors via guanine nucleotide binding proteins, the so-called G-proteins. Members of this family posses seven stretches of relatively hydrophobic amino acids which have been proposed to form alpha-helical transmembrane spanning regions linked by loops of varying size (O'Dowd, 1992) (fig. 4). In this model the amino terminal end of the receptor is extracellular, the polypeptide chain crosses the cellular membrane seven times and the carboxyl terminal end is intracellular (see Todd and O'Malley, 1993). There is relatively high sequence identity between receptor types in the transmembrane (Tm) regions, while the hydrophilic regions have much more sequence diversity among family members. Most of the known receptors have a long cytoplasmic loop between transmembrane regions 5 and 6 (3rd intracellular loop), which is thought to be involved in binding to G-proteins, whereas Tm 3 and 5 (and perhaps Tm 2 and 7) are thought to be involved in dopamine binding (Dahl *et al.*, 1991). The G-protein

coupled dopamine receptors respond to the binding of their cognate neurotransmitter by changing the metabolic machinery of the postsynaptic cell through a mechanism involving the formation of intracellular second messengers, i.e. cyclic AMP. The receptor and the cyclase do not interact directly, but are coupled by transducer proteins that shuttle through the membrane. There are several kinds of G-proteins: stimulatory (G_s) or inhibitory (G_i). These proteins bind GTP or GDP. When the transmitter is bound to the receptor and the complex is associated with G_s, GTP displaces GDP at the nucleotide site on the transducer. The G-protein now associates with the catalytic subunit of adenylate cyclase, stimulating it to catalyze the conversion of ATP to cyclic AMP. The GTP-G-protein and the catalytic subunit of the cyclase together constitute the active form of cyclase. When GTP is bound to G_i, the complex inhibits cyclase activity. The duration of cyclic AMP synthesis is regulated by the GTPase activity of G_s. Its potency as an activator of the cyclase is again restored by interacting with the transmitter-receptor complex at the external surface of the cell.

Numerous G protein-coupled receptors described to date have proven to be intronless within their coding regions (c.f. Gingrich and Caron, 1993). As far as the dopamine receptors are concerned, the receptors of the dopamine D_1-like family also are intronless. However, the receptors of the D_2-like family are an exception to this rule and belong to the category of intron-containing G protein-coupled receptors (O'Malley *et al.*, 1990). The dopamine D_2-receptor is interrupted by five to six introns (Giros *et al.*, 1989; Monsma *et al.*, 1989), the D_3 by five (Sokoloff *et al.*, 1990), and the D_4 by four (van Tol *et al.*, 1991). The presence of introns within the coding region of the D_2-like receptors allows for the possibility of alternate splicing of the exons. Such splice variants have been found in other receptor systems, where such splice variants form functionally different receptor proteins. The D_2-receptor has been found to exist in two isoforms ($D_{2(short)}$, $D_{2(long)}$), generated by alternate splicing of the 87 base pair exon between introns 4 and 5 (Giros *et al.*, 1989; Monsma *et al.*, 1989; O'Malley *et al.*, 1990; Gandelman *et al.*, 1991). These isoforms differ, therefore, by 29 amino acids in the putative third intracellular loop (fig. 4). Despite the fact that the 3rd intracellular loop is thought to be involved in the function of G protein recognition, thus far no functional differences have been reliably ascribed to the D_2-receptor splice variants. It has now been extensively demonstrated that both the $D_{2(short)}$ and $D_{2(long)}$ forms of the cloned D_2-receptor both act through the inhibition of adenylate cyclase and the activation of K^+ channels. So far, no differences in coupling efficiency have been observed between the $D_{2(short)}$ and $D_{2(long)}$ forms. The D_3 gene can also undergo alternative splicing to form several shorter variants (Bouthenet *et al.*, 1991). Similar to the D_2-receptor, one of these variants is 63 bases shorter and codes for a functional receptor that lacks a 21 amino acid stretch in the 3rd cytoplasmic loop. D_3 mRNA has a unique distribution in the brain, including prominent expression in limbic areas such as the islands of Calleja, the nucleus accumbens and the mammillary nuclei. It is also expressed in many classical D_2-receptor rich regions such as the striatum (Sokoloff *et al.*, 1990). The D_4-receptor is the most recently described D_2-like sequence (van Tol *et al.*, 1991). In contrast to the D_2- and D_3-receptor genes, the D_4 gene is relatively small consisting of 4 known exons and 3 introns spanning about 3500 bases. No alternate RNA splicing has been detected for the D_4-receptor (van Tol *et al.*, 1991). However, the (human) D_4 gene itself appears to be highly polymorphic in human populations (O'Dowd, 1992; Seeman *et al.*, 1992). When expressed in transfected cell lines the splice variants are functional and can be distinguished pharmacologically (Seeman *et al.*, 1992).

Scope of the present study

Dopamine is involved in many aspects of brain functioning and abnormalities in the function of dopaminergic neurons have been implicated in many neurologic and psychiatric disorders, like Parkinson's disease, Tourette syndrome, schizophrenia, depression, and mania. Consequently, the role of dopamine in brain functioning has been extensively studied with the focus on locomotor activity, positive reinforcement, spatial memory, higher cognitive functions and neurohormone release. In order to get more insight in the (dys)functioning of dopamine in these neurologic diseases, there always has been a keen interest in the anatomy, physiology, and especially the pharmacology of dopaminergic systems in the CNS. Since most of the dopamine-linked neurologic and psychiatric disorders appear to be associated primarily with the telencephalic and diencephalic areas of the brain, the overwhelming majority of the dopamine studies have focussed on these areas, rather than on the brain stem and spinal cord. Furthermore, the majority of the dopaminergic neurons are located in these rostral parts of the brain and are largely absent in the brainstem and spinal cord. The finding that a small group of dopaminergic neurons in the caudal diencephalon projected to the spinal cord and brainstem indicated that dopamine was also involved in modulating the functions of these lower parts of the central nervous system. Previous findings had shown that other members of the monoamine group of transmitters, especially serotonin and noradrenaline, exerted strong effects on motor, sensory and autonomic transmission in the brain stem and spinal cord. Attempts to identify these dopaminergic projections in detail proved difficult, since (nor)adrenergic and dopaminergic fibers could not be reliably distinguished. The development of antibodies that identified exclusively dopaminergic fibers and terminals combined with data suggesting a close interaction of dopamine with other monoamines which are directly involved in brain stem and spinal cord functions as well as in higher cognitive functions, have prompted the initiation of the studies described in this thesis. In these studies, we have attempted to compare the dopamine distribution with that of (nor)adrenaline and serotonin, since these are chemically related molecules and show a similar mode of projection: large parts of the brain are

innervated through collaterals derived from a relatively small number of neurons. Apart from the dopaminergic innervation itself, the distribution of the different dopamine receptors must also be considered in order to understand the role of dopamine in the brainstem and spinal cord. In the present studies we have identified the localization of the D_2 receptors in spinal cord and brain stem. The localization of other dopamine receptor subtypes was not studied, mainly due to a lack of reliable antibodies. However, by comparing our data with more recent data from the literature, a comprehensive picture of the dopaminergic system in brain stem and spinal cord is obtained.

In the first part of this thesis (chapter II-III), the distribution of the neurotransmitter dopamine and its D_2-receptor is described in spinal cord. In Chapter II, the distribution of dopamine in rat, cat, and monkey spinal cord is documented employing an antibody directed against glutaraldehyde bound dopamine. In chapter III, the distribution of dopamine D_2-receptor in rat spinal cord is analyzed, using immunocytochemistry and *in situ* hybridization. The localization of its D_2-receptor is established using an anti-peptide antibody raised against the putative 3rd intracellular loop of the D_2-receptor. In the second part of this thesis (chapter IV-V), the distributions of dopamine and its D_2-receptor in rat brain stem are described. For this purpose the same immunocytochemical techniques were used as described in chapter II-III. In the last chapter (chapter VI) the main findings obtained in the studies of this thesis are discussed.

The Distribution Of Dopamine Immunoreactivity In The Rat, Cat And Monkey Spinal Cord

Jan C. Holstege, Henk van Dijken, Ruud M. Buijs*, Erika Goedknegt, Taco Gosens and Corlinda M.H. Bongers
Journal of Comparative Neurology, 376: 631-652 (1996)

* The Netherlands Institute for Brain Research Meibergdreef 33
1105 AZ Amsterdam, The Netherlands

The Distribution Of Dopamine Immunoreactivity In The Rat, Cat And Monkey Spinal Cord

Abstract

In the present study, the distribution of dopamine was identified light microscopically in all segments of the rat, cat and monkey spinal cord by using immunocytochemistry with antibodies directed against dopamine.

Only fibers and (presumed) terminals were found to be immunoreactive for dopamine. Strongest dopamine labeling was present in the sympathetic intermedio-lateral cell column (IML). Strong dopamine labeling, consisting of many varicose fibers, was found in all laminae of the dorsal horn, including the central canal area (region X), but with the exception of the substantia gelatinosa, which was only sparsely labeled, especially in rat and monkey. In the motoneuronal cell groups dopamine labeling was also strong, showing a fine granular appearance. The sexually dimorphic cremaster nucleus and Onuf's nucleus (or its homologue) showed a much stronger labeling than the surrounding somatic motoneurons. In the parasympathetic area at sacral levels, labeling was moderate. The remaining areas, like the intermediate zone (lamina VI-VIII), were only sparsely innervated. The dorsal nucleus (column of Clarke) showed the fewest dopamine fibers as did the central cervical nucleus, suggesting that cerebellar projecting cells were avoided by the dopamine projection. In all species, the descending fibers were located mostly in the dorsolateral funiculus, but laminae I and III also contained many rostro-caudally oriented fibers.

It is concluded that dopamine is widely distributed within the spinal cord, with few differences between species, emphasizing that dopamine plays an important role as one of the monoamines that influences sensory input as well as autonomic and motor output at the spinal level.

Introduction

The existence of dopamine (DA, also known as oxytyramine or hydroxytyramine) in the brain was already known for some time (Blaschko, 1939), when it was recognized in the late 1950's that dopamine could function as an independent catecholamine transmitter (Montagu, 1957), possibly involved in Parkinson's disease (Carlsson, 1959). Initially dopamine was localized with the Falck-Hillarp formaldehyde histofluorescence technique, which was used by Dahlström and Fuxe (1964) for a detailed mapping of dopamine cell groups, fibers and terminals in the brain. With respect to the spinal cord they concluded that, in contrast to noradrenaline, dopamine was not present. However, with the histofluorescence technique (and its modifications) it is difficult to differentiate between noradrenergic and dopaminergic fibers and terminals, especially in areas where they are intermingled. This problem does not occur with biochemical techniques, which showed that after transecting the rat spinal cord (Magnusson, 1973) or traumatizing the spinal cord of dogs (Hedeman *et al.*, 1974), the noradrenaline concentration caudal to the lesion declined at a different rate than the dopamine concentration. These findings suggested a transmitter role for dopamine, independent from noradrenaline, even though the dopamine concentration in the spinal cord is five to ten times lower than the concentration of noradrenaline (Hedeman *et al.*, 1974; Commissiong *et al.*, 1978; Karoum *et al.*, 1981; Basbaum *et al.*, 1987). Furthermore, destruction of the locus coeruleus led to a decrease of the noradrenaline concentration in the spinal cord, while the concentration of dopamine remained unchanged (Commissiong *et al.*, 1978). Similarly, (partial) depletion of noradrenaline by 6-hydroxydopamine did not lead to a change in the dopamine concentration in the spinal cord (Mouchet *et al.*, 1982). The use of neurotoxins to deplete noradrenaline made it also possible to identify dopamine containing structures with the histofluorescence technique, since the labeling observed after such treatment could only be attributed to the presence of dopamine. Using this approach it was found (Skagerberg *et al.*, 1982) that in the spinal cord, dopamine was present only in fibers and varicosities, located mainly in the dorsal horn, the area around the central canal and in the IML, with only a few scattered fibers in the ventral horn. The existence of a dopamine projection to the spinal cord, separate from noradrenaline, was also suggested by experiments in rat, combining the retrograde transport of a fluorescent tracer with histofluorescence (Björklund and Skagerberg, 1979) or tyrosine hydroxylase (TH) immunocytochemistry (Hökfelt *et al.*, 1979). These experiments showed the existence of a catecholaminergic projection from the diencephalon to the spinal cord (diencephalospinal tract), originating in the A11 cell group and, in the rabbit, the A13 cell group (Blessing and Chalmers, 1979). The existence of a dopamine projection from the paraventricular hypothalamic nucleus to the spinal cord has also been suggested (Swanson *et al.*, 1981), but this was not confirmed in later studies (Skagerberg and Lindvall, 1985; Skagerberg *et al.*, 1988). Since neurons in the A11 cell group do not contain dopamine-β-hydroxylase (Swanson and Hartman, 1975), the enzyme synthesizing noradrenaline from dopamine, they were considered as dopaminergic. Further investigations on the diencephalo-spinal system (Skagerberg and Lindvall, 1985) showed that relatively few cells in the A11 cell group provided the dopamine innervation for

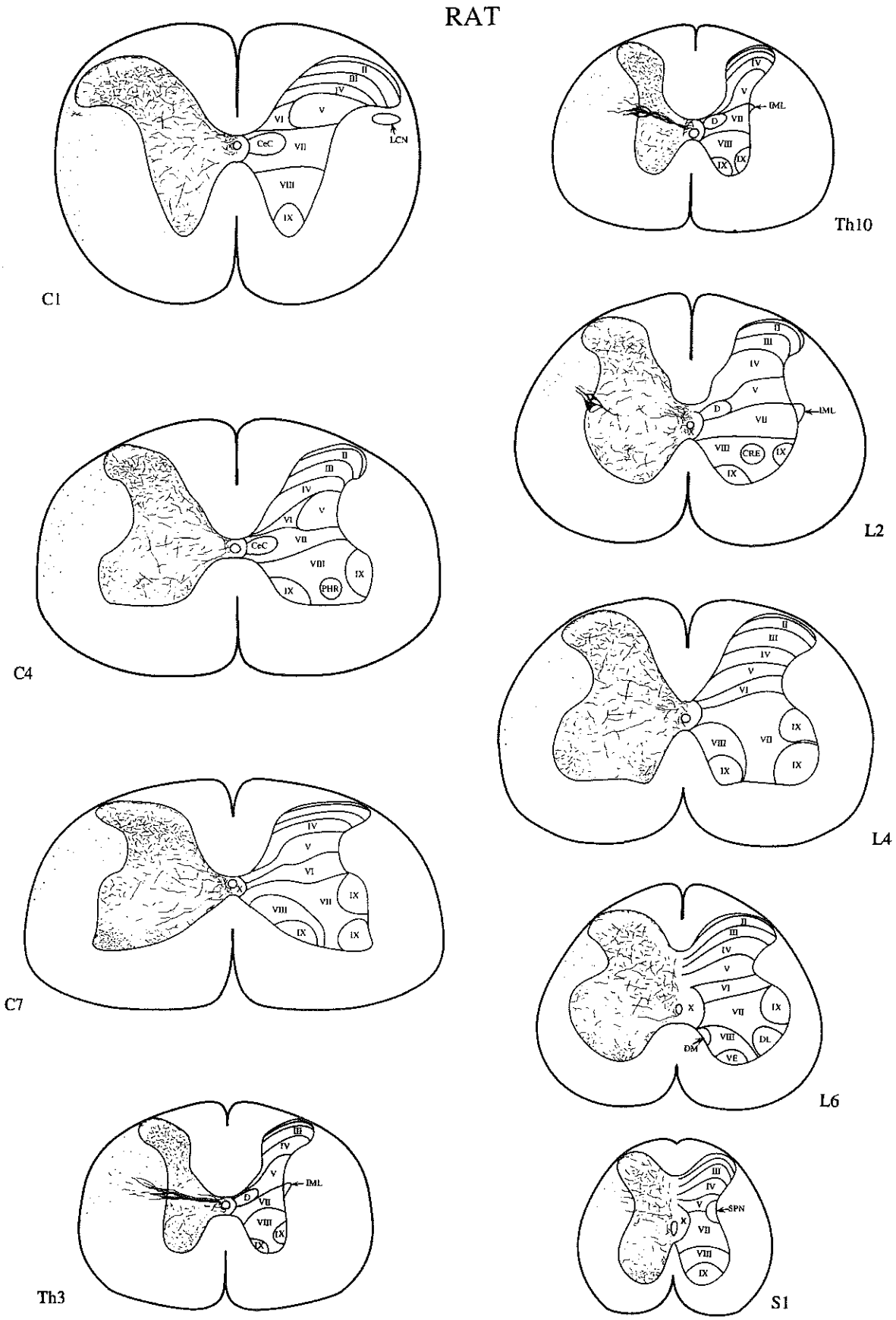


Figure 1: Schematic representation of the dopamine innervation in selected segments of the rat spinal cord. For details see text. Abbreviations: CeC, central cervical nucleus; CRE, cremaster nucleus; D, dorsal nucleus (column of Clarke); DL, dorsolateral nucleus; DM, dorsomedial nucleus; IML, intermedio-lateral nucleus; LCN, lateral cervical nucleus; PHR, nucleus of the phrenic nerve; SPN, sacral parasympathetic nucleus; VE, ventral nucleus.

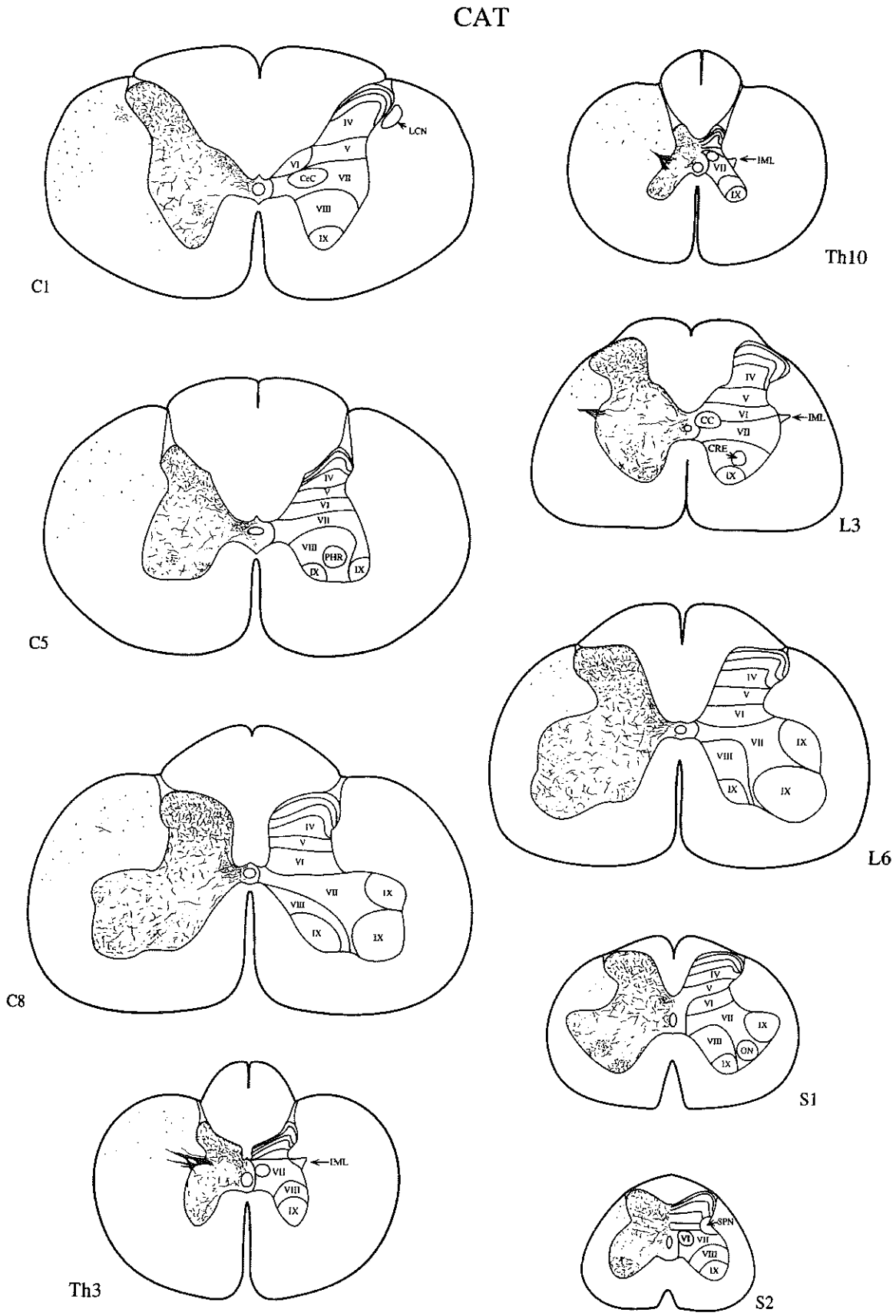


Figure 2: Schematic representation of the dopamine innervation in selected segments of the cat spinal cord. For details see text. Abbreviations: CC, column of Clarke (dorsal nucleus); ON, Onuf's nucleus; for other abbreviations see fig. 1.

the entire spinal cord through a highly collateralized system. Thus the presence of dopamine as a transmitter in the spinal cord and the existence of a dopamine projection to the spinal cord originating in the hypothalamus are now well established.

For an analysis of the laminar distribution of the dopamine fibers within the spinal cord, TH immunocytochemistry was used after destroying the noradrenaline terminals with 6-hydroxydopamine treatment (Dietl *et al.*, 1985). This study showed the highest density of TH immunoreactive dopamine fibers in lamina III, IV, X and the thoracic IML, with few fibers elsewhere in the spinal cord. The identification of dopamine using high performance liquid chromatography (HPLC) with electrochemical detection applied to micro-dissected spinal cord areas (Basbaum *et al.*, 1987) showed that highest levels of dopamine were present in the IML, the dorsal horn and the central canal region with somewhat lower levels in laminae VI and VII. A limited, but consistent amount of dopamine was also found in the motoneuronal cell groups, indicating a direct dopamine innervation of spinal motoneurons. The latter finding confirmed the results obtained with specific uptake of 3H-dopamine, which showed radioactively labeled terminals in the dorsal as well as the ventral horn (Kondo *et al.*, 1985). When it became possible to produce specific antibodies directed against small transmitter molecules (Storm-Mathisen *et al.*, 1983), including dopamine and noradrenaline (Geffard *et al.*, 1984; Geffard *et al.*, 1986; Buijs *et al.*, 1989), these transmitters could be directly visualized by immunocytochemistry. With this technique the presence of dopamine fibers and terminals in the spinal cord was confirmed (Yoshida and Tanaka, 1988; Shirouzu *et al.*, 1990; Mouchet *et al.*, 1992; Ridet *et al.*, 1992). However, some uncertainty remained with respect to the intensity of the dopamine innervation of the motoneuronal cell groups. The present study was undertaken to investigate in detail the localization of dopamine in all spinal segments of the rat, cat and monkey spinal cord by using immunocytochemistry with highly specific antibodies against dopamine. We found dopamine immunoreactivity in all laminae of the spinal cord, without major differences between species. Labeling was concentrated in the dorsal horn and the IML. In addition we found many lightly stained varicosities in the motoneuronal cell groups, confirming the existence of a prominent dopamine projection to spinal motoneurons.

Materials and methods

A detailed analysis was performed on material obtained from adult male Wistar rats, three female cats, one female monkey (*Macaca fasicularis*) and one male monkey (*Macaca arctoides*). All the animals were obtained through the institutional animals facility, housed and handled according to national guidelines, supervised by an institutional animal care and usage committee. The male monkey was bred by a pharmaceutical company and had not been involved in any previous testing. The female monkey, which had been obtained from an institutional research facility, had been involved in behavioral experiments, without any known pharmacological testing. They were kept in our institutional facility for at least 1 year in good health. The other animals were obtained from approved breeders through the institutional experimental animals facility. Before perfusion, the animals were deeply anaesthetized with pentobarbital (70 mg/kg) intraperitoneally.

Immunocytochemical procedures

The animals were perfused transcardially with 50 ml (rats), 150 ml (cats) or 500 ml (monkeys) of saline containing 10 mM Ascorbic Acid, pH 7.4, followed by 1 l (rats), 2.5 l (cats) or 4 l (monkeys) of 10 mM Ascorbic Acid in 0.05 M Acetate Buffer, pH 4.0 (AAAB), containing 5% glutaraldehyde. After perfusion the spinal cords were dissected and all the spinal segments were identified and cut with a razor blade. They were kept overnight at 4°C in AAAB, containing 30% sucrose. The following days, 30 µm sections were obtained on a sliding freezing microtome from all cervical, lumbar and sacral as well as half of the thoracic and the rostral coccygeal spinal segments. The free floating sections were collected in Tris-buffered saline, pH 7.4, containing 10 mM Ascorbic acid (TBSA). Some spinal cords segments were cut in horizontal or sagittal sections. The free floating sections were rinsed in TBSA and kept for several days in the same liquid at 4°C. Next they were treated with TBSA, containing 1% borohydrate ($NaBH_4$) for 30 min at room temperature. They were then rinsed several times in TBSA and incubated overnight at 4°C with a polyclonal dopamine antibody from rabbit ("Jannes", Buijs *et al.*, 1984; Voorn and Buijs, 1987) diluted 1:4000 in Tris-buffered saline, pH 7.4 (TBS), containing 0.5% Triton X-100 and 10 mM Ascorbic Acid. In rat and cat several sections were also treated with a different dopamine antibody (Steinbusch and Tilders, 1987; Steinbusch *et al.*, 1991) (SanBio B.V, Uden), diluted 1:2000 in Tris-buffered saline. After the primary dopamine antibody, the sections were rinsed thoroughly with TBS and a goat-anti rabbit (GAR) antibody ("Betsy", diluted 1:100, 1 hour incubation) was applied. Subsequently the sections were rinsed with TBS and incubated with a rabbit PAP complex (Nordic, 1:1000, 1 hour), rinsed in TBS followed by phosphate buffer (0.1 M, pH 7.4). These incubations were performed at room temperature. Then the sections were reacted with 0.05% 3,3'-diaminobenzidine in the presence of 0.01% hydrogen peroxide in phosphate buffer (0.1 M, pH 7.4). After rinsing in phosphate buffer, the sections were mounted onto glass slides with a chromealum-gelatine solution, and some were subsequently counterstained with 0.05% thionin. Finally, all sections were dehydrated, coverslipped and examined with bright and darkfield illumination. As a control, some of the sections were processed after substitution of the primary antiserum with TBS. This resulted in no contrast staining.

DSP-4 experiments

In order to deplete noradrenaline in the spinal dorsal

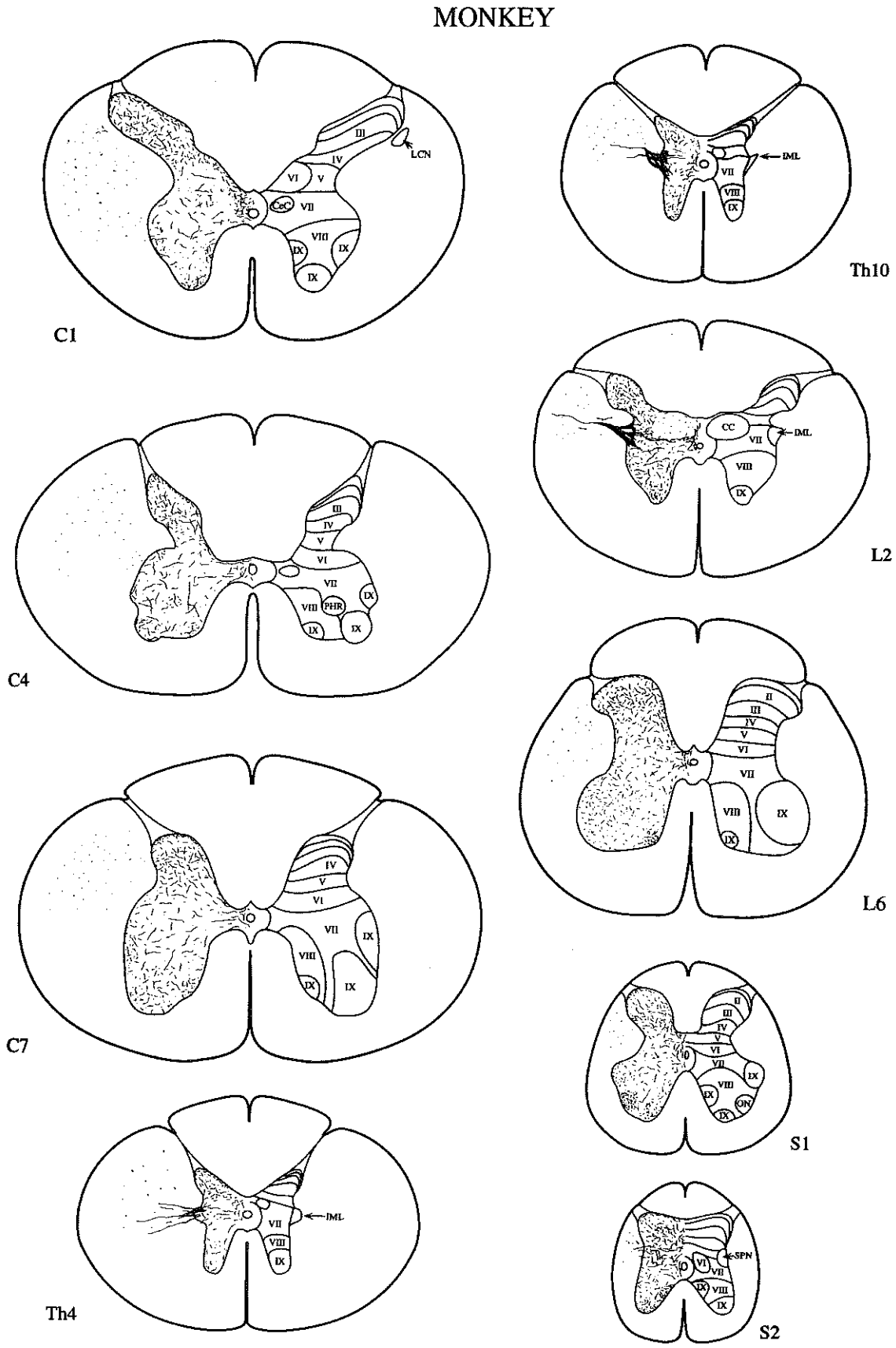


Figure 3: Schematic representation of the dopamine innervation in selected segments of the monkey spinal cord. For details see text. Abbreviations: see fig. 2.

horn, 4 rats received an intraperitoneal injection (50 mg/kg) of N-(2-chloroethyl)-N-ethyl-2-bromobenzyl-amine (DSP-4) (Jonsson, 1983), while 2 rats received an intraperitoneal saline injection. After a period of two weeks the rats were deeply anaesthetized and perfused for dopamine immunocytochemistry as described above. Alternate 30 µm frozen sections were processed for either dopamine or dopamine-β-hydroxylase (DBH) immunocytochemistry. For dopamine immuno-cytochemistry the same procedure was used as described above except for the GAR antibody (Vector, 1:200, 1 hour incubation) and the rabbit-PAP complex (Nordic, 1:800, 1 hour incubation). For DBH immunocytochemistry the sections were preincubated in a solution of 5% normal goat serum (NGS) in phosphate buffer (PB; 0.1 M, pH 7.4) containing 0.9% NaCl and 0.3% Triton X-100 for 90 minutes. Next they were incubated for 24-48 hours at 4 °C with rabbit-anti DBH (Eugene Tech; 1:750) in 0.1 M PBS (pH 7.4) containing 0.3% Triton-X100 and 2% NGS. Hereafter, the sections were rinsed and incubated (1-2 hour) with biotinylated GAR (Vector; 1:200). After a few rinses, biotin avidin complex (Vector) was applied (1-2 hour). Finally, the sections were reacted with 3,3'-diaminobenzidine in the presence of 0.01% hydrogen peroxide, rinsed several times in PB, mounted on glass slides with chromealum, dehydrated, coverslipped and examined with bright and darkfield illumination.

Results

The distribution of dopamine immunoreactivity was examined light microscopically in transverse, horizontal and sagittal sections from nearly all segments of the rat, cat and monkey spinal cord (figs. 1-11). Dopamine immunoreactivity was present almost exclusively in fibers and (presumed) terminals. Labeled neuronal cell bodies were found in all species only in the rostral part of C1. Labeled neuronal cells (0-5 cells per section) were found in the ventrolateral funiculus and in the area immediately dorsal of the central canal. In all likelihood these cells represented the caudal extension of the A1 and A2 cell group respectively as described by Dahlström and Fuxe (1964) in the lower medulla. Labeled neuronal cell bodies were never found in other areas of the spinal cord. Dopamine fibers and varicosities were found in all laminae of the spinal grey matter at all spinal levels, but with considerable differences in regional density.

The following detailed descriptions were made from sections treated with the "Jannes" dopamine antibody for which the method was optimized. An identical labeling pattern was obtained using the Sanbio dopamine antibody. The distribution of the dopamine immunoreactivity will be described separately for the dorsal horn, the ventral horn and various "specialized areas" in the spinal cord. In addition, the location of the descending fibers in the white and grey matter will be described.

Dorsal horn

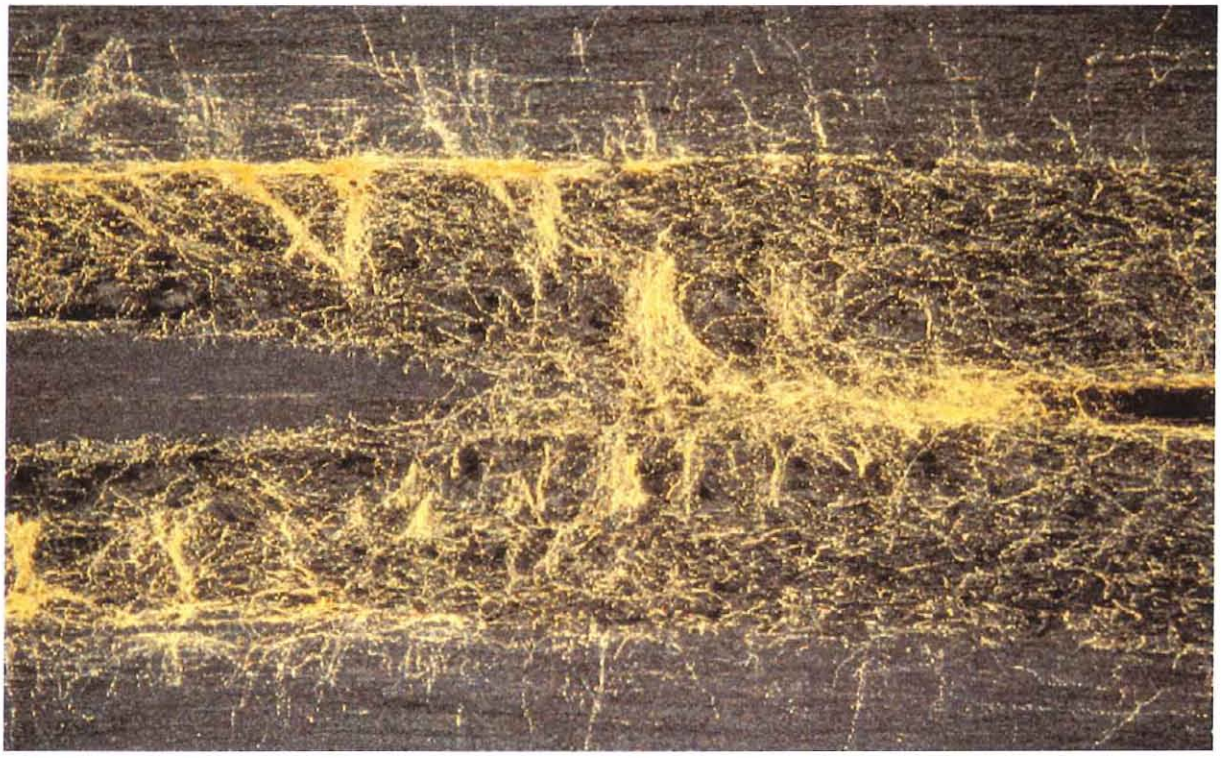
Within the dorsal horn of the rat, cat and monkey spinal cord, dopamine immunoreactivity was found in all laminae (figs. 1-7 and 8). As a rule, labeling was moderate in lamina I, sparse (rat and monkey) to moderate (cat) in lamina II (substantia gelatinosa) and strong in laminae III-V. In laminae I of rat and monkey there were many short fibers with varicosities and occasional patches of labeling consisting of many small caliber fibers and varicosities, which sometimes seemed to exceed the width of lamina I in dorsal or ventral direction. Only a few varicose fibers were traversing lamina II. In the cat the difference in labeling intensity between lamina I and II was less pronounced. In lamina III, IV and V, including the neuropil of the lateral reticular part of these laminae, thick fibers with clear varicosities formed a dense network. Horizontal and parasagittal sections (fig. 11) showed that most fibers in lamina III, as well as those in laminae I and II, were oriented predominantly in a rostrocaudal direction, while in laminae V and to a lesser extent in lamina IV this rostro-caudal orientation of the fibers was not observed; instead they were oriented more randomly. The area around the central canal contained only a few rostro-caudally oriented fibers. In transverse sections it appeared that many dorsoventrally oriented fibers at the medial border of the dorsal horn terminated in the central canal region or crossed to the other side, mostly into the dorsal grey matter. In addition to these general aspects of dopamine immunoreactivity in the dorsal horn, there were also differences between species as described below.

Rat dorsal horn. In rat (figs. 1, 6, 7 and 8), labeling was strongest in lamina III and slightly less prominent in lamina IV and V. In lamina I the intensity of the labeling was clearly less as compared to the deeper laminae. The lowest amount of labeling was seen in lamina II where immunoreactivity was almost absent, apart from some traversing fibers, which ran mostly in a rostro-caudal direction (fig. 11). At the lower cervical as well as the lower lumbar levels, there was less labeling in the medial part of laminae III-V as compared to the lateral part (fig. 8). The overall labeling of the dorsal horn was less dense in the lumbo-sacral cord than in the cervical cord, although the general pattern of the labeling was the same at both levels. The lateral spinal nucleus (not indicated in fig. 1) was moderately innervated throughout the spinal cord.

Cat dorsal horn. In cat (figs. 2 and 4), the differences in labeling intensity between laminae was much less as compared to the rat. Strongest labeling was found not only in lamina III-V, but also in lamina I. Lamina II was moderately innervated. Differences in labeling between the medial and lateral part of laminae III-V at low cervical levels were less pronounced than in rat and monkey. At thoracic levels, the overall labeling intensity became less than at cervical levels and labeling in lamina II was sparse, containing mostly rostro-caudally oriented fibers. Going further caudally to lumbar levels, the labeling intensity increased again, being strongest at L7. However, like in rat, labeling was still less intense as compared to the cervical cord.

Monkey dorsal horn. In monkey dorsal horn (figs. 3 and 5-*top*) the overall labeling intensity was somewhat less than in the rat and cat. In the *Macaca fasicularis*, at

Figure 4: Photomicrograph with darkfield illumination of a section from the cat L6 segment showing dopamine immunoreactive fibers and varicosities. Note the difference in type of labeling in the dorsal and ventral horn, the sparse labeling in the substantia gelatinosa (lamina II, see fig. 2) and in the ventral horn intermediate zone (laminae VII and VIII, see fig. 2). Also note the descending fibers in the dorsolateral funiculus close to the dorsal horn (see also fig. 2). 1 cm = 120 μm.



cervical levels, lamina I was moderately labeled with fine caliber fibers, while in lamina III and IV several thick strongly labeled fibers were present, mainly in the medial part of this area. Lamina II was sparsely labeled, containing a few rostro-caudally oriented fibers. At thoracic levels, overall labeling intensity was decreased, with very few labeled fibers in lamina II. Going further caudally to lumbosacral levels, the intensity of the labeling increased again, but lamina II remained sparsely innervated. The same pattern, including a sparsely innervated lamina II was also found at sacral levels. In the *Macaca arctoides* the labeling pattern was rather different from the other two monkeys, because of a sparse to moderate labeling of lamina III and IV, while lamina I was strongly labeled and labeling in lamina II was sparse (fig. 5-*top*). This typical pattern of dorsal horn labeling was especially apparent at cervical levels and was not seen in rats and cats, or in the other monkey.

Ventral horn

The dopamine immunoreactivity in the ventral horn (figs. 1-4, 6-10 and 11) had a very different appearance as compared to the dorsal horn. For a major part this difference was due to the type of innervation of the motoneuronal cell groups. This area (lamina IX) received a dense innervation of immunoreactive varicosities, many of which appeared to be smaller than in other laminae of the spinal cord. In addition, intervaricose segments were less pronounced, giving the labeling of the motoneuronal cell groups a fine granular (punctate) appearance, especially at lower magnifications (compare figs. 4, 6 and 9 with fig. 7). Interestingly, this type of labeling was found in transverse, horizontal and sagittal sections, indicating that the innervation pattern is organized without a predominant orientation, except for some "specialized areas" (see below). In all species a very strong labeling of the motoneuronal cell groups innervating the tail muscles was observed at the sacral (fig. 6) and coccygeal segments. This labeling was also of a fine granular appearance. In the intermediate zone (laminae VII and VIII) labeling was sparse with only a few traversing fibers.

Rat ventral horn. In the rat ventral horn (figs. 1, 6, 7 and 10) dopamine immunoreactivity generally appeared as indicated above. With respect to regional differences, there was a slightly increased labeling in the ventrolateral group of motoneurons mainly at the C7 segment (Matsushita and Ueyama, 1973). Dopamine immunoreactivity in the medial and lateral motoneuronal cell groups was equally strong both at low cervical and low lumbar spinal levels. At caudal thoracic and especially at lumbar levels, there was a slight increase in varicose fibers in the motoneuronal cell groups and, as a result, the fine granular appearance of the dopamine immunoreactivity became less conspicuous.

Cat ventral horn. In cat (figs. 2 and 4), as in rat, dopamine immunoreactivity was much stronger in the motoneuronal cell groups as compared to the intermediate zone. However, the differences in the type of labeling between the two areas, as found in rat, was much less apparent in cat. This was due on the one hand to an increased number of labeled fine caliber fibers and varicosities in the intermediate zone, while on the other hand the number of labeled large varicosities and fibers in the motoneuronal cell groups was increased. At low cervical levels there was especially strong labeling in the ventral motor nucleus (Matsushita and Ueyama, 1973), at the C7-8 segments. At thoracic and high lumbar levels the intermediate zone was almost devoid of labeling, while labeling of the motoneuronal cell groups remained strong.

Monkey ventral horn. In the monkey ventral horn (figs. 3 and 9) the fine granular aspect of the dopamine immunoreactivity in the motoneuronal cell groups was in sharp contrast to the thick varicose fibers in the dorsal horn. Thick varicose fibers were also found in the intermediate zone, but here the number of fibers and varicosities was much less. The difference in density and intensity of the labeling between the intermediate zone and the motoneuronal cell groups became less apparent at the cervical and lumbar enlargements because of a stronger labeling in the intermediate zone. Also in these areas the labeling in the motoneuronal cell groups was somewhat stronger.

Specialized areas

Central cervical nucleus. This area, just dorsolateral to the central canal, is present mainly at high cervical levels, but is often difficult to delineate. It received a very sparse dopamine innervation, consisting mainly of a few fibers traversing the nucleus.

Lateral cervical nucleus. This nucleus, which is located at the segments C1-C3, is most prominent in the cat and monkey. It received a moderate innervation with dopamine fibers and varicosities.

Region around the central canal. This area, also referred to as lamina X, received a strong dopamine

Figure 5: Top: photomicrograph with darkfield illumination of a section from the monkey L2 segment showing dopamine immunoreactive fibers and varicosities. Note only very sparse labeling in the dorsal nucleus (column of Clarke, see fig. 3) and very strong labeling in the IML nucleus. 1 cm = 60 μm. **Bottom**: photomicrograph with darkfield illumination of a horizontal section from the rat Th6-7 segment showing dopamine immunoreactive fibers and varicosities. Note in the midline on the left the central canal and on the right the dorsal funiculus, indicating that the horizontal section is oriented slightly dorsoventrally. This makes it possible to see the strong labeling in the IML nucleus on the right, the labeling in the intercalatus nuclei in the middle and the central autonomic nuclei on the left. Also note that neurons of the dorsal nucleus (right, close to the dorsal funiculus, relatively small at this level) are avoided by the dopamine fibers. In the lateral funiculus (located along the entire length of the top and the bottom of the micrograph) some rostro-caudally oriented fibers can be seen as well as laterally running fibers, which probably innervate laterally running dendrites of IML neurons. 1 cm = 140 μm.

innervation, consisting of both thick and fine caliber fibers and varicosities (figs. 1-6). This innervation was limited to the region directly surrounding the central canal and a somewhat wider area extending bilaterally in a dorsolateral direction. This area also contained several crossing dopamine fibers, which were located in the dorsal grey commissure and appeared to run towards the contralateral dorsal horn rather than the ventral horn.

Phrenic nucleus. In transverse sections of the C4 - C6 segments, a characteristic group of motoneurons, corresponding to the nucleus of the phrenic nerve (Kuzuhara and Chou, 1980), was observed ventromedially in the ventral horn (figs. 1-3 and 10). Dopamine immunoreactivity in the phrenic nucleus showed a fine granular aspect, similar to that of surrounding motoneurons. In longitudinal and sagittal sections the phrenic motoneurons were easily distinguished by their longitudinally organized dendrites. In contrast to the findings in the transverse sections, dopamine immunoreactivity was stronger in the area containing these longitudinal dendrites than in the surrounding neuropil (fig. 10). It was, therefore, concluded that the dopamine labeling of the phrenic nucleus was somewhat stronger than the surrounding motoneurons and organized predominantly in a longitudinal fashion.

Sympathetic preganglionic neurons. These neurons are located in the spinal segments between the caudal C8 and L3. The large majority of the sympathetic preganglionic neurons are located in the IML and a few neurons are present near the central canal (central autonomic nucleus) or the area in between (nucleus intercalatus) (Strack *et al.*, 1988). All these areas, but especially the IML, received a very strong dopamine innervation (fig. 5). Cell bodies often stood out as small unlabeled areas in a massively innervated region. The area between the IML and the central canal, which contains many dendrites of sympathetic neurons, also received a strong dopamine innervation, which sometimes appeared as a heavily labeled band between the IML and the central canal. In horizontal sections (fig. 5) the dopamine immunoreactivity was clearly seen to be organized in a "ladder like" pattern (Hosoya *et al.*, 1991).

Dorsal nucleus (column of Clarke). This area contains large cells, projecting to the cerebellum, and is located in segments Th1 to L3 (Grant *et al.*, 1982). Dopamine innervation was nearly absent with only an occasional fiber and some terminal-like structures (fig. 5); most fibers seemed to avoid the nucleus.

Cremaster nucleus. The cremaster nucleus, a sexually dimorphic motor nucleus innervating the cremaster muscle, is located primarily in the segments L1 and L2 and is most prominent in the male (Nagy and Senba, 1985). In the male Arctoides monkey the cremaster nucleus received a very strong dopamine innervation (fig. 9). In transverse sections the same strong innervation was sometimes seen in medio-laterally oriented dendritic bundles, which are known to belong to cremaster motoneurons (fig. 9). In male rats and female cats, the cremaster nucleus also received a stronger innervation in comparison with surrounding motoneurons, but this labeling was less prominent than in the male monkey.

Lumbo-sacral motoneurons. The motoneurons which innervate, through fibers in the pudendal nerve, the anal and urethral sphincter muscles and related muscles form a special group of motoneurons (Schrøder, 1980). They are somewhat smaller than the surrounding (somatic) motoneurons and they are sexually dimorphic (McKenna and Nadelhaft, 1986). In different species these motoneurons are located in different areas of the ventral horn. In rat two different groups can be distinguished, namely the nucleus dorsomedialis (DM) (also known as the spinal nucleus of the bulbocavernosus, SNB) and nucleus dorsolateralis (DL) (Schrøder, 1980). In cat the DM and DL nuclei, as distinguished in the rat, are fused to form a ventrolaterally located nucleus, designated (like in man) as Onuf's nucleus (Sato *et al.*, 1978; Ueyama *et al.*, 1984). In monkey the organization is similar to the cat (Nakagawa, 1980). The DM and DL nuclei in the rat and Onuf's nuclei in the cat and monkey received a strong dopamine innervation, which was much stronger than dorsolaterally located motoneurons, which innervate muscles associated with the foot. Sometimes prominent dendritic bundles were seen radiating laterally and medially from Onuf's and DL nuclei and laterally from the DM nucleus. These bundles also received a strong dopamine innervation. The intensity of the labeling in the sexually dimorphic nuclei was similar to that of the tail-muscle motoneurons, which first appear ventromedially in the ventral horn, at the S1 segment. In addition, a ventral group of motoneurons, innervating the levator ani muscle, also received a strong dopamine innervation, similar to Onuf's nucleus.

Sacral parasympathetic area. This area, located mainly in the segments S1-S4 (depending on species) contains the parasympathetic nucleus. This area showed a stronger dopamine innervation than the surrounding intermediate zone (fig. 6), but it could not be determined whether the dopamine fibers terminated specifically on the preganglionic parasympathetic neurons.

Location of the descending dopamine fibers

In all animals the majority of the descending dopamine fibers was located in the dorsolateral funiculus (figs. 1-6) and, to a limited extent, in the lateral and ventrolateral funiculi at cervical levels. An occasional fiber was seen in the ventromedial and dorsal funiculi. In sagittal and horizontal sections it was found that the funicular fibers showed few varicosities and appeared as relatively straight structures. Occasionally a fiber gave rise to a collateral, often at a right angle, which ran to the lateral part of the dorsal horn. Collateral fibers, which originated from stem fibers in the dorsolateral and lateral funiculi, were rarely seen to reach the ventral horn. Apart from the fibers in the funiculi there were also rostrocaudally oriented fibers in the dorsal horn, most notably in laminae I and III, but also in lamina II (fig. 11). In the region around the central canal there were a large number of fibers, including several rostro-caudally oriented fibers. However, it appeared from

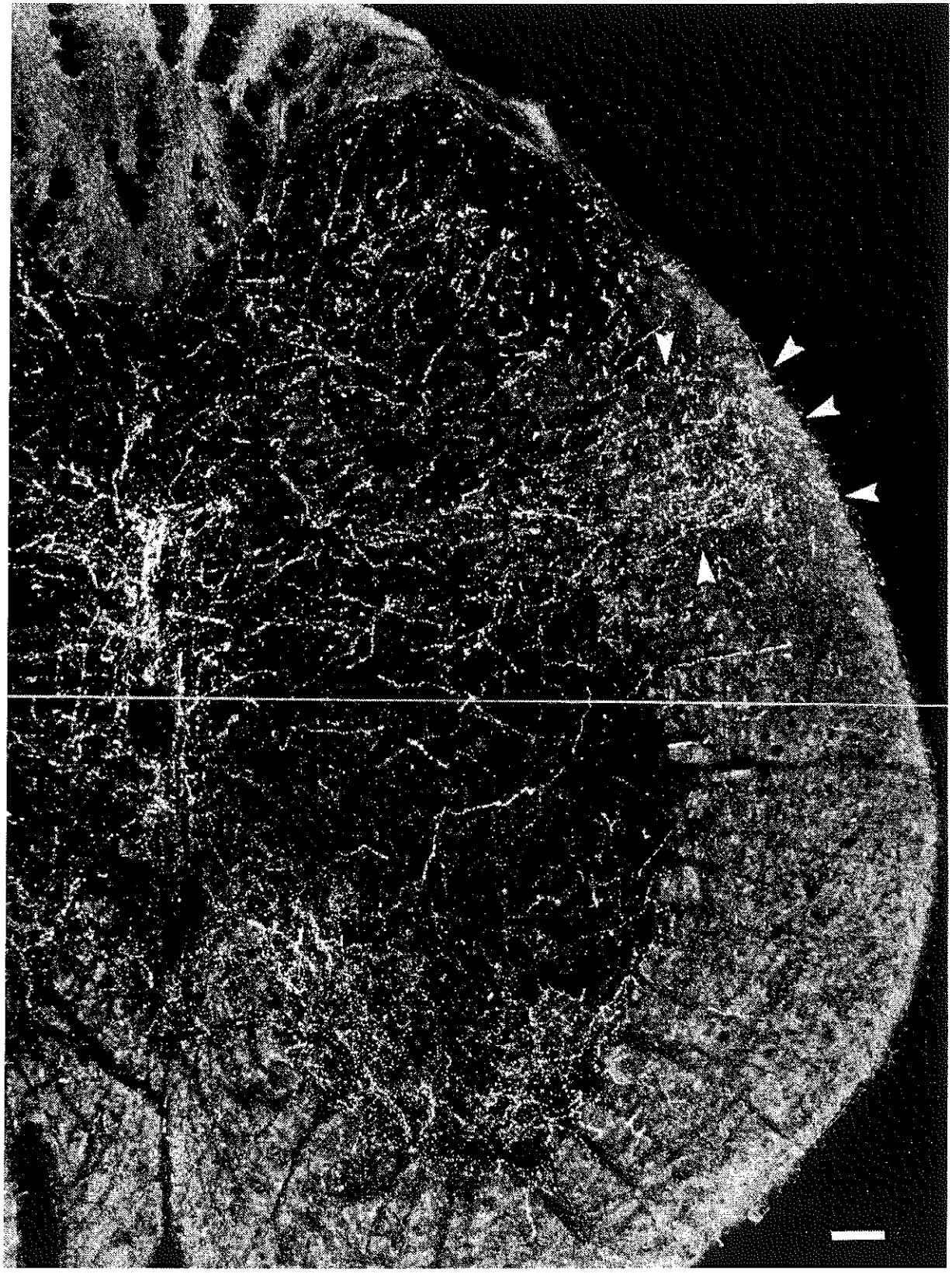


Figure 6: Photomicrograph with darkfield illumination of a section from the rat S1 segment showing dopamine immunoreactive fibers and varicosities. Note the labeling in the area of the parasympathetic preganglionic neurons just medial of the descending fibers in the dorsolateral funiculus (white arrowheads). Also note the strong, punctate labeling in the motoneuronal cell groups. 1 cm = 70 µm.

sagittal and horizontal sections that these fibers form a minority and hence do not contribute significantly to the descending dopamine system.

DSP-4 experiments

Sections from the spinal cords of rats treated with DSP-4, which only affects the noradrenaline fibers in the dorsal horn (Jonsson, 1983), were processed for DBH immunocytochemistry (fig. 8), while alternate sections from the same (DSP-4 treated) rat were processed for dopamine immunocytochemistry (fig. 8). In these DSP-4 treated rats there were very few DBH-immunoreactive fibers in the dorsal horn, while the ventral horn contained many DBH immunoreactive fibers. These results are similar to those described previously (Fritschy and Grzanna, 1989; Lyons *et al.*, 1989). In contrast, the alternate sections showed many dopamine immunoreactive fibers and varicosities throughout the spinal grey matter, including the dorsal horn, with the same pattern and intensity as in untreated rats. This demonstrated that the absence of (nor)adrenergic fibers, as visualized by the lack of DBH immunoreactivity, did not influence the dopamine immunoreactivity. Alternate sections from saline injected control rats, treated for DBH (fig. 8) or dopamine immunocytochemistry as the DSP-4 treated rats, showed many immunoreactive fibers and varicosities throughout the dorsal and ventral horn in a similar pattern as found in the present study for dopamine or described elsewhere for DBH (Westlund *et al.*, 1983; Fritschy and Grzanna, 1990; Rajaofetra *et al.*, 1992b)

Discussion

The present study shows the distribution of dopamine in the spinal cord of the rat, cat and monkey. It provides a detailed study of all parts of the spinal cord, including the various specialized areas. Below the specificity of the dopamine antibodies that we used will be discussed. Then the topographical distribution of the labeling, as compared with the other monoamines, will be discussed, followed by some functional considerations.

Methodological aspects

In the present study the distribution of dopamine in the rat, cat and monkey spinal cord was determined by using immunocytochemistry with highly specific antibodies directed against (glutaraldehyde-bound) dopamine.

Nevertheless the possibility that the dopamine antibody reacted with adrenergic and/or noradrenergic terminals should be considered, not because of cross-reactivity of the antibodies (see below), which have been extensively tested and used, but rather because the dopamine antibodies may recognize dopamine, which is present as a precursor in (nor)adrenergic terminals. However, biochemical studies in areas with a high noradrenaline and a low dopamine innervation, like the cerebellum and hippocampus, have shown that the dopamine concentration is only 1-3% of the noradrenaline concentration (Commissiong *et al.*, 1978; Westerink and Mulder, 1981; Verhage *et al.*, 1992). These studies further showed that destruction of the locus coeruleus, and consequently the noradrenaline innervation of several brain areas, including the cerebellum and hippocampus, had no effect on dopamine levels in those areas (Westerink and De Vries, 1985). This would indicate that even in areas with a relatively high noradrenaline and a low dopamine concentration, dopamine represents the transmitter pool rather than the "precursor pool" of noradrenaline. Subsequent biochemical and/or anatomical studies in cerebellum (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992) and hippocampus (Verney *et al.*, 1985; Verhage *et al.*, 1992; Gasbarri *et al.*, 1994) confirmed the existence of a dopamine projection independent from the noradrenaline innervation. Furthermore we have found (van Dijken and Holstege, 1995) that in the external cuneate nucleus and the area postrema of the lower brainstem there was a strong immunoreactivity for DBH, while labeling for dopamine was virtually absent (using the same "Jannes" antibody as we have used in the present study). This indicated that the dopamine "precursor pool" in (nor)adrenergic terminals was too low to be identified by the dopamine antibody, probably due to a very high turnover rate of dopamine in these terminals. These findings further showed that there is no cross-reaction of the dopamine antibody with noradrenaline, confirming the high specificity of the antibody. On the other hand it should be mentioned that (nor-)adrenergic cell somata, like those in the locus coeruleus, show some immunoreactivity for dopamine, probably because more dopamine is present as a precursor in the somata than in the terminals of (nor-)adrenergic neurons (see also Geffard *et al.*, 1984).

Our control experiments for the spinal cord with DSP-4 treated rats, in which DBH labeled (nor) adrenergic terminals in the dorsal horn had disappeared almost completely, showed that the dopamine immunoreactivity was still present in those rats. Moreover, the intensity and the pattern of the dopamine labeling were identical to those in untreated rats. A similar finding was obtained in a study (Mouchet *et al.*, 1992) using rats treated with 6-OHDA, which causes a specific degeneration of noradrenergic terminals, leaving the dopaminergic terminals largely unaffected. These experiments showed that in 6-OHDA treated rats, dopamine immunoreactive fibers were still present, while in other 6-OHDA treated rats the noradrenaline immunoreactivity had disappeared. Taken together, the various data demonstrated that the dopamine antibody did not recognize dopamine in noradrenergic terminals, nor did it crossreact with noradrenaline.

It may be concluded that in the spinal cord, including the motoneuronal cell groups, a dopamine innervation is present, which is independent of the (nor)adrenergic innervation. Moreover, the dopamine labeling in the spinal cord as found in the present study represented exclusively dopamine fibers and terminals.

Topographical distribution

The results of the present study show that in rat, cat and monkey dopamine fibers and varicosities are present in all laminae of the spinal grey matter throughout the spinal cord, but with clear regional differences. The

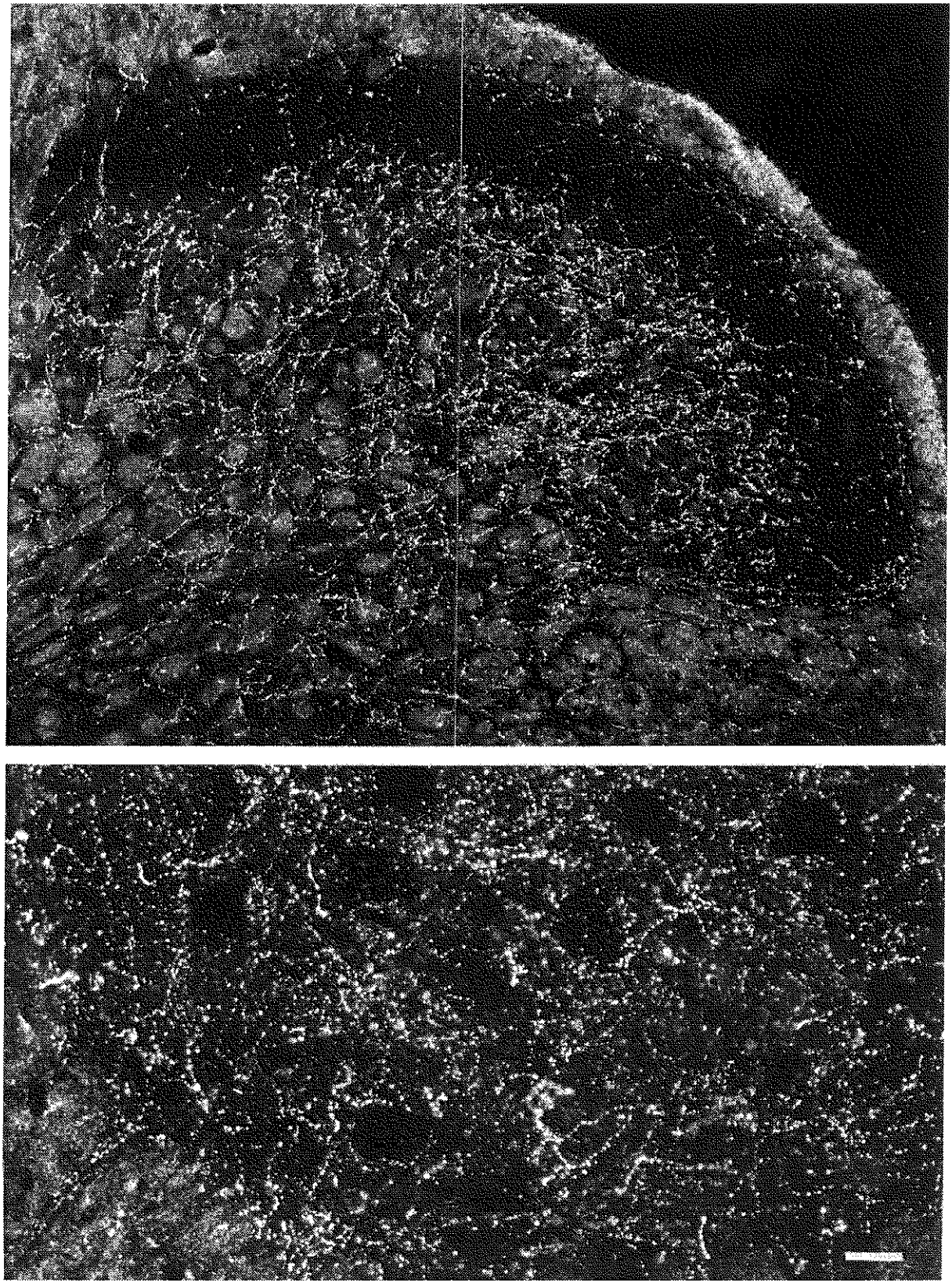
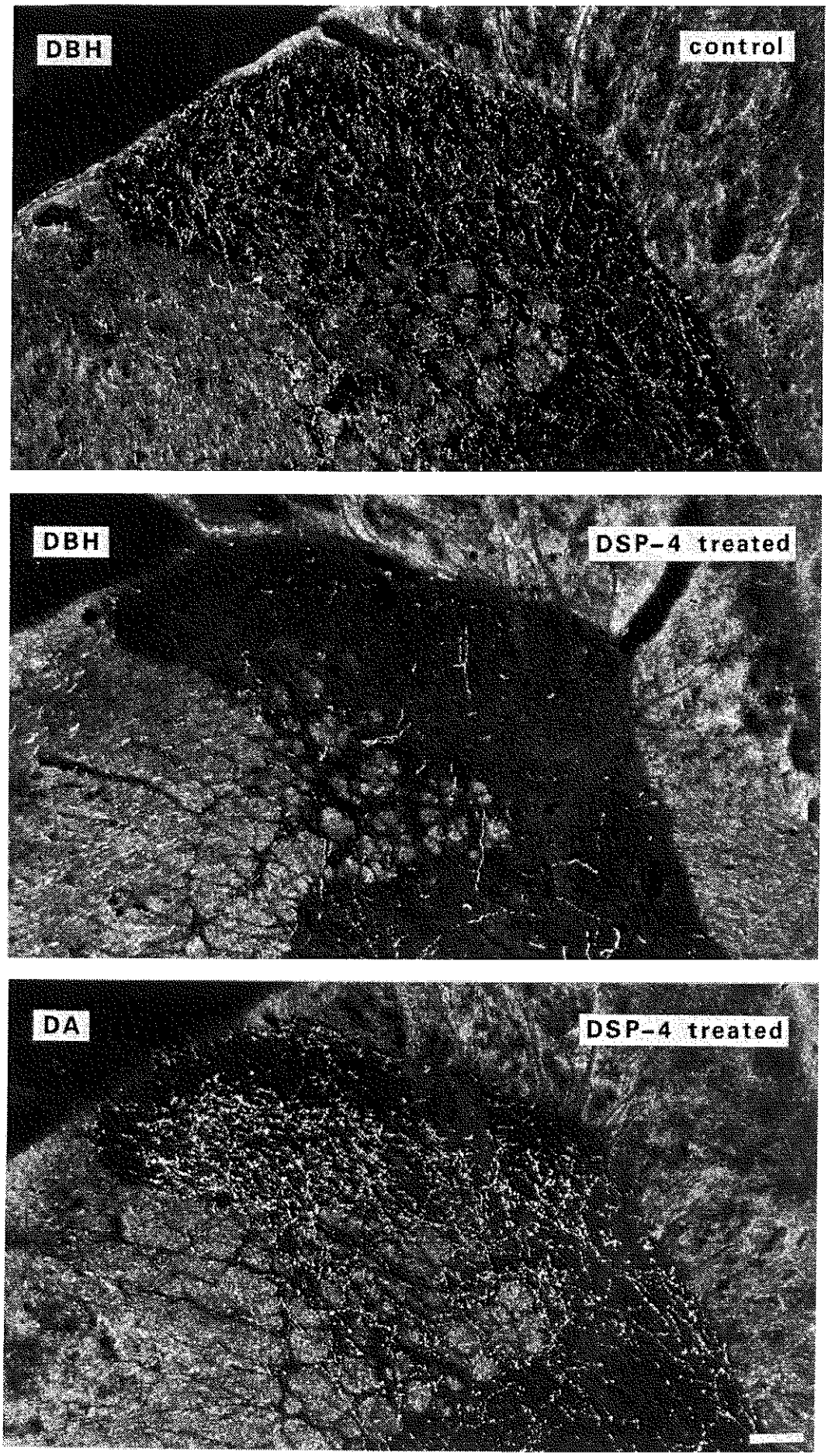


Figure 7: Top: photomicrograph with darkfield illumination of a section from the rat C1 segment showing dopamine immunoreactive fibers and varicosities in the dorsal horn. Note the strong labeling in laminae III-V and the moderate labeling in lamina I, while labeling in lamina II (the substantia gelatinosa) is only sparse. See fig. 1 for laminar arrangement. 1 cm = 65 µm. **Bottom:** photomicrograph with darkfield illumination of the motoneuronal cell groups from a section from the rat L5 segment showing dopamine immunoreactive fibers and varicosities. Note the abundance of small varicosities, presumably terminals, with a paucity of intervaricose fibers, although the position of many varicosities suggests that they are interconnected. The motoneurons appear as "black holes". The ventral funiculus can be seen below and left. 1 cm = 35 µm.


DBH
control
DBH
DSP-4 treated
DA
DSP-4 treated

general pattern of labeling of the dopamine fibers and terminals is similar in the species investigated. Dopamine labeled neuronal cell bodies were only found in the rostral C1 segment, probably representing the caudal extension of the medullary A1 and A2 cell groups. Although many neurons in the A1 and A2 cell group contain DBH, some of them were found to be immunoreactive for dopamine (Maqbool *et al.*, 1993) or TH (Mouchet *et al.*, 1986) and not for DBH, indicating that they actually represented dopaminergic neurons. Labeled (neuronal) cell bodies were never identified below rostral C1, which is in contrast with the finding (Mouchet *et al.*, 1986) of TH immunoreactive neurons at the S1 segment. If both observations were correct it must be assumed that these TH-labeled cells are not able to convert *L*-DOPA (the TH product) into dopamine, as has been shown to occur in the hypothalamus (Meister *et al.*, 1988; Steinbusch *et al.*, 1991).

Dorsal horn. In the dorsal horn, the lack of innervation of the substantia gelatinosa is the most conspicuous finding, which is consistent throughout all species, but least obvious in the cat. Previous studies in rat, using immunocytochemistry with an antibody directed against dopamine (Yoshida and Tanaka, 1988; Mouchet *et al.*, 1992; Ridet *et al.*, 1992), have described the same pattern of innervation of the dorsal horn as in the present study. Similar to the distribution of dopamine, serotonin also showed a widespread innervation of the dorsal horn including a sparse innervation of the substantia gelatinosa, especially in its inner part. In rat (Marlier *et al.*, 1991), cat (Ruda *et al.*, 1982) and monkey (Kojima and Sano, 1983; LaMotte and Lanerolle, 1983) serotonin immunoreactive labeling in the dorsal horn was found to be strongest in lamina I and outer lamina II, whereas there was only sparse labeling in the inner part of lamina II. Laminae III-VI were only moderately innervated. The distribution of the dopamine labeling in laminae III-V is slightly different than the serotonergic innervation since the dopamine labeling is stronger (rat) or similar (cat and monkey) as compared to the labeling in lamina I. An extensive innervation of the dorsal horn has also been described for noradrenaline or DBH immunoreactive fibers and terminals in the rat (Westlund *et al.*, 1983; Fritschy and Grzanna, 1990; Hagihira *et al.*, 1990; Rajaofetra *et al.*, 1992b), cat (Doyle and Maxwell, 1991) and monkey (Westlund *et al.*, 1984). The low innervation of lamina II, which is typical for dopamine and serotonin, is less obvious in case of noradrenaline, although a stronger innervation of the outer part of lamina II versus the inner part has been suggested (Westlund *et al.*, 1983; Hagihira *et al.*, 1990; Rajaofetra *et al.*, 1992b). Also in our study with DBH, the difference between dopamine and noradrenaline with respect to the innervation of the substantia gelatinosa was very clear (compare fig. 8 *top* and *bottom*). In several areas the dopamine innervation of lamina I appeared as patches occasionally located outside the boundaries of this lamina. Whether these patches actually exceeded the border between laminae I and II or whether there was a local widening of lamina I, cannot be determined with certainty in the present material. The finding in one monkey of only sparse labeling in lamina II and deeper laminae, while lamina I was strongly labeled (fig. 5-*top*), was rather surprising. This finding showed that regional density can vary, not only between, but also within species.

The intermedio-lateral cell column (IML). The IML, which is located from caudal C8 to L3 (Strack *et al.*, 1988), received a massive dopamine innervation and this was also true for the nuclei that may be considered as the medial extensions of the IML towards the central canal: the nucleus intercalatus and the central autonomic nucleus, located just dorsal and dorsolateral to the central canal. These findings are in agreement with previous reports on the dopamine innervation of the spinal cord (Skagerberg *et al.*, 1982; Yoshida and Tanaka, 1988; Mouchet *et al.*, 1992; Ridet *et al.*, 1992). The IML is characterized by a patchy organization with dendritic bundles interconnecting the sympathetic subnuclei, forming a "ladder-like" pattern when viewed in horizontal sections (Romagnano and Hamill, 1984; Hosoya *et al.*, 1991). The dopamine innervation exactly mimics this discontinuous organization, indicating that the dopamine fibers are aimed at the sympathetic preganglionic neurons and their dendrites. A similar "ladder-like" pattern of innervation was found with many other transmitters, including noradrenaline, serotonin, adrenaline and various peptides like substance P, oxytocin, vasopressin, neurotensin, somatostatin, neuropeptide Y and enkephalin (Swanson and McKellar, 1979; Glazer and Ross, 1980; Holets and Elde, 1982; Westlund *et al.*, 1983; Romagnano and Hamill, 1984; Krukoff, 1987; Fuxe *et al.*, 1990; Hosoya *et al.*, 1991; Rajaofetra *et al.*, 1992b).

The dorsal nucleus (column of Clarke). This nucleus is located in the same segments as the IML. It projects mainly to the cerebellum (Grant *et al.*, 1982). In contrast to the IML, the dorsal nucleus received very few, if any, dopamine immunoreactive fibers. The serotonergic innervation of the nucleus dorsalis is also

Figure 8: Photomicrograph with darkfield illumination of the dorsal horn from the rat C7 segment. **Top:** DBH immunoreactive fibers and varicosities in a control (saline-injected) rat. Note that, in comparison with the dopamine labeling (bottom), the density of the DBH labeling is similar in all laminae of the dorsal horn (including lamina II) and the relatively fine caliber of the fibers. 1 cm = 90 µm. **Middle:** DBH immunoreactive fibers and varicosities two weeks after treatment with the neurotoxin DSP-4. Note that most DBH labeling has disappeared after DSP-4 treatment (compare with control animal in top micrograph). 1 cm = 90 µm. **Bottom:** Dopamine immunoreactive fibers and varicosities two weeks after treatment with the neurotoxin DSP-4 (same rat as in middle micrograph). Note that the dopamine immunoreactivity has not changed significantly in the absence of (nor)adrenaline (see middle micrograph), providing direct proof that the dopamine immunoreactivity is not located in (nor)adrenergic fibers and varicosities. 1 cm = 90 µm.

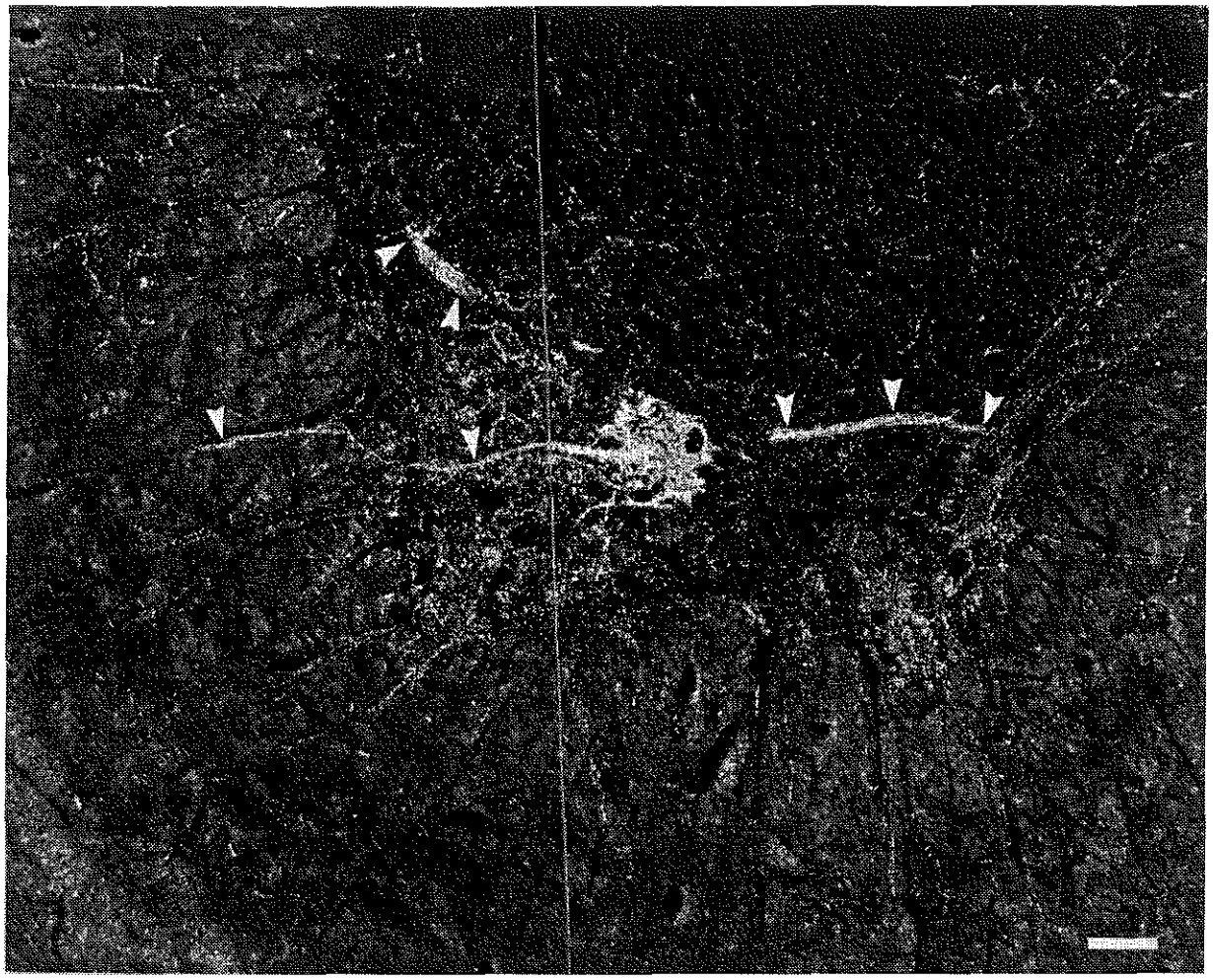


Figure 9: Photomicrograph with darkfield illumination of a section from the monkey L3 segment showing a very large number of dopamine immunoreactive fibers and varicosities around the motoneurons of the cremaster nucleus. Note that this strong innervation is also present along (presumed) dendritic bundles (white arrowheads) that radiate in several directions. Also note the fine granular labeling in the other (medial and lateral) motoneuronal cell groups, while the intermediate zone is only sparsely innervated. 1 cm = 100 μm.

low but not absent (Kojima *et al.*, 1983a; Polistina *et al.*, 1990). It is interesting to note that the central cervical nucleus, which also projects to the cerebellum (Matsushita and Hosoya, 1979), received a very sparse dopamine innervation as well. This might suggest that cerebellar-projecting neurons are avoided by the dopamine fibers. On the other hand, there are many more neurons, apart from the nucleus dorsalis and the central cervical nucleus, that project to the cerebellum (Grant *et al.*, 1982). Since these cells are scattered over different locations in the spinal cord, it will be difficult to determine whether or not they receive a monoaminergic innervation. The reason for the avoidance by dopamine of spinal nuclei projecting to the cerebellum is still unclear.

The ventral horn. The presence of dopamine in the motoneuronal cell groups of the ventral horn was discovered much later than in the dorsal horn (Yoshida and Tanaka, 1988; Shirouzu *et al.*, 1990; Ridet *et al.*, 1992). The most conspicuous aspect of the dopamine labeling in the motoneuronal cell groups was its fine granular, punctate-like aspect, which was especially apparent in the rat. This appearance, that is probably caused by a large number of small, lightly stained terminals, may have been the reason why initially the existence of dopaminergic terminals in the ventral horn was denied or neglected. Also in our material, after a less than optimal fixation or immunocytochemical procedure, the dopamine labeling in the ventral horn was the first to disappear. Thus, sensitive techniques are necessary for the anatomical identification of dopaminergic terminals in the ventral horn, which is in line with the biochemical observation that the dopamine concentration in the ventral horn is significantly lower than in the dorsal horn (Fleetwood-Walker and Coote, 1981; Basbaum *et al.*, 1987). Apparently, the fine granular network in the motoneuronal cell groups of the ventral horn is derived mostly from fibers in the dorsal horn, which are running through the intermediate zone to reach the motoneuronal area. Other monoaminergic transmitters, like serotonin and noradrenaline, have also been reported to show a finer appearance in the motoneuronal cell groups than in the dorsal horn (Kojima *et al.*, 1983a; Fritschy *et al.*, 1987). The

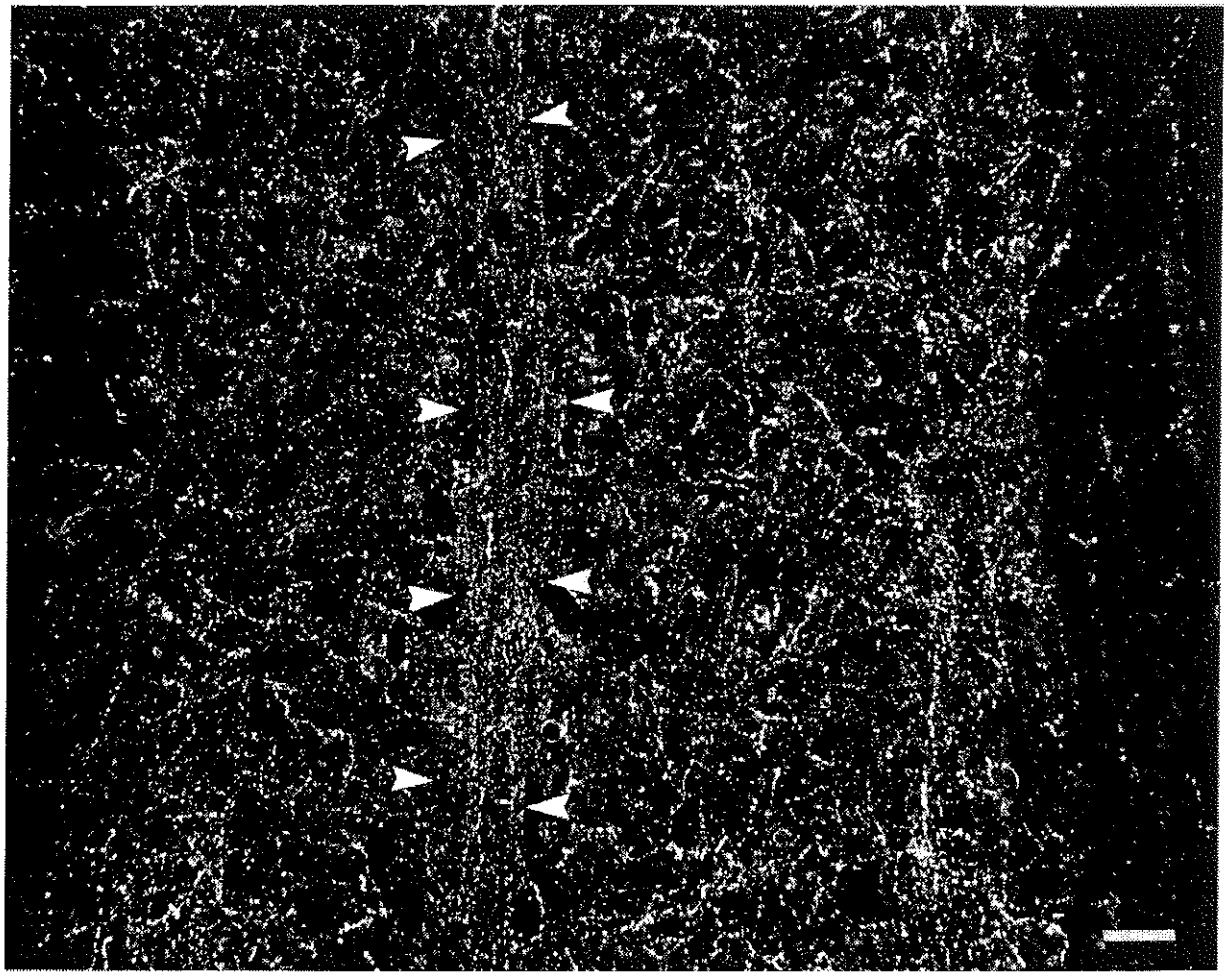


Figure 10: Photomicrograph with darkfield illumination of a horizontal section through the ventral horn of the rat C4-C5 segments. The ventrolateral funiculus is located on the left side of the micrograph and the ventromedial funiculus is located on the right side. Note the longitudinally oriented dopamine labeling in the area of the phrenic nucleus (white arrowheads), presumably alongside dendrites of phrenic motoneurons. Also note that the longitudinally oriented labeling appears somewhat stronger than the labeling in the surrounding neuropil. 1 cm = 55 μm.

strongest labeling in the motoneuronal cell groups was found in the cremaster nucleus, situated ventromedially at rostral lumbar levels, and in Onuf's nucleus (in cat and monkey) and its homologue in the rat (see results) at the lumbo-sacral level. These sexually dimorphic nuclei also receive a strong innervation of serotonin, noradrenaline and various peptides (Kojima *et al.*, 1983b; Kojima and Sano, 1984; Kojima *et al.*, 1985; Nagy and Senba, 1985; Uda *et al.*, 1986; Newton and Hamill, 1988; Wang *et al.*, 1989; Newton, 1990; Poulat *et al.*, 1992; Rajaofetra *et al.*, 1992a). We are presently investigating the dopamine innervation of these nuclei in more detail, especially with respect to possible differences in innervation between males and females.

Dopamine and glutamate. In most parts of the spinal cord, the distribution of the dopamine immunoreactivity is very similar to the distribution of noradrenaline and serotonin. In this respect it is interesting to note that immunoreactivity for glutamate or glutaminase (a glutamate synthesizing enzyme) was present in serotonergic neurons of the raphe nuclei (Kaneko *et al.*, 1990; Nicholas *et al.*, 1992; see also Holstege, 1996) and noradrenergic neurons in the locus coeruleus area (Kaneko *et al.*, 1990; Fung *et al.*, 1994; Liu *et al.*, 1995), all of which give rise to descending spinal projections. These findings suggest an extensive colocalization of glutamate with serotonin and with noradrenaline. In view of the many similarities between the different monoamines, it may be speculated that the dopaminergic terminals in the spinal cord also contain glutamate, as was also suggested by the presence of glutaminase in dopaminergic neurons in the A11 cell group (Kaneko *et al.*, 1990) from which the spinal dopaminergic terminals originate.

Functional considerations

Dopamine fibers and terminals are located throughout the spinal grey matter. Consequently it is to be expected that dopamine will influence both sensory and motor systems as well as various autonomic functions. Below

the effects of dopamine on some major spinal cord functions, and the receptors involved, will be discussed.

Dopamine effects on sensory transmission. Initially the effects of dopamine on sensory processing of peripheral sensory information in the spinal cord were studied by measuring the latencies of several (spinal) reflexes, like the hot-plate and tail-flick reflexes, following application of dopamine and various dopamine agonists (Barasi *et al.*, 1987; see Jensen, 1986 for review). These studies generally showed an increase in latency, suggesting an inhibition of sensory (pain) transmission. In most cases these effects could be reversed by dopamine D_2-receptor antagonists. However, effects on spinal reflexes may also be produced by influencing the motor component of the reflex. This problem does not occur with direct recordings from spinothalamic tract neurons in the superficial or deep dorsal horn. These studies showed that in monkey (Willcockson *et al.*, 1984) dopamine decreased the response of dorsal horn cells to glutamate and to noxious pinch. In rat and cat (Fleetwood-Walker *et al.*, 1988) dopamine or a D_2-agonist, iontophoretically applied to dorsal horn neurons, selectively inhibited the responses of these neurons to noxious stimuli, while the responses to nonnociceptive stimuli were unaffected. The same results were obtained after stimulating the A11 dopamine cell group, from where the dopamine spinal projections originate. All these effects were reversed by a D_2-receptor antagonist. More recently, evidence was found that spinal dopamine is involved in the antinociceptive effects of morphine (Weil-Fugazza and Godefroy, 1991) and cocaine (Kiritsy-Roy *et al.*, 1994). These effects could also be antagonized by D_2-antagonists. However, the exact mechanisms involved are still unclear.

The above studies all indicate a prominent role of D_2-receptors in mediating the antinociceptive effects of dopamine. This is in agreement with ligand binding studies on dopamine receptors in the spinal cord which all show the existence of D_2-receptors in the dorsal horn (for details and references see van Dijken *et al.*, 1996). Furthermore, recent *in situ* hybridization and immunocytochemical studies on the localization of spinal D_2-receptors in our laboratory (van Dijken *et al.*, 1996) have also shown the existence of neurons in the dorsal horn, including lamina I, expressing D_2-receptors. Taken together the various data indicate that dopamine exerts an inhibitory effect on nociceptive transmission in the spinal dorsal horn, mediated by D_2-receptors, while the effects on non-nociceptive transmission and the involvement of D_1-receptors may be less prominent.

Dopamine effects on motor control. At present only a few studies have investigated the effects of dopamine on spinal motor output. In an early study in cat (Barasi and Roberts, 1977), an increase in the field potential of antidromically activated motoneurons was found, after iontophoretic application of dopamine in the motoneuronal cell groups. This effect was blocked by a (nonspecific) dopamine receptor antagonist. These results suggested that dopamine produced an increase in motoneuron excitability. This effect may be accomplished by enhancing glutamate activated currents, as shown in cultured embryonic chick motoneurons (Smith *et al.*, 1995). A similar mechanism, i.e. the facilitation of motoneuronal excitation by other transmitters, like glutamate, has also been proposed for serotonin and noradrenaline (White and Neuman, 1980; White, 1985). Other studies have investigated the effect of dopamine or dopamine receptor agonists and antagonists on various monosynaptic and polysynaptic spinal reflexes with contradictory results. In some cases facilitation was found (Dupelj and Geber, 1981), while others reported an inhibitory effect (Carp and Anderson, 1982; Ono and Fukuda, 1984; Pehek *et al.*, 1989). In addition it was found in spinal rats (Maitra *et al.*, 1993) that intravenously applied apomorphine, a non-selective dopamine receptor agonist, had an inhibitory influence on Renshaw cell bursts elicited by electrical stimulation of the ventral roots, while a similar concentration of apomorphine had no effect on the monosynaptic reflex from the dorsal root. A recent study on the monosynaptic reflex in acutely spinalized rats using various dopamine agonists and antagonists like apomorphine and bromocriptine (a D_2-agonist) (Kamijo *et al.*, 1993) showed a depression of this reflex, which was mediated by neither D_1- nor D_2-receptors.

In a separate line of experiments, mostly performed in the late sixties (see Grillner, 1975 for review) the effects of 3,4-dihydroxyphenylalanine (DOPA) were investigated. An intravenous injection of DOPA produced walking on a treadmill in the spinal cat and had powerful, but complicated, effects on various other reflexes (Andén *et al.*, 1966b; Commissiong and Sedgwick, 1974; Grillner, 1975). Since DOPA is a precursor in the dopamine and noradrenaline synthesis and active only after decarboxylation (Andén *et al.*, 1966a), it was generally assumed that the synthesis of dopamine from DOPA and the subsequent formation and release of noradrenaline, were responsible for the effects of DOPA in the spinal cord. This assumption was based on pharmacological experiments (Jurna and Lundberg, 1968) and the supposition that dopamine was present only as a precursor for noradrenaline (see introduction). However, there are indications that dopamine may also play a role in mediating the effects of DOPA (Carp *et al.*, 1989). A recent study (Skoog and Noga, 1995) in cat and guinea-pig on the effects of iontophoretically applied dopamine on group II afferent fibers showed a significant depression of the monosynaptic field potential of electrically stimulated group II fibers, while there were no effects on group I fibers. It was further suggested that the effects in the dorsal horn were mediated by D_1- as well as D_2-receptors, while those in the intermediate zone were not mediated by dopamine receptors. This latter finding would fit with the low amount of dopamine that we have found in the intermediate zone. The strong innervation of the sexually dimorphic nuclei in the spinal cord suggests a specific function of dopamine in this special group of motoneurons. The finding (van

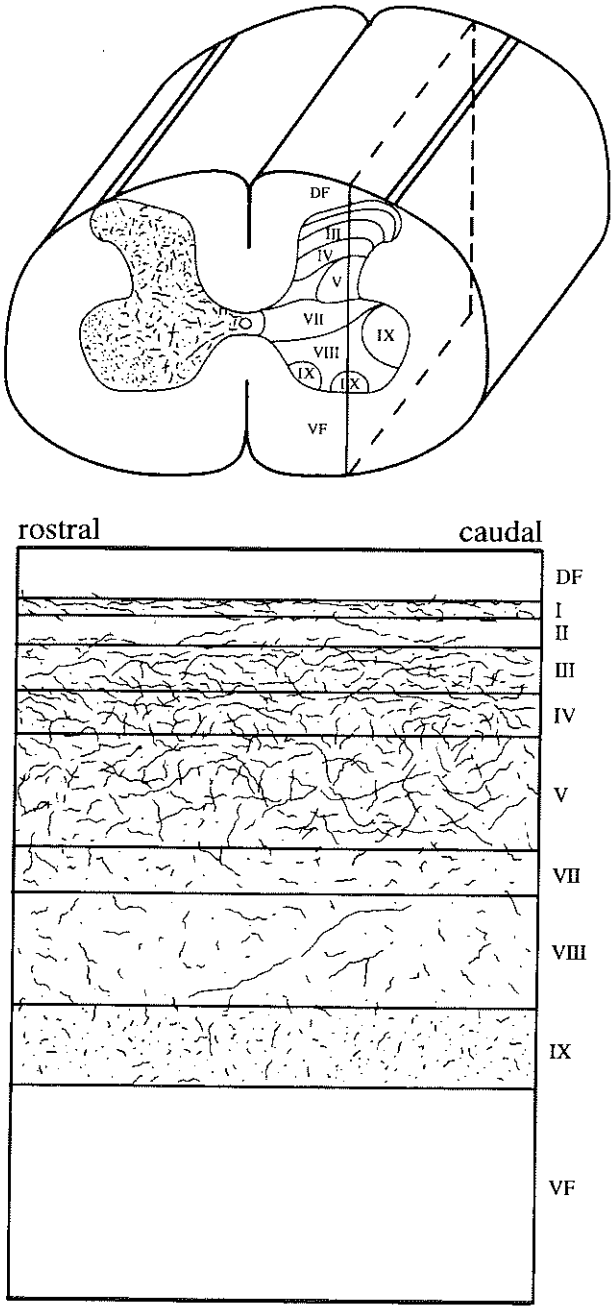


Figure 11: Schematic drawings of the dopamine labeling in the rat low cervical spinal cord. **Top:** three-dimensional drawing of the low cervical spinal cord. On the left the labeling as it appears in a transverse section, on the right the plane of section of the drawing in B) is indicated. **Bottom:** labeling in a sagital section as indicated above. Note the rostro-caudally oriented fibers in laminae I-III, the more random orientation in laminae IV and especially lamina V, the paucity of labeling in laminae VII and VIII and the punctate labeling in lamina IX.

Dijken *et al.*, 1996) that D_2-receptors were present in the (the homologue of) Onuf's nucleus in the rat would strengthen this idea, although the specific function of dopamine in these cases is unclear. Taken together it may be concluded that the effects of dopamine on motor output are diverse and exerted on the motoneuronal as well as on the interneuronal level. It seems likely that different types of dopamine receptors are involved, while some effects may be produced by non-dopamine receptors. Also a presynaptic action should not be excluded (Maitra *et al.*, 1993). More detailed analyses of the effects of dopamine on individual (motor) neurons, rather than on groups of cells or on reflexes, are needed to broaden our understanding of the effects of dopamine on the motor system, and to compare the cellular physiology of dopamine with the actions of noradrenaline and serotonin (for review see White *et al.*, 1996). In view of

the presence of dopamine in the motoneuronal cell groups the question arises whether part of the disturbed motor performance in Parkinson's disease may be attributed to changes in dopamine neurotransmission at the spinal level (Lindvall *et al.*, 1983). However, there is no evidence that A11 dopaminergic neurons are affected in Parkinson's disease and in addition it was shown (Scatton *et al.*, 1986) that the dopamine concentration in the spinal cord of Parkinsonian patients was the same as compared to healthy subjects. Therefore, it seems at present unlikely that changes in spinal dopamine are involved in producing the motor deficits associated with Parkinson's disease.

Dopamine effects on autonomic functions. The dense dopamine innervation of the preganglionic neurons in the IML, suggests that dopamine is strongly involved in regulating the sympathetic outflow. Studies on the effects of dopamine have focussed mainly on blood pressure and heart rate, which are controlled by the preganglionic sympathetic neurons in the upper thoracic area (Lahlou *et al.*, 1990). Intrathecal administration of D_1- and D_2-receptor agonists showed that hypotensive effects could be obtained after D_1 and also after D_2-receptor activation, while bradycardia was related only to D_2-receptor activation (Pellissier and Demenge, 1991). Our recent study (van Dijken *et al.*, 1996) using *in situ* hybridization and immunocytochemistry also showed the presence of D_2-receptors in the IML. Iontophoretic application of dopamine onto a very limited number of preganglionic sympathetic neurons at the Th-2 segment induced an increase in firing rate of these neurons (Lewis and Coote, 1990), suggesting an increase in blood pressure and heart rate. However, it was shown in the same study that the effects of noradrenaline, which were investigated in much more detail than those of dopamine, were inhibitory in high doses and excitatory in low doses. If the same mechanism also applies to the effects of dopamine, it may explain that the effects of dopamine on the sympathetic preganglionic neurons regulating blood pressure and heart rate, are not straightforward. The effects of dopamine may depend not only on the types of receptors that are involved, but also on the concentration of the transmitter released and on the interaction with many other transmitters that are present in the IML.

The dopamine innervation of the sacral cord suggests a specific innervation of the preganglionic parasympathetic neurons, although this innervation is much less pronounced than the innervation of the sympathetic preganglionic neurons in the IML. With respect to the effects of dopamine on the parasympathetic nuclei there are no data available. The high degree of collateralization in the dopamine diencephalo-spinal projection would imply that dopamine is released simultaneously in many different areas of the spinal cord, including the sympathetic and parasympathetic preganglionic neurons. If the release of dopamine is part of a specific type of behavior, it is to be expected that dopamine will exert opposite effects on sympathetic and parasympathetic preganglionic neurons. Whether this is indeed the case is presently unclear.

Conclusion

The present study shows the existence of a distinct and extensive dopamine innervation of the sensory, motor and autonomic areas of the spinal cord of the rat, cat and monkey. Although the concentration of dopamine in the spinal cord is much less than that of noradrenaline and serotonin, evidence is now accumulating for specific effects of dopamine in these spinal cord areas, i.e. an inhibitory effect on sensory transmission, a mainly facilitory (but probably mixed) effect on motor transmission and a complicated, probably mainly inhibitory, effect on sympathetic outflow. However, more detailed studies are needed to determine the effects of dopamine in specific areas of the spinal cord with more certainty. The distributions of dopamine, noradrenaline and serotonin are very similar, which strengthens the idea that the monoamines have a similar mode of operation and may have similar effects, although their activity may be regulated differently, depending on the behavioral context.

This study emphasizes that besides noradrenaline and serotonin, dopamine should also be included when considering the monoaminergic effects on spinal processing. In fact, the interaction between the various monoamines may be of even greater importance than the effect of the respective transmitters alone. Investigations along this line may prove worthwhile.

Acknowledgements

The authors thank Dr. J. Voogd for reading the manuscript and Mr. E. Dalm for his help with the photography. Supported by Grant 900-550-072 from the Netherlands Research Organization (NWO).

Localization Of Dopamine D_2 Receptor In Rat Spinal Cord Identified With Immunocytochemistry And In Situ Hybridization

Henk van Dijken, Jan Dijk[1], Pieter Voorn[2], and Jan C. Holstege.

[1]Department of Medical Biochemistry, Sylvius Laboratory, University of Leiden, and
[2]Department of Anatomy and Embryology, Vrije Universiteit, Amsterdam, The Netherlands.

European Journal of Neuroscience, 8: 621-628 (1996)

Localization Of Dopamine D_2 Receptor In Rat Spinal Cord Identified With Immunocytochemistry And In Situ Hybridization

Abstract

In the present study the distribution of dopamine D_2-receptors in rat spinal cord was determined by means of immunocytochemistry using an anti-peptide antibody, directed against the putative 3rd intracellular loop of the D_2-receptor, and *in situ* hybridization using a [^{35}S]UTP labeled anti-sense riboprobe.

With the immunocytochemical technique, labeling was confined to neuronal cell bodies and their proximal dendrites. Strongest labeling was present in the parasympathetic area of the sacral cord and in two sexually dimorphic motor nuclei of the lumbosacral cord, the spinal nucleus of the bulbocavernosus, and the dorsolateral nucleus. Moderately labeled cells were present in the intermediolateral cell column, the area around the central canal and lamina I of the dorsal horn. Weak labeling was present in the lateral spinal nucleus and laminae VII and VIII of the ventral horn. Except for the two sexually dimorphic motor nuclei of the lumbosacral cord labeled motoneurons were not encountered.

With the ISH technique radioactive labeling was present in many neurons, indicating that they contained D_2-receptor mRNA. The distribution of these neurons was very similar to the distribution obtained with immunocytochemistry, but with ISH additional labeled cells were detected in laminae III and IV of the dorsal horn, which were never labeled with immunocytochemistry.

The present study shows that the D_2-receptor is expressed in specific areas of the rat spinal cord. This distribution provides anatomical support for the involvement of D_2-receptors in modulating nociceptive transmission and autonomic control. Our data further indicate that D_2-receptors are not directly involved in modulating motor functions with the exception, possibly, of some sexual motor functions.

Introduction

Biochemical studies in the seventies (Magnusson, 1973; Commissiong and Neff, 1979) first indicated that dopamine in the spinal cord acted as a neurotransmitter and not solely as a precursor in the synthesis of other catecholamines. Anatomically, the presence of dopamine fibers in the spinal cord was first demonstrated by histofluorescence techniques (Björklund and Skagerberg, 1979; Hökfelt *et al.*, 1979; Skagerberg *et al.*, 1982). More recently, immunocytochemical studies using dopamine specific antibodies at the light and electron microscopical level revealed that dopamine fibers and terminals existed in virtually all laminae throughout the spinal cord (Holstege *et al.*, 1990; Shirouzu *et al.*, 1990; Mouchet *et al.*, 1992; Ridet *et al.*, 1992). Dopamine immunoreactivity was most pronounced in the dorsal horn, in the area around the central canal, in the sympathetic intermediolateral cell column (IML), and in the motoneuronal cell groups. Retrograde tracing techniques combined with histofluorescence (Björklund and Skagerberg, 1979) or immunohistochemistry of catecholamine-synthesizing enzymes, like tyrosine hydroxylase (Hökfelt *et al.*, 1979; Skagerberg *et al.*, 1988) and aromatic *L*-amino decarboxylase (Skagerberg *et al.*, 1988), indicated that the spinal dopamine innervation originated exclusively from the A11 catecholamine cell group, which is situated in the periventricular posterior hypothalamus. The paraventricular nucleus has also been implicated as a minor source for the spinal dopamine innervation (Swanson *et al.*, 1981). Thus, the existence of an extensive dopamine innervation of the spinal cord, mainly originating from the A11 group, is now well established.

Over the years, biochemical, pharmacological, and electrophysiological studies (Barasi and Roberts, 1977; Coote *et al.*, 1981; Fleetwood-Walker *et al.*, 1988) provided evidence for specific effects of dopamine within the spinal cord which were mediated by dopamine receptors. Presently, dopamine receptors are subdivided in two receptor families based on pharmacological and biochemical criteria (for reviews see Civelli *et al.*, 1991; Gingrich and Caron, 1993). These receptor families include D_1-like receptors, which are stimulatory coupled to adenylate cyclase, and D_2-like receptors, which are either inhibitory or not coupled to this enzyme. Subtypes belonging to the two dopamine receptor families have been cloned and sequenced: D_1-like receptors include the D_1 and D_5 dopamine receptors, whereas D_2-like receptors include the D_2, D_3, and D_4 dopamine receptors. By alternate splicing of the D_2-receptor gene two isoforms (D_{2short} and D_{2long}) are generated, which differ by a splice insert of 29 amino acids in the putative 3rd intracellular loop of the D_2-receptor. This insert is present in the long isoform, but is lacking in the short isoform.

The specific distribution of dopamine receptors in the central nervous system has been studied with receptor ligand binding, ISH, and more recently immunocytochemistry. So far, the few studies that were focussed on the distribution of dopamine receptors in the spinal cord were mainly based on receptor ligand

binding. Binding studies on homogenized rat spinal cord first suggested the presence of dopamine D_1-receptors in the spinal cord (Bhargava and Gulati, 1990) especially in its ventral part (Dubois *et al.*, 1986). In contrast, D_2-receptor binding sites were found mainly in the dorsal horn of the spinal cord (Demenge *et al.*, 1980; Dubois *et al.*, 1986; Bouthenet *et al.*, 1987), especially laminae II and III, and near the central canal (Scatton *et al.*, 1984). More recently, an extensive binding study on the D_2-receptor with the highly selective compound YM-09151-2 confirmed and extended these findings (Yokoyama *et al.*, 1994) by showing additional binding in the IML and motoneuronal cell groups of the ventral horn. The presence of D_2-receptor mRNA in the spinal gray matter has also been reported, but no anatomical details were provided (Bouthenet *et al.*, 1991). However, the interpretation of the results obtained with the ligand binding technique is often difficult because the selectivity of the ligands and the anatomical resolution are limited. The immunocytochemical method provides a better anatomical resolution and may also be a more accurate tool to distinguish between different dopamine receptors and their subtypes than the current ligand binding techniques. Only after the discovery of the sequence of the D_2-receptor (Bunzow *et al.*, 1988) it became possible to prepare specific antibodies, using fusion proteins or synthetic peptides corresponding to a specific part of the D_2-receptor. These peptide fragments have been used successfully for raising polyclonal antibodies against the D_2-receptor (McVittie *et al.*, 1991; Ariano *et al.*, 1993; Boundy *et al.*, 1993b; Boundy *et al.*, 1993a; Chazot *et al.*, 1993; Sesack *et al.*, 1994; Smiley *et al.*, 1994) and other dopamine receptors, including the D_1- and D_3-receptor (Ariano and Sibley, 1994; Smiley *et al.*, 1994). Some of these immunocytochemical studies also reported the presence of dopamine receptors in the spinal cord (Ariano *et al.*, 1993; Smiley *et al.*, 1994), however, no anatomical details were provided. Taken together, the available data, mostly based on receptor ligand binding, suggest that dopamine D_1-receptors are predominantly located in the ventral horn, while D_2-receptors are mainly localized in the dorsal horn, but may also be found in motoneuronal cell groups of the ventral horn.

In the present study we have used a polyclonal antipeptide antibody (Plug *et al.*, 1992) for mapping of the D_2-receptor in rat spinal cord. In addition we have applied the ISH technique to verify and extend the data obtained with immunocytochemistry, using an anti-sense [^{35}S]UTP labeled riboprobe for detection of D_2-receptor mRNA.

Materials and methods

Preparation and characterization of the D_2-receptor antibody

Preparation and characterization of the antibody used in the present study has been described previously (Plug *et al.*, 1992). Briefly, a polyclonal antiserum (pAb2) was obtained from rabbits after injecting a synthetic peptide coupled to keyhole limpet hemocyanin. The peptide was derived from an amino acid sequence (amino acids 301 - 315) in the 3rd intracellular loop of the rat D_2-receptor, which is present in both the short and long isoform. For the purpose of the present study two different affinity gels were used to obtain purified antibodies from this antiserum: (1) Protein A-Sepharose 6MB (Pharmacia) and (2) synthetic peptide coupled directly to CNBr-activated Sepharose 4B. In each case 500 µl antiserum was incubated with 500 µl of the corresponding gel in a total volume of 5 ml 10 mM Tris-HCl (pH 7.4), 250 mM NaCl. After 16 h at 4°C the gel suspension was transferred to a 3 ml disposable column and washed extensively with Tris-HCl buffer. Specific antibodies were eluted with 3 column volumes of 0.1 M glycine-chloride (pH 2.5) and neutralized immediately by collecting them in 0.3 column volume 1 M Tris-HCl (pH 8.0), followed by addition of bovine serum albumin solution to a final concentration of 1 mg/ml. The collected purified antisera were stored frozen at -80°C until use. The antibodies obtained with the protein A-sepharose 6MB gel gave the most intense staining in all regions of the spinal cord, and antibodies obtained with this method were mainly used for the purpose of this study.

Immunocytochemistry

Adult, male Wistar rats were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 50 ml saline, followed by 500-750 ml 4% (wt/vol) freshly made paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). In some cases up to 0.05-0.2 % (vol/vol) glutaraldehyde was added to the fixative and best results were obtained with 0.1 % glutaraldehyde. The fixation liquid was followed by 150 ml of 10-15% (wt/vol) sucrose in phosphate buffer. After perfusion the brains and spinal cords were removed and stored overnight at 4 °C in phosphate buffer containing 20-25% (wt/vol) sucrose. The next day 30 µm frozen sections were cut from all spinal segments and selected parts of the brain and collected in 0.1 M Tris buffered saline (pH 8.6). All subsequent procedures were performed on free-floating sections at room temperature except where specified otherwise. After each step sections were thoroughly rinsed with Tris buffered saline. Sections were first incubated with a solution of 5% (vol/vol) normal goat serum (Gibco) in Tris buffered saline containing 0.3% (vol/vol) Triton X-100 for 90 minutes to reduce background staining. Next, sections were incubated for 24-48 hours at 4 °C with polyclonal D_2-receptor antipeptide antibody (1:500) followed by biotinylated goat-anti-rabbit (1:200, 120 minutes) and the ABC procedure (ABC Elite, Vector). The peroxidase complex was visualized with 0.05% (wt/vol) 3,3'-diaminobenzidine (Sigma) in 0.05 M Tris-HCl solution (pH 7.6) in the presence of 0.01% (vol/vol) hydrogen peroxide, which yields a brown precipitate. In some cases 0.01% (wt/vol) nickel ammonium sulphate solution was added, which resulted in a dark blue precipitate. After completion of the immunocytochemical procedure sections were mounted onto glass slides with gelatin-chrome alum adhesive, and allowed to dry at room temperature. Next, sections were dehydrated in graded ethanol baths, transferred to xylene, and coverslipped with Permount. Some slides

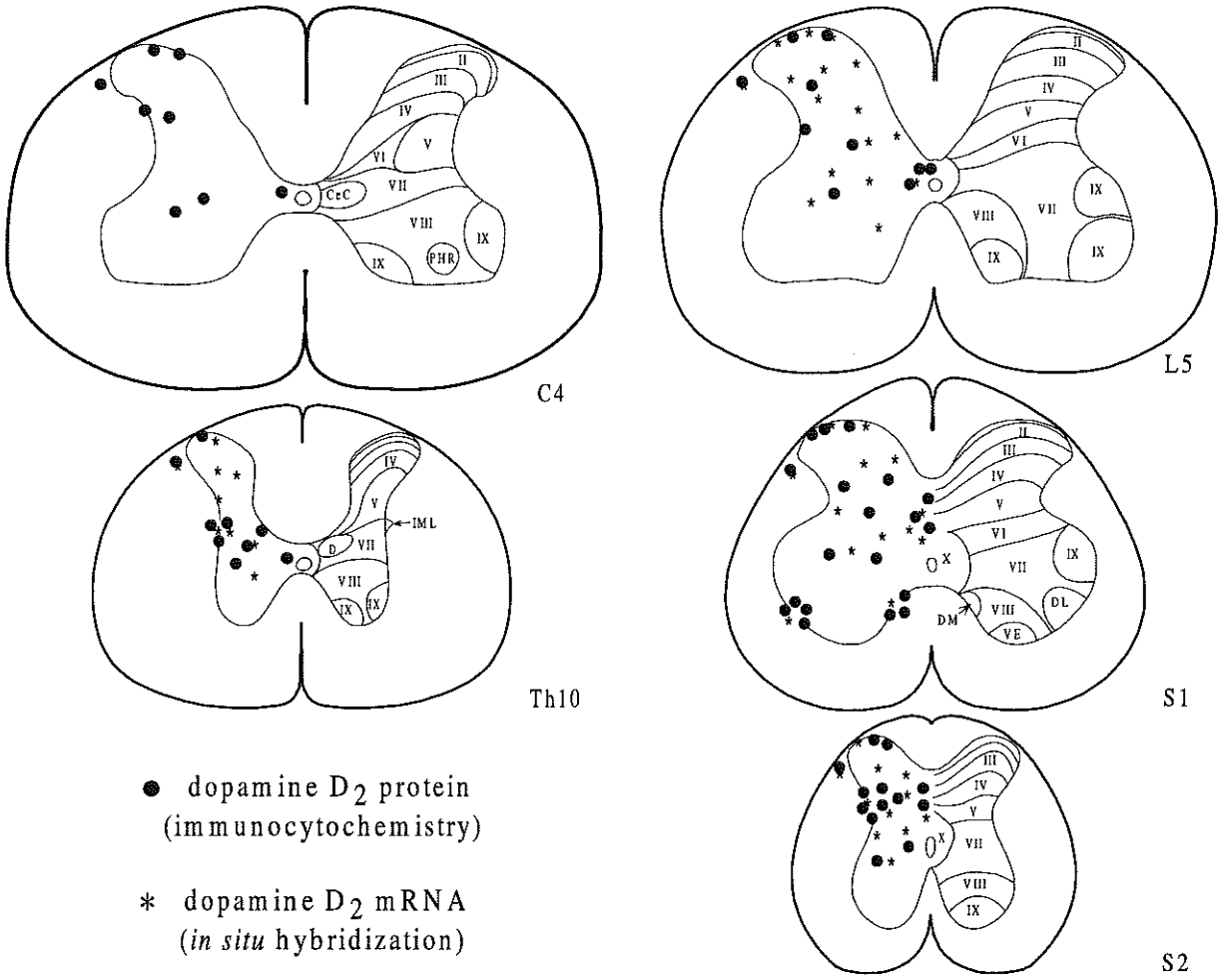


Figure 1: Schematic representation showing the distribution of neurons immunoreactive for dopamine D_2 receptor protein (filled dots; 1 dot = 1-3 cells) or radioactively labelled for D_2 receptor mRNA (stars; 1 star = 3-5 cells), as revealed by immunocytochemistry and *in situ* hybridization. Note that no stars were drawn in the first segment (C4), because high cervical levels were not processed for *in situ* hybridization.

were counterstained with cresyl violet. Sections were examined light microscopically with bright-field illumination.

Immunocytochemical controls

Controls for the immunocytochemical methods included: (i) substitution of the anti-peptide antiserum with preimmune serum, (ii) substitution of the primary antiserum with saline, and (iii) preadsorption of the antiserum with an excess of its peptide antigen (1 mg/ml antiserum) prior to incubation. Omission of the primary antibody or substitution of preimmune serum for the anti-peptide antibody resulted in clean sections without staining. Preadsorption of the antiserum with an excess of its peptide antigen prior to incubation prevented neuronal staining, except for some white matter glia, which is, therefore, considered as background staining. Adding glutaraldehyde to the perfusion fixative resulted in reduction of background staining, including the white matter glia, while the labeling in the areas described remained, albeit less intense.

In the brain D_2-receptor immunoreactivity was found in many areas, among which the cerebral cortex, striatum, substantia nigra pars compacta, hippocampus, ventral tegmental area, superficial layers of superior colliculus, and hypothalamus. The labeling obtained in brain was very similar to previous anatomical studies on the localization of D_2-receptor using ISH (Weiner *et al.*, 1991; Brouwer *et al.*, 1992), receptor ligand binding (Dubois *et al.*, 1986; Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994), and immunocytochemistry (Brock *et al.*, 1992; Ariano *et al.*, 1993; Levey *et al.*, 1993). We therefore concluded that the immunocytochemical labeling of neuronal elements obtained in the present study represented D_2-receptor protein.

In situ hybridization

Rats were decapitated and their spinal cords were quickly removed and frozen on dry ice. Cryostat sections (10 μm) from thoracic, lumbar and sacral levels of the spinal cord were made and collected on gelatin-coated slides, air dried at room temperature and fixed with 4% (wt/vol) paraformaldehyde solution in

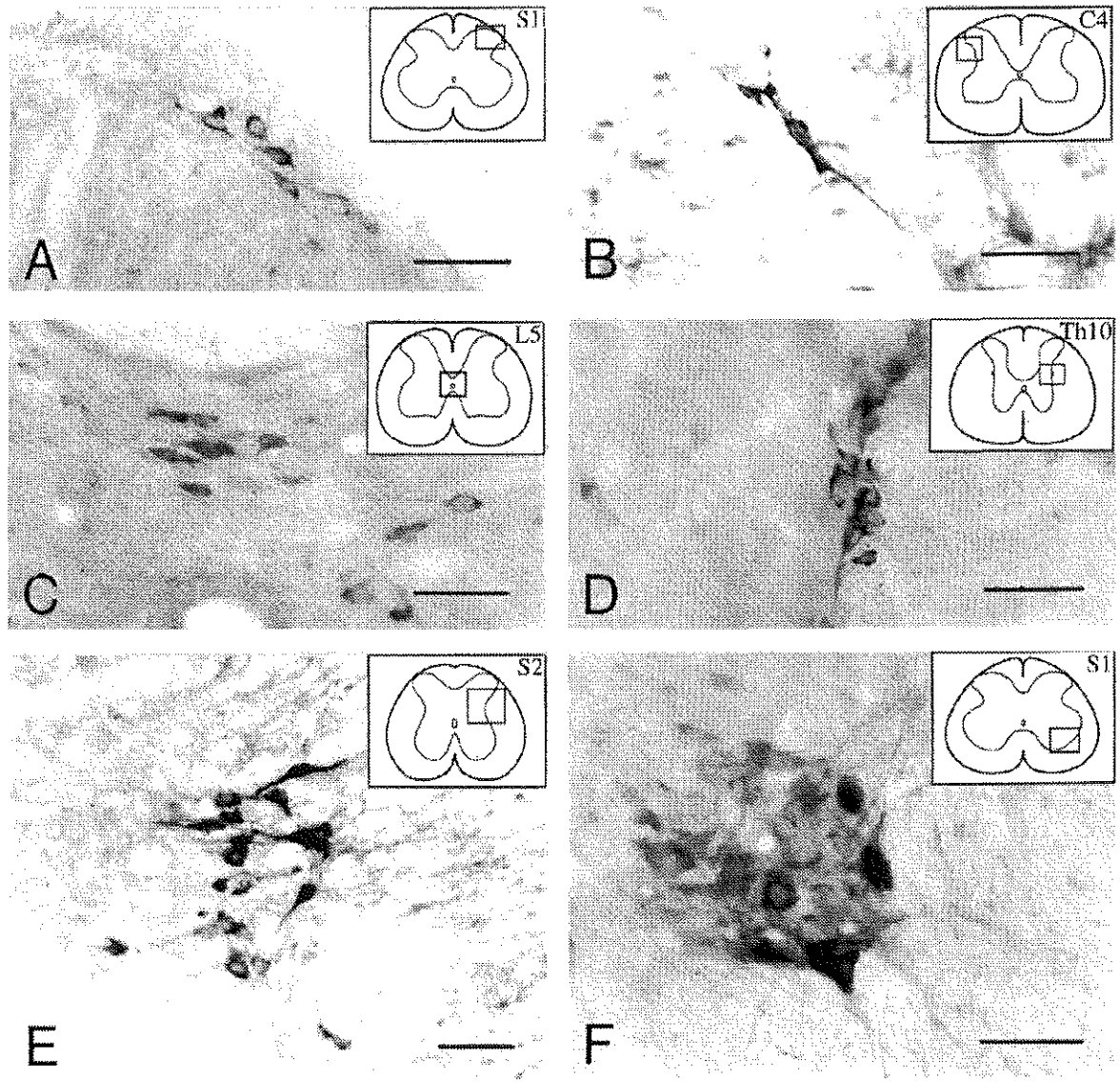


Figure 2: A-F. Light micrograph of dopamine D_2 receptor protein immunoreactive cells in different areas of the rat spinal cord as indicated schematically in each inset. (A) Lamina I; (B) Lateral spinal nucleus of the dorsal horn; (C) Area around the central canal, (D) Preganglionic cells of the intermediolateral cell column of the thoracic cord, (E) Parasympathetic area of the sacral cord; (F) Sexually dimorphic motoneurons in the dorsolateral nucleus of the ventral lumbosacral cord. Scale bar = 50 µm.

0.05 M phosphate buffered saline (pH 7.4). After three rinses in phosphate buffered saline, sections were treated with acetic anhydride, dehydrated in graded ethanol baths, followed by two successive chloroform baths for ten minutes each. Next, slides were allowed to air dry and were stored at -70 °C until further processing.

Sections were hybridized with [^{35}S]UTP labeled anti-sense riboprobe for D_2-receptor as described (Jongen-Rêlo *et al.*, 1994). After hybridization and stringency washing steps the sections were air dried, dipped into nuclear photographic emulsion (Kodak NTB-2) and stored in the dark (4 °C). After 12-16 weeks exposure the sections were developed, fixed, and counterstained with cresylviolet. The distribution of labeled neurons was analyzed light microscopically with dark-field illumination.

Specificity controls for the *ISH* included hybridization with "sense" probe and tissue treatment with RNAse prior to antisense probe hybridization. Both methods resulted in absence of labeling and confirmed the specificity of the hybridization signal generated by the radioactively labeled riboprobe.

Results

Immunocytochemistry

In all positive-staining regions examined in the central nervous system the DAB reaction product, indicating the presence of D_2-receptor protein, was mostly associated with neuronal cell bodies and their proximal dendrites, but in some cases the dendritic processes

could be followed for some distance within a section. Application of the antibody on spinal cord sections resulted in a distinct pattern of labeled neurons. In comparison with most brain areas, labeled neurons in the spinal cord were few, and weakly labeled. However, some strongly labeled neurons were also observed (see below).

At all levels of the spinal cord labeling was found exclusively in cell bodies and their proximal dendrites. The immunoreactivity was virtually absent from other neuronal processes, like axons, small dendrites nor was there any indication for localization of immunoreactivity in terminals. The labeled neurons were located in specific areas throughout the spinal cord, including the superficial dorsal horn, the region surrounding the central canal, the sympathetic IML and the sacral parasympathetic area and, to a lesser extent, in the ventral horn (fig. 1).

In the dorsal horn densely labeled neurons were present in lamina I (fig. 2A). In every section examined, an average of 1-3 labeled cells were observed. They were found at all levels of the spinal cord and were distributed both medially and laterally within lamina I. In the lateral spinal nucleus, located in the lateral funiculus of the spinal cord, moderately labeled cells were occasionally present (fig. 2B). At high cervical levels no labeled cells were seen in the lateral cervical nucleus, although it was sometimes difficult to delineate its boundary with the lateral spinal nucleus. In each spinal cord section, from high cervical to low sacral levels, on average 1 or 2 cells were labeled in the lateral spinal nucleus. No labeled cells were observed in lamina II and III of the dorsal horn. In lamina IV and V some scattered weakly labeled cells were present, and occasionally a moderately labeled cell was encountered. In general, when going from lamina IV to laminae V and VI more labeled cells were observed and the labeling became more intense, although they were still not strongly labeled. In the area around the central canal moderately and weakly labeled cells were present at all levels of the spinal cord, but they were somewhat more intense at the lumbar level (fig. 2C). The labeled cells were scattered around the central canal and clusters of moderately labeled cells were present in the area immediately dorsal of the central canal.

In the IML of the spinal cord, at thoracic and high lumbar levels, clusters of 5-6 moderately labeled cells were present (fig. 2D) in many, but not all sections. The labeling extended into the proximal dendrites radiating towards the lateral funiculus. A high proportion of the neurons of the IML were immunoreactive. Additional labeled cells were located in related areas towards the central canal (intercalated nuclei), but these cells were less intensely labeled.

Strongest labeling within the spinal cord was found in cells of the parasympathetic area of the sacral cord (fig. 2E), and a high proportion of the neurons in this area were immunoreactive. The labeled cells showed a dense staining of their cell bodies and proximal dendrites. They were arranged in clusters of 4-5 cells, with their dendrites radiating towards the lateral funiculus. In addition to these strongly labeled cells also several moderately labeled cells were present in this area.

In the ventral horn, a few moderately and weakly labeled cells were present in laminae VII and VIII. In general, when going more ventrally, staining of labeled cells became faint. Labeled motoneurons were found only in the lumbosacral spinal nucleus of the bulbocavernosus (SNB) and dorsolateral nucleus (DLN) (fig. 2F), which innervate different target muscles: the DLN innervates the external urethral sphincter, while the SNB innervates the external anal sphincter and the bulbocavernosus. Motoneurons of the SNB and DLN were labeled equally strong, and their proximal dendrites were also densely labeled. A light staining of the neuropil surrounding the SNB and DLN was observed, probably due to faint labeling of small dendrites, which are present in longitudinally oriented bundles. Labeled motoneurons were never observed at other levels of the spinal cord.

In situ hybridization

The presence of D_2-receptor mRNA was detected with ISH. Radioactively labeled riboprobe was hybridized to D_2-receptor mRNA, which resulted in a distinct accumulation of silver grains over many cell bodies (fig. 3A-B).

In general, labeling of neuronal cell bodies was found in the same areas of the spinal gray matter as described for immunocytochemistry (fig. 1 and fig. 3A), but in many of these areas a larger number of neurons were labeled with the ISH technique. Labeled cells were present in lamina I of the superficial dorsal horn and in the lateral spinal nucleus, while no labeling was seen in lamina II. Some labeled cells were detected in lamina III and IV of the dorsal horn (fig. 3A), which is in contrast with the immunocytochemical findings. In the base of the dorsal horn labeled cells were scattered throughout lamina V and VI. In the area around the central canal labeled cells were present in clusters, predominantly located in the area immediately dorsal of the central canal. In the IML of the thoracic cord clusters of 5-6 labeled cells were observed, and additional labeled cells were present in the intercalated nuclei towards the central canal (fig. 3B). In the sacral parasympathetic area labeled cells were also present. Furthermore, in the ventral horn some labeled cells were scattered throughout lamina VII and VIII. In the motoneuronal cell groups, however, no labeling was detected except for some labeled cells in the lumbosacral SNB and DLN.

Discussion

In the present study, for the first time the regional distribution of D_2-receptor protein and mRNA was identified in rat spinal cord using immunocytochemistry and ISH. The results showed a characteristic distribution of D_2-receptors throughout the spinal cord, providing anatomical support for the involvement of D_2-receptors in the effects of dopamine in the spinal cord.

Technical considerations

The observation that D_2-receptor immunoreactivity was

located mainly within the somata and proximal dendrites of labeled neurons is in agreement with other studies using different D_2-receptor antibodies (Brock *et al.*, 1992; Ariano *et al.*, 1993). A subsequent study (Fisher *et al.*, 1994) at the electron microscopical level showed that in neostriatal neurons the D_2-receptor immunoreactivity was uniformly present throughout the somatic cytoplasm and became less strong towards the periphery. The immunoreactivity was not associated with any cellular organelle. Some axon terminals contained traces of immunoreactivity, but labeled postsynaptic densities were not described. These findings may suggest that the antibody only recognized D_2-receptor protein before its incorporation as a functional receptor into the neuronal membrane or during its degradation. If this also holds true for the antibody used in the present study, it would explain that only cell somata and proximal dendrites were stained. On the other hand, if the antibody would recognize both functional and nonfunctional receptors, our results would indicate that the highest proportion of D_2-receptor protein is located within the cell body and proximal dendrites while there are only minute amounts of functional receptors in more distal dendrites.

The long exposure times (up to 3 months) that were needed to visualize the radioactively labeled riboprobe indicated that the hybridization signal was relatively low. Similarly, with immunocytochemistry it was noted that, compared to other parts of the central nervous system, the spinal cord contained relatively few cells that were immunoreactive for the D_2-receptor and that most of them were not strongly labeled. Thus, it seems likely that in comparison with other brain areas the level of D_2-receptors in rat spinal cord is low both at the protein and mRNA level. Due to these low expression levels the total number of cells expressing D_2-receptors may have been underestimated.

Distribution of D_2-receptors in spinal cord

The results of the present study showed that the D_2-receptor is expressed in several areas of the rat spinal cord. In all spinal segments investigated with immunocytochemistry or ISH, the distribution obtained with each technique was very similar, i.e. all areas with neurons immunoreactive for D_2-receptor protein also showed neurons expressing D_2-receptor mRNA, although more labeled cells were detected with ISH.

With immunocytochemistry, strongest labeling was present in neuronal cell bodies located in the sacral parasympathetic area and in the lumbosacral SNB and DLN. Moderately labeled cells were present in the IML, in the area around the central canal, and in lamina I of the superficial dorsal horn, while weak labeling was present in neuronal cell bodies located in the lateral spinal nucleus and lamina VII and VIII of the ventral horn. The results obtained with ISH confirmed this distribution and extended the immunocytochemical findings by showing additional labeled cells in lamina III and IV of the dorsal horn, an area which was never labeled with immunocytochemistry. This difference may be explained by the fact that in this region the concentration of D_2-receptor protein may be very low and, therefore, undetectable with immunocytochemistry. As a consequence our findings indicate that for detection of D_2-receptor expression in the spinal cord the ISH technique may be a more sensitive technique than the immunocytochemistry technique. Finally, it should be noted that the identification of the spinal laminae and nuclei was based on cytological and topographical criteria. As a consequence, in the sacral parasympathetic area there is no direct evidence that neurons expressing the D_2-receptor actually represent parasympathetic preganglionic neurons, as strongly suggested by their location. This can only be ascertained by using double-labeling studies which combine D_2-receptor localization with the appropriate technique for identifying specific neurons.

Previous studies on the regional distribution of D_2-receptors in spinal cord all used the receptor ligand binding technique. Ligand binding was reported in the dorsal horn (Scatton *et al.*, 1984; Dubois *et al.*, 1986; Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994), the area around the central canal (Scatton *et al.*, 1984; Yokoyama *et al.*, 1994), the IML and in motoneurons (Yokoyama *et al.*, 1994). Our findings are in agreement with these binding studies, but differed from the reported binding in the substantia gelatinosa and in the motoneuronal cell groups, areas which showed neither immunoreactivity nor hybridization signal. However, it must be kept in mind that the binding pattern may differ from the cellular distribution obtained with ISH or immunocytochemistry, because the functional D_2-receptors, i.e. the binding sites, may be located on dendrites of neurons which have their cell somata in another lamina of the spinal cord. With respect to the binding found in the substantia gelatinosa our findings indicate that the parent cell bodies of these D_2-receptor binding sites are located outside the substantia gelatinosa of the dorsal horn (lamina II). On the other hand, the ligands may also bind to other receptors of the D_2-receptor family, due to a lack of pharmacological selectivity which is inherent to the receptor ligand binding technique. This may be true especially for the reported ligand binding in spinal motoneurons (Yokoyama *et al.*, 1994), because neither binding studies nor *in situ* hybridization studies have described the presence of D_2-receptors in motoneurons. Moreover, we never observed D_2-receptor labeling in motoneurons outside the lumbosacral SNB and DLN. Therefore, we consider the presence D_2-receptors in spinal motoneurons, except the SNB and DLN, most unlikely.

Functional aspects

The distribution of D_2-receptors in rat spinal cord, as demonstrated with immunocytochemistry and ISH, suggests that D_2-receptors may play a role in the transmission of sensory information and autonomic processing. Our results also indicate that D_2-receptors are not directly involved in modulation of motoneurons, with the exception of motoneurons in the SNB and DLN of the lumbosacral cord.

Several studies indicate that spinal dopamine and, more specifically, D_2-receptors have an inhibitory role in the transmission of nociceptive stimuli. It has been reported that intrathecally administered D_2-receptor

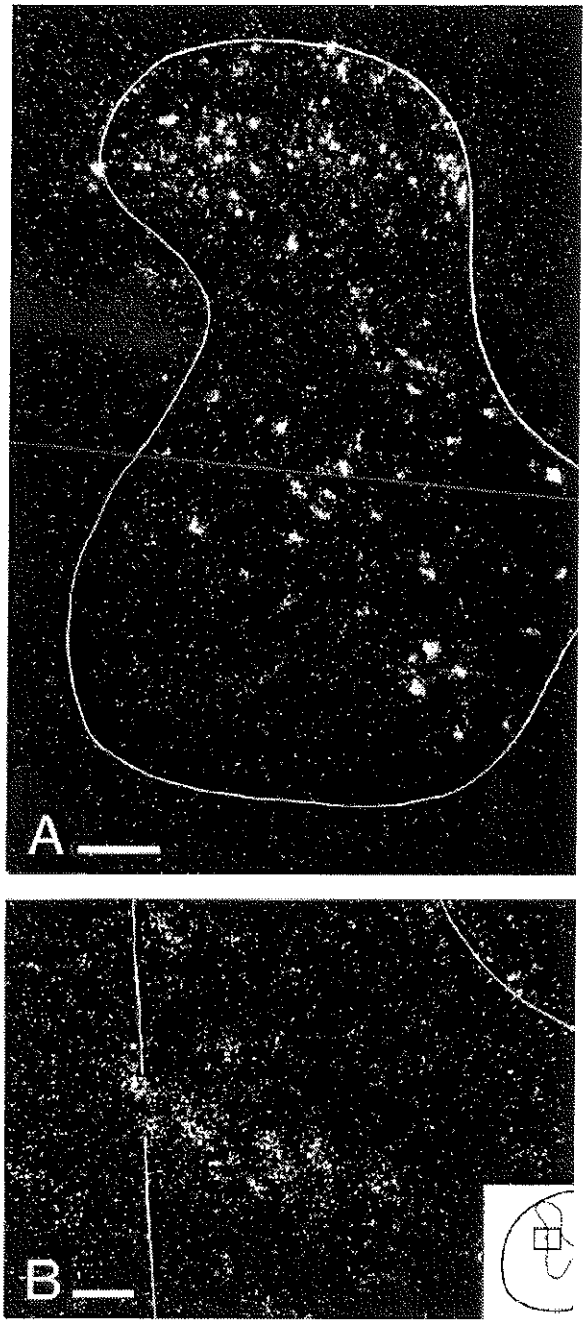


Figure 3: A-B. Darkfield microautoradiographs of selected parts of the rat spinal cord showing accumulation of silver grains over neurons containing detecting dopamine D_2 receptor mRNA hybridized to 35S-labeled riboprobe. (A) Lumbar cord; (B) Lateral horn of thoracic cord. Scale bar = 200 µm and 50 µm, respectively.

agonists produced a reversible reduction of thalamic nociceptive responses to noxious stimuli (Clatworthy and Barasi, 1987). Furthermore, focal electrical stimulation in the region of the A11 cell group selectively suppressed nociceptive responses of spinal multireceptive neurons (Fleetwood-Walker *et al.*, 1988). This inhibition was rapidly reversed by ionophoresis of the D_2-receptor antagonist sulpiride in the superficial and deeper dorsal horn. Similarly, D_2-receptor antagonists antagonized the antinociceptive effects induced by intrathecally applied dopamine (Liu *et al.*, 1992). In contrast, neither the D_1-receptor agonist SKF38393 nor D_1-receptor antagonist SCH23390 showed any effect on the dopamine-induced antinociception (Barasi *et al.*, 1987; Liu *et al.*, 1992). These findings suggest that the dopamine-induced antinociceptive effects are mediated via spinal D_2-receptors located in the superficial and deeper dorsal horn. This is in good agreement with the regional distribution of D_2-receptors in the dorsal horn as described in the present study.

The presence of D_2-receptors in the IML of the thoracic and high lumbar cord is in agreement with its dense dopamine innervation (Yoshida and Tanaka, 1988; Shirouzu *et al.*, 1990; Mouchet *et al.*, 1992; Ridet *et al.*, 1992; Holstege *et al.*, 1996). Apomorphine and other, more selective, D_2-receptor agonists administered intrathecally down regulated autonomic functions, resulting in hypotension and bradycardia (Petitjean *et al.*, 1984; Clatworthy and Barasi, 1987; Pellissier and Demenge, 1991; Lahlou and Demenge, 1993). Our findings suggest that the inhibition by dopamine of preganglionic cells of the IML is mediated by D_2-receptors.

D_2-receptors were found to be most prominent in neurons of the sacral parasympathetic area and the lumbosacral SNB and DLN, which innervate anatomically distinct perineal muscles that are involved in functionally distinct copulatory reflexes. Previous studies have shown that dopamine agonists inhibit specific sexual motor functions like penile reflexes (Stefanick *et al.*, 1982; Pehek *et al.*, 1989). Our results provide some anatomical support for the involvement of D_2-receptors located in the lumbosacral cord in the regulation of sexual motor behavior at the spinal level.

There is also some evidence favoring the idea that dopamine is involved in the control of spinal motor functions. Although dopamine is present in the ventral cord, including motoneuronal cell groups, our results indicate that D_2-receptors are not directly involved in modulating motoneuron activity. The reported effects of dopamine on somatic motor functions at the spinal level are contradictory. Dopamine receptor mediated depression of the spinal monosynaptic transmission has been reported (Carp and Anderson, 1982; Ono and Fukuda, 1984) and denied (Kamijo *et al.*, 1993). Recently, it has been reported that dopamine enhances glutamate-activated currents in cultured chick embryo spinal motoneurons. The enhancement was diminished by the D_1-receptor antagonist SCH23390 and mimicked by the (partial) D_1-receptor agonist SKF38393 (Smith *et al.*, 1995). These findings suggest that in the motoneuronal cell groups the presence of dopamine and, more particularly, the absence of D_2-receptor labeling in motoneurons may indicate that other dopamine receptors, like D_1-receptors, are more important in this part of the spinal cord.

It may be concluded that the D_2-receptors are expressed in specific areas of the rat spinal cord. Although the level of D_2-receptor in spinal cord may be

much lower than in brain, several physiological studies indicate that activation of spinal D_2-receptors has important functional consequences. Their distribution provides anatomical support for the involvement of spinal D_2-receptors in the modulation of nociceptive transmission and autonomic control. Our data further indicate that spinal D_2-receptors are not directly involved in modulating motor functions at the motoneuronal level with the possible exception of some sexual motor functions. However, it should be mentioned that motor functions may be modulated indirectly via D_2-receptors located in the dorsal horn or at supraspinal levels. In order to further elucidate the role of dopamine in modulating spinal cord functioning further investigation of various types of dopamine receptors in the spinal cord needs to be undertaken.

Acknowledgments

The authors thank Prof. Dr. Jan Voogd for reading the manuscript. We also thank Henri Klop and Allert Jan Jonker for their technical assistance with the ISH study. The technical advice of Erika Sabel-Goedknegt and Richard Hawkins is acknowledged. The assistance of Eddie Dalm in the preparation of the photomicrographs is appreciated.

The Distribution Of Dopamine Immunoreactive Fibers And Presumptive Terminals In The Rat Brain Stem. A Comparison With The Distribution Of Dopamine-ß-Hydroxylase.

The Distribution Of Dopamine Immunoreactive Fibers And Presumptive Terminals In The Rat Brain Stem. A Comparison With The Distribution Of Dopamine-ß-Hydroxylase.

Abstract

In the present study the distribution of fibers and terminals immunoreactive for dopamine was identified light microscopically in rat brain stem and compared with the distribution of fibers and terminals immunoreactive for dopamine-ß-hydroxylase (DBH), the enzyme essential for synthesizing noradrenaline and adrenaline.

The dopaminergic fibers and terminals showed a characteristic distribution within the brain stem. They were present in many brain stem nuclei with various labeling densities. Strongest dopamine labeling was found in the dorsal motor nucleus of the vagus, principal nucleus of the inferior olive and dorsal raphe nucleus. Other areas, like the nucleus of the solitary tract, raphe pallidus and obscurus, lateral parabrachial nuclei, and the periaqueductal gray, were strongly labeled, whereas the sensory trigeminal complex, the medial parabrachial nucleus, the locus coeruleus and several other (nor)adrenergic cell groups were labeled moderate to strong. Furthermore, dopamine immunoreactive fibers and terminals were present in many other areas of the brain stem, including the inferior and superior colliculi, deep mesencephalic nuclei, pontine reticular nuclei, prepositus hypoglossal nuclei, cochlear nuclei, cuneate and gracile nuclei. In most nuclei, dopamine was not distributed throughout the nucleus, but aimed at specific subnuclei. DBH containing fibers and terminals in the brain stem were distributed more ubiquitously. In most areas there was much overlap with the dopaminergic innervation, but the regional innervation pattern differed. In other areas like area postrema, cerebellum, anterior portion of the ventral cochlear nucleus and external cuneate nucleus, which were all sparsely innervated by dopaminergic fibers, the (nor)adrenaline synthesizing enzyme was abundantly present.

The results of the present study demonstrate a substantial dopaminergic innervation of many brain stem nuclei, which is independent from the (nor)adrenergic innervation. So far, relatively few studies have focussed on the effects of dopamine in the brain stem. However, our anatomical findings suggest that dopamine is involved in modulating information processing in many brain stem areas and should be considered equally important as other monoamines like (nor)adrenaline and serotonin.

Introduction

The brain stem is a highly complex structure that includes numerous neuronal circuits for processing, controlling, and integrating a variety of sensory, autonomic and motor functions. In order to understand how the brain stem exerts all these different functions, it is essential that the connections within the brain stem are known, including the neurotransmitters therein. At present, the distributions of most classical neurotransmitters within the brain stem have been described, among which the biogenic amines or monoamines, which constitute a special group of transmitters. Originally, monoaminergic neurons and pathways in the brain stem were visualized by the Falck-Hillarp formaldehyde histofluorescence technique. Using this technique, Dahlström and Fuxe were the first to describe the distribution of monoaminergic neurons in the brain (Dahlström and Fuxe, 1964). In their pioneering study they reported the localization of catecholaminergic and serotonergic cell groups in the rat brain stem, which they designated A1-A12 and B1-B9, respectively. Since the Falck-Hillarp formaldehyde histofluorescence technique and its subsequent modifications have only limited specificity, the different types of monoamines were distinguished mainly by means of pharmacological tools, like enzyme inhibitors (for review see Björklund and Lindvall, 1984). Moreover, the histofluorescence method preferentially identified dopaminergic projection systems with a high dopamine content, such as the nigro-striatal and mesocorticolimbic pathways. Therefore, in areas like the brain stem, where the dopaminergic innervation is less abundant and densely intermingled with noradrenaline- and adrenaline-containing neuronal structures, the visualization of individual catecholamines within fibers and terminals is not possible with this method alone.

A great step forward was the introduction of highly sensitive immunocytochemical labeling techniques for detecting the major catecholamine synthesizing enzymes, like tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH), and phenylethanol-N-methyltransferase (PNMT) (see Hökfelt *et al.*, 1984). Since these enzymes play a key role in the catecholamine biosynthetic pathway, the presence of a particular catecholamine within a neuronal structure could be determined indirectly by comparing subsequent sections processed with antibodies against the respective catecholamine enzymes. TH, which is present in all catecholaminergic neurons, catalyses the conversion of tyrosine to dihydroxyphenylalanine (DOPA), which is subsequently converted to dopamine by aromatic *L*-amino acid decarboxylase. In

(nor)adrenergic structures, where dopamine is not the end product, DBH catalyses the conversion of dopamine to noradrenaline, which in turn is converted to adrenaline by PNMT in adrenergic structures. Thus, dopaminergic structures contain TH, noradrenergic structures contain TH and DBH, and adrenergic structures contain TH, DBH, and PNMT. Accordingly, the enzymatic profile of a structure will indicate which catecholamine is used as a neurotransmitter: adrenergic structures are detected by the presence of PNMT; noradrenergic structures contain DBH, but lack PNMT, while dopaminergic structures contain TH, but lack DBH (and PNMT). Consequently, a structure has to be screened for the presence of at least two enzymes in order to determine whether it is dopaminergic or noradrenergic. This is technically difficult especially for smaller structures, like fibers and terminals. Furthermore, in several studies the enzymatic profile was found to be inconsistent. For example, PNMT, which is generally considered as a specific marker for adrenergic structures, has been detected in hypothalamic neurons that do not contain TH (Ross *et al.*, 1984; Ruggiero *et al.*, 1985), and therefore may not be able to synthesize adrenaline precursors. In these structures, PNMT alone is not a reliable marker for adrenaline. Neuronal structures immunoreactive for TH but not for *L*-amino acid decarboxylase have also been reported. Since the latter enzyme is essential for the conversion of *L*-DOPA to dopamine, these structures probably may be considered as *L*-DOPAergic and not dopaminergic (Jaeger *et al.*, 1984). In order to avoid the drawbacks of the indirect labeling techniques, but to preserve the advantage of high sensitivity, antibodies against the catecholamines themselves were required.

When antibodies directed against dopamine and noradrenaline became available (Geffard *et al.*, 1984), they were used extensively for direct immunocytochemical detection of dopaminergic and noradrenergic neuronal structures in the central nervous system of different species (Steinbusch, 1981; Geffard *et al.*, 1984; Smiley *et al.*, 1992). The advantage of direct detection with high specificity and sensitivity makes it the method of choice for investigating the dopaminergic innervation in the brain stem, especially in areas where the dopaminergic innervation is less abundant or intermingled with other catecholamines.

Most studies on the distribution of dopaminergic fibers and terminals have focused on areas outside the brain stem, i.e. the striatum, cerebral cortex (Berger *et al.*, 1991; Smiley *et al.*, 1992), and cerebellum (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992), while most studies on dopamine within the rat brain stem concentrated on the distribution of dopaminergic neurons (Armstrong *et al.*, 1982; Kalia *et al.*, 1985). As a result, the dopaminergic innervation of the brain stem and its functional role may have been underestimated. Therefore, in the present study, a detailed mapping of the dopaminergic fibers and terminals in the brain stem was undertaken throughout medulla oblongata, pons, and mesencephalon caudal to the substantia nigra. A second objective of the present study was to compare the distribution of dopamine in the brain stem with that of other catecholamines. For this purpose, an antibody raised against DBH was used to identify both noradrenergic and adrenergic structures. Successful immunocytochemical detection of dopamine requires that the animals are perfused with a fixative that contains a relatively high concentration of glutaraldehyde, while for detection of catecholamine biosynthetic enzymes, glutaraldehyde is generally avoided for obtaining good results and paraformaldehyde is used (Armstrong *et al.*, 1982; Kalia *et al.*, 1985; Aldes *et al.*, 1988; Klepper and Herbert, 1991; Herbert and Saper, 1992; Asan, 1993). However, we found that detection of DBH was feasible in our glutaraldehyde fixed material. This made it possible to compare the distributions of dopamine and (nor)adrenaline within adjacent sections of the same animal.

Materials and methods

Animals and tissue preparation

Adult male Wistar rats were deeply anaesthetized with pentobarbital (70 mg/kg) and perfused transcardially with 50 ml of 0.9 % saline, 0.8 % sucrose and 0.4 % D-glucose in 0.05 M phosphate buffer, pH 7.4, followed by 750-1000 ml 5% glutaraldehyde in 0.05 M acetate buffer, pH 4.0. Ascorbic acid (10 mM) was added to both perfusion liquids to prevent oxidation of the catecholamine ring of the dopamine molecule.

The brain stems were removed immediately after fixation and left overnight at 4 °C in acetate buffer containing ascorbic acid and 20-25% sucrose. Next, serial transverse sections (30 µm) were cut on a freezing microtome and collected in 0.1 M Tris buffered saline, pH 7.6 (TBS), containing 10 mM ascorbic acid. Free floating sections were rinsed and kept in the same liquid overnight at 4 °C. The next day, alternate sections were processed for either dopamine or DBH immunocytochemistry.

Antisera and specificity controls

The polyclonal rabbit anti-dopamine antibody used in this study was kindly provided by Dr. R.M. Buijs, Netherlands Institute for Brain Research, Amsterdam, The Netherlands. The production of the antibody and its characteristics have been described in detail elsewhere (Buijs *et al.*, 1984; Voorn and Buijs, 1987). A polyclonal rabbit anti-DBH antibody (SanverTech) was used for detection of dopamine-ß-hydroxylase.

In order to test whether the dopamine antibody showed cross-reactivity with noradrenergic structures, the noradrenaline depleting neurotoxin DSP-4 was used, since it crosses the blood-brain barrier and induces the loss of a subset of noradrenergic neural structures as indicated by various noradrenaline markers without affecting dopamine structures (Fritschy and Grzanna, 1989; 1991). One group of rats received a single i.p. injection of DSP-4 (50 mg/kg) dissolved in saline prior to use, while another group of rats received no treatment at all. After a period of 2-3 weeks all rats were sacrificed and alternate sections were processed for dopamine or DBH immunocytochemistry, respectively.

Dopamine and DBH immunocytochemistry

Until application of the secondary antibody, ascorbic acid (10 mM) was added to all solutions. Tissue sections were placed for 30 minutes in 0.1 M TBS, pH 7.6, containing 1% sodium borohydride. Sections were thoroughly rinsed in TBS. Alternate brain stem sections, obtained from the same animal, were incubated for 24-72 hours at 4 °C with, either anti-dopamine (1:4000), or anti-DBH (1:1000) in TBS containing 0.5 % Triton-X100. Dopamine sections were processed for peroxidase-antiperoxidase (PAP) immunocytochemistry and DBH sections were processed for ABC immunocytochemistry. After application of the dopamine antibody, the dopamine sections were incubated for 90 minutes in goat-anti-rabbit antibody ("Betsy", diluted 1:200) and then placed in rabbit PAP complex (1:800, Nordic) for 60 minutes. Between steps the sections were thoroughly rinsed with TBS. After application of the DBH antibody, the DBH sections were incubated for 90 minutes in biotinylated goat-anti-rabbit antibody (Vector, diluted 1:200) and then placed for 60 minutes in freshly prepared ABC Elite solution (Vector). In between steps the sections were thoroughly rinsed with TBS.

In both cases, the immunocytochemical reaction product was revealed with 0.05% diaminobenzidine in the presence of 0.01% hydrogen peroxide in 0.05 M Tris-HCl, pH 7.6, under visual control. Upon completion of the immunocytochemical staining procedure the sections were mounted onto glass slides with gelatin-chrome alum adhesive, dehydrated in graded alcohol baths, transferred to xylene, and cover slipped with Permount. The distribution of dopamine and DBH immunoreactive structures were examined light microscopically under darkfield and brightfield illumination.

Results

The objective of the present immunocytochemical study was to identify the anatomical localization of dopamine and DBH immunoreactive fibers and presumptive terminals (appearing as punctuate profiles) within the rat brain stem. Neuronal cell bodies in the brain stem immunoreactive for either dopamine or DBH were mostly located in catecholaminergic cell groups. Since their location has already been described extensively in previous studies (Dahlström and Fuxe, 1964; Armstrong *et al.*, 1982; Kalia *et al.*, 1985), they were not included in the present investigation.

DSP-4 experiments

DSP-4 is a noradrenaline-depleting neurotoxin whose reported effects are restricted to areas in the central nervous system receiving their noradrenergic afferents from the locus coeruleus proper (LC), i.e. the cerebellum, the inferior and superior colliculi, the cochlear nuclei, the vestibular nuclei, the dorsal column nuclei, the sensory spinal trigeminal nucleus, and the spinal dorsal horn (Fritschy and Grzanna, 1989; 1991). Following systemic administration of DSP-4, there was a nearly complete loss of DBH staining in the above areas, whereas in the same areas of the alternate sections there was no change in dopamine staining.

General appearance and morphology of dopamine and DBH immunoreactive structures

The morphology of dopamine immunoreactive fibers was relatively uniform. Generally they were thin and possessed relatively large, regularly spaced, varicosities. Some morphological differences were present between dopamine immunoreactive structures located in different regions. Most notably in the motor nuclei the intervaricose fibers were only lightly stained, giving them a dot-like, granular appearance at low magnifications. In the DBH preparations, the morphology of DBH immunoreactive fibers was not as uniform as the dopamine immunoreactive ones. Different morphological types of DBH immunoreactive fibers could be distinguished. Generally they formed an intermingling network consisting of relatively "coarse" fibers with large varicosities and relatively "smooth" fibers with small intervaricose segments and a large number of small varicosities. The morphology of the "coarse" fibers was similar to the morphology found in the dopamine preparations. However, the "smooth" fibers were typically present in DBH preparations and were never found in dopamine preparations. Similar to the dopamine preparations, in some of the brain stem motor nuclei the intervaricose DBH fibers were lightly stained, leading to a granular appearance.

Distribution of dopamine and DBH immunoreactive structure

Dopamine and DBH immunoreactive fibers and terminals were present in many brain stem nuclei with varying staining densities. In order to compare the staining densities for different brain stem areas, 7 density levels were distinguished defined as nearly or completely absent (level 0), weak (level 1), weak to moderate (level 2), moderate (level 3), moderate to strong (level 4), strong (level 5) and very strong (level 6). The results are represented schematically in fig. 1. Strongest dopamine labeling (level 6) was found in the nucleus of the solitary tract, dorsal motor nucleus of the vagus, and lateral parabrachial nucleus. Other areas like the medial parabrachial nucleus, locus coeruleus, periaqueductal gray and all raphe nuclei were strongly labeled (level 5). The principal nucleus of the inferior olive was also strongly labeled. Less dense staining (level 1-4) was observed in many other areas of the brain stem, like the inferior and superior colliculi, deep mesencephalic nuclei, pontine reticular nuclei, both sensory and motor trigeminal nuclei, prepositus hypoglossal nucleus, cochlear nuclei, cuneate and gracile nuclei, and spinal trigeminal complex. A limited number of dopamine fibers were always present in the reticular formation throughout the brain stem. Other brain stem regions like area postrema, cerebellum, anterior portion of the ventral cochlear nucleus and external cuneate nucleus, were virtually devoid of dopaminergic fibers with only an occasional traversing fiber (level 0).

In contrast to the more restricted organization of the dopamine immunoreactive fibers and terminals, DBH immunoreactive fibers and terminals were distributed more ubiquitously throughout the brain stem. Thus, most areas with a significant dopamine innervation also

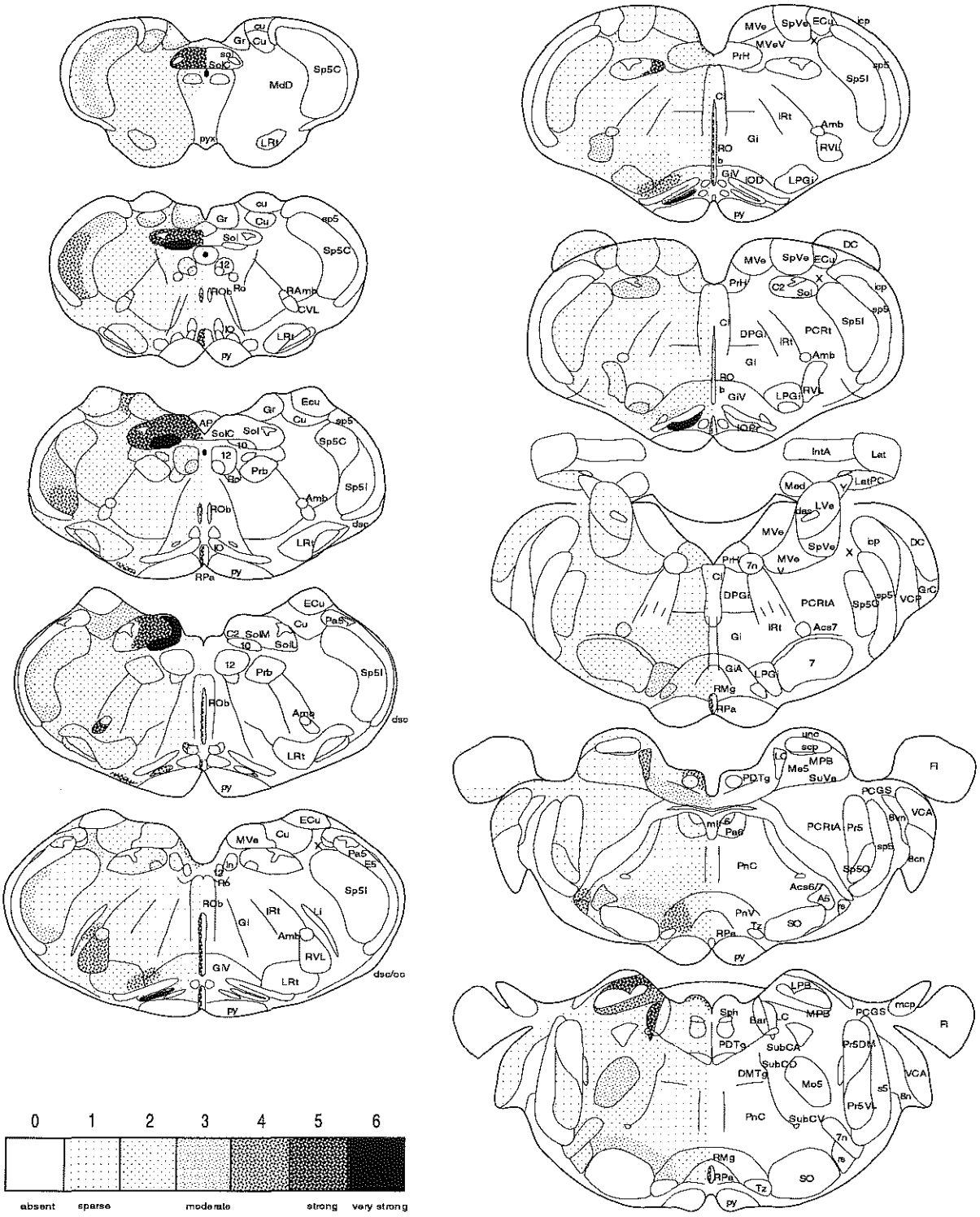


Figure 1: Schematic representation of the dopamine innervation - consisting of dopamine immunoreactive fibers and (presumptive) terminals - in the rat brain stem, including the medulla oblongata, the pons, and the mesencephalon caudal to the substantia nigra. Note that neuronal cell bodies immunoreactive for dopamine are not included in this schematic representation. For details see text; for abbreviations see list of abbreviations.

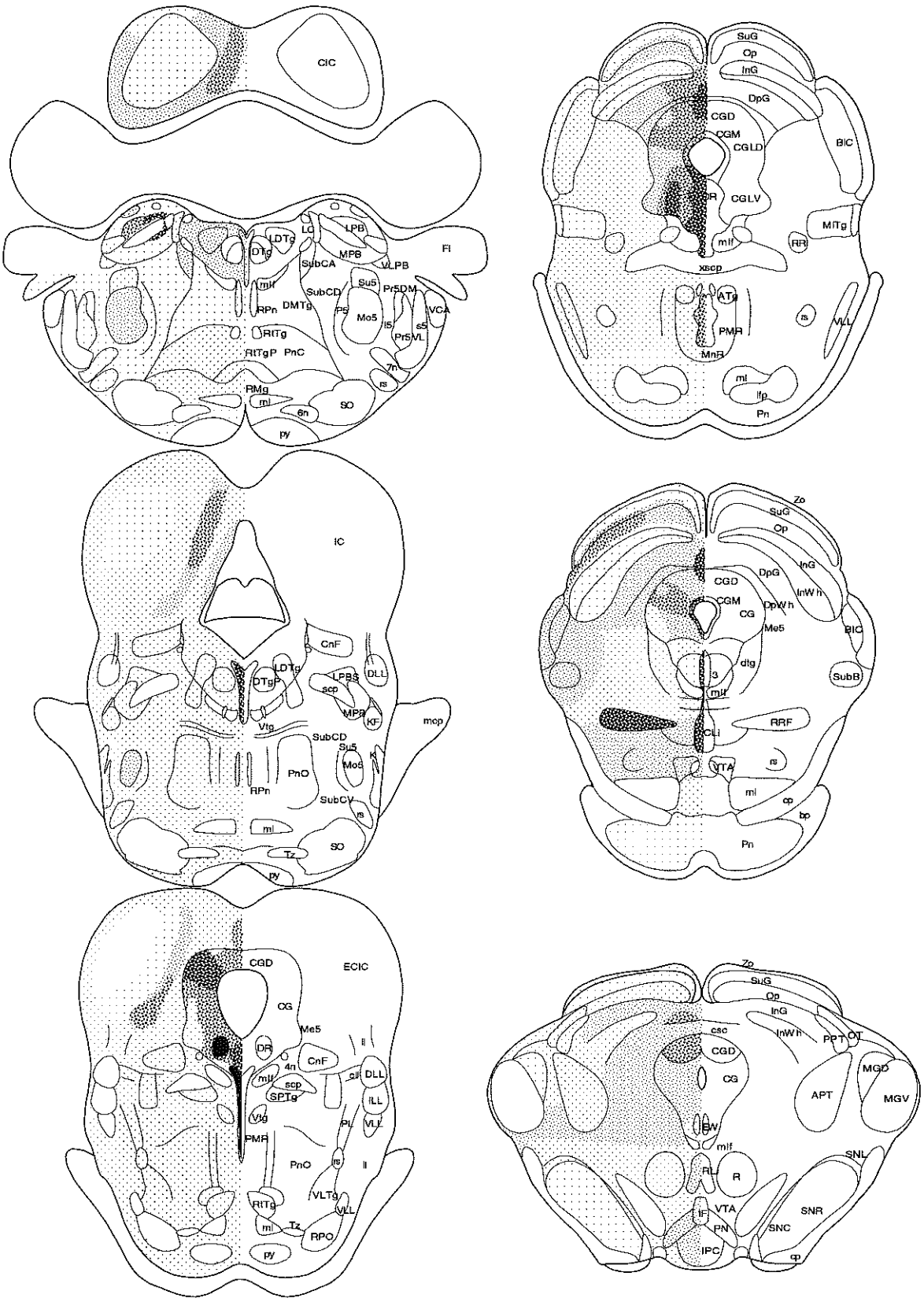


Figure 1: *(Continued)*

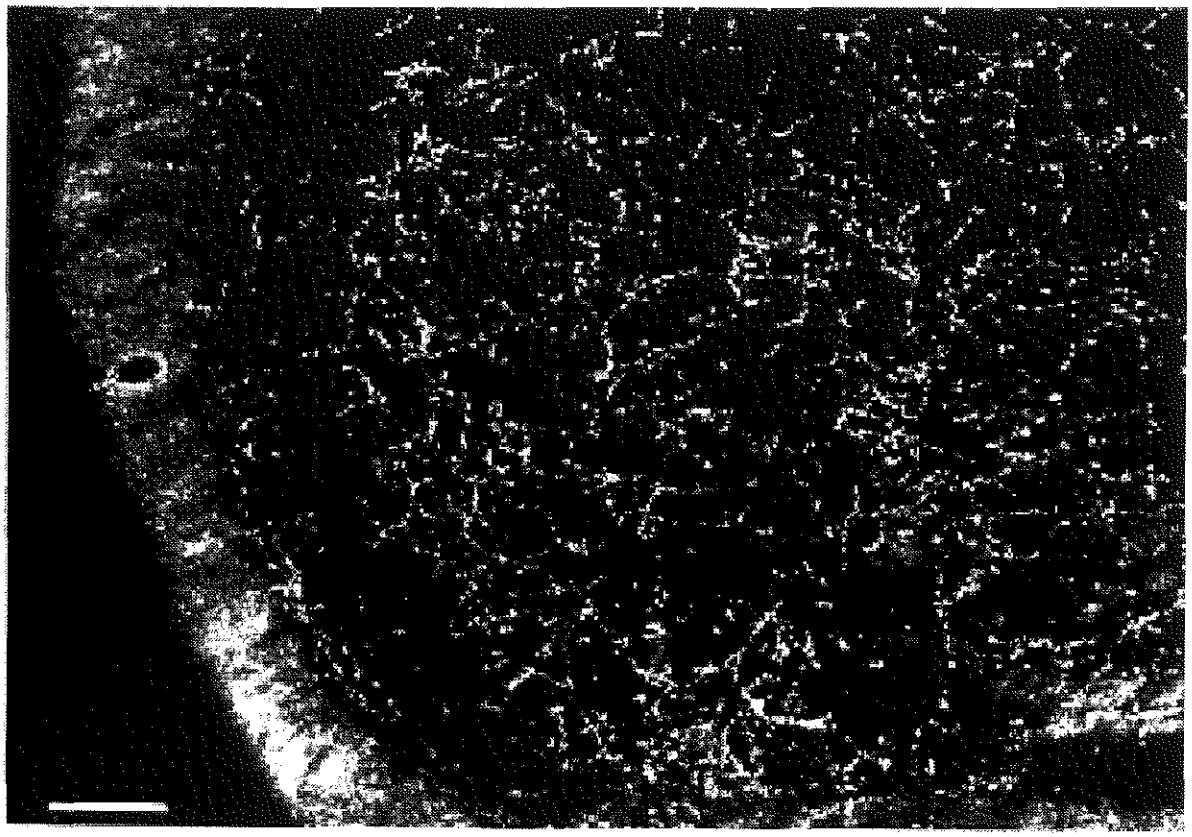


Figure 2: Photomicrographs with darkfield illumination of a section from the caudal medulla after processing for dopamine immunoreactivity, showing the ventral part of the spinal trigeminal nucleus, caudal division. Note that the labeling in lamina II (substantia gelatinosa) is less than the other laminae. Scale bar = 100 µm.

received a DBH innervation, but the reverse was not always the case.

Below, the distribution of fibers and terminals in the rat brain stem immunoreactive for dopamine (fig. 1) and DBH will be described in detail. The description has been subdivided as follows: 1) trigeminal complex, 2) medulla oblongata (remaining nuclei), 3) pons and mesencephalon, and 4) motor nuclei of the cranial nerves.

Trigeminal complex

The trigeminal sensory nuclei are located dorsolaterally in the brain stem and extend from the cervical spinal cord to the midbrain. They are divided into (1) the spinal trigeminal nucleus (Sp5), which can be subdivided into caudal (Sp5C), interpolar (Sp5I), and oral (Sp5O) subnuclei; (2) the main or principal trigeminal nucleus (Pr5), and (3) the mesencephalic trigeminal nucleus (Me5). The trigeminal complex also contains the trigeminal motor nucleus (Mo5), which is located in the pons next to the Pr5 (see below). Some small nuclei, like the paratrigeminal nucleus (Pa5), are also considered part of the trigeminal complex (Waite and Tracey, 1995).

The spinal trigeminal nucleus (Sp5)

Dopamine labeled fibers were distributed throughout the Sp5 (fig. 1). The distribution pattern showed rostrocaudal, dorsoventral, and mediolateral gradients, which were observed throughout the Sp5. Dopamine labeling was strongest in the caudal part of the Sp5, except for the transition zone with the spinal cord, and diminished in a rostral direction. The ventral and lateral parts of the Sp5 generally displayed the strongest dopamine labeling. The weak labeling in the dorsal part of the Sp5 disappeared completely at more rostral levels.

In the alternate sections there was a widespread DBH staining in all parts of the Sp5. Compared to dopamine, the DBH staining was distributed more uniformly throughout the Sp5. As a result, there were no obvious gradients in the distribution of DBH.

The caudal subnucleus (Sp5C)

The Sp5C is the most caudal part of the Sp5. Sp5C is located at levels between the upper cervical cord and the obex. As in the dorsal horn of the spinal cord, Sp5C has a laminar arrangement with a marginal layer (lamina I), a substantia gelatinosa (lamina II), and a magnocellular layer, with an organization analogous to the spinal laminae III to V. The distribution of dopamine in the Sp5C was very similar to the distribution that we have observed in the cervical dorsal horn (Holstege *et al.*, 1996) (fig. 2). The marginal layer

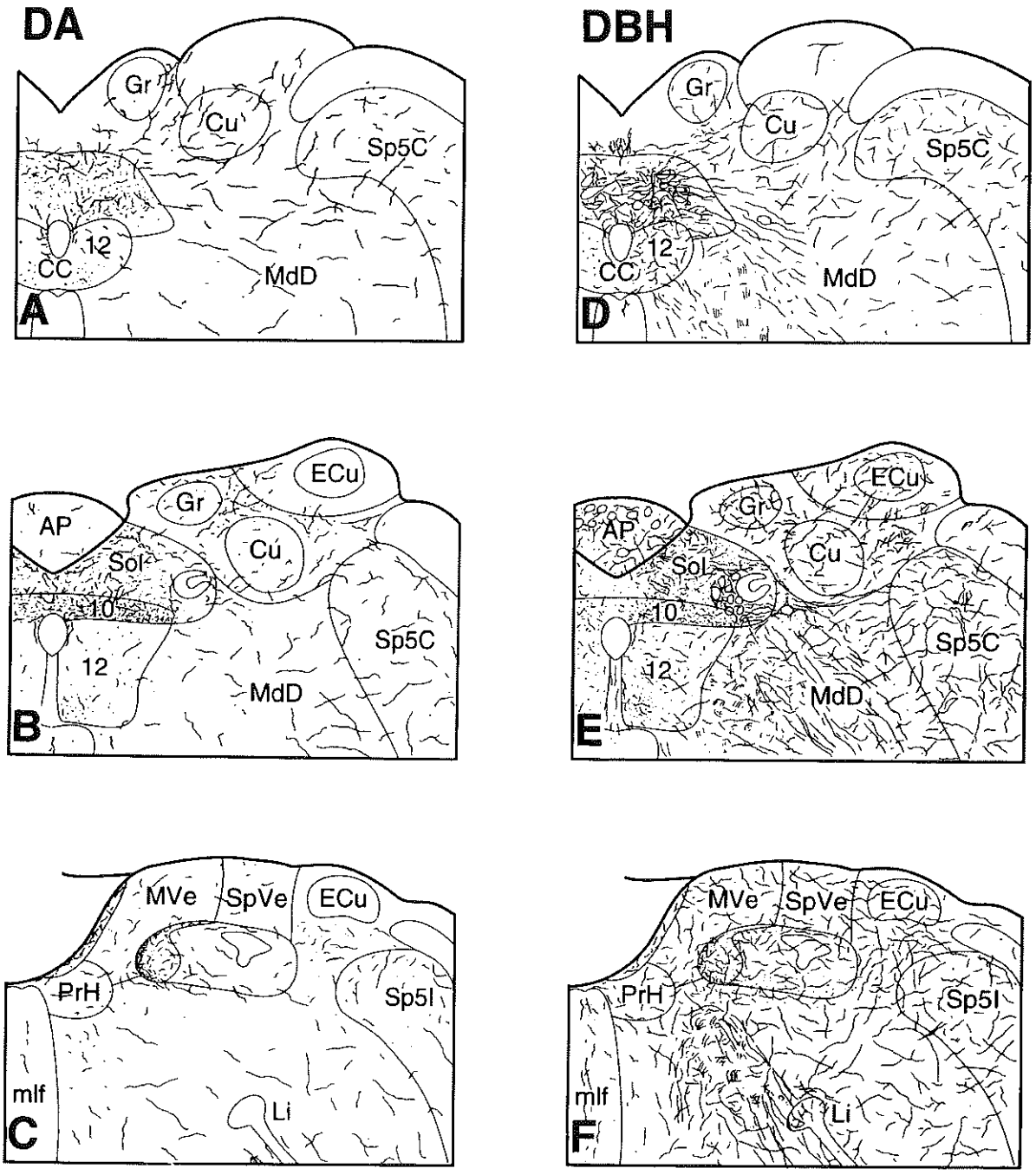


Figure 3: Schematic representation of dopamine and DBH immunoreactive fibers and terminals in the rat nucleus of the solitary tract at different levels of the brain stem. Note that the external cuneate nucleus and area postrema are both sparsely innervated by dopamine, whereas the (nor)adrenaline synthesizing enzyme DBH is abundantly present. For details see text; for abbreviations see enclosed list of abbreviations.

of Sp5C was sparsely to moderately labeled with dopamine fibers. The lowest amount of dopamine immunoreactivity was found in the substantia gelatinosa of the Sp5C, which only contained some traversing fibers. Moderate dopamine labeling was present in the peripheral part of the magnocellular layer, in agreement with the findings in lamina III of the spinal cord. Similarly, the medial part of the magnocellular layer - the analogue of lamina IV and V of the spinal dorsal horn - was moderately labeled with more radially oriented dopamine fibers. At levels of the caudal pole of the inferior olive dopamine labeling became moderate to strong in the periphery of Sp5C and slightly more rostrally, at the level where the substantia gelatinosa had disappeared completely, dopamine fibers were running dorsoventrally along the outer border of the magnocellular layer. In the medial part of Sp5C, dopamine fibers were sparse and oriented more or less randomly. The paratrigeminal nucleus (Pa5), located at the dorsolateral medullary surface

within the spinal trigeminal tract, was sparsely to moderately labeled with dopamine fibers.

The distribution of DBH fibers in Sp5C was also very similar to the distribution of DBH fibers in the spinal dorsal horn. In the marginal layer and substantia gelatinosa of Sp5C the DBH labeling was moderate to strong. The inner part of the substantia gelatinosa was stained somewhat lighter than its outer part. The magnocellular layer was moderately labeled with DBH fibers.

The interpolar subnucleus (Sp5I)

The Sp5I is the rostral continuation of the Sp5C. It is located rostral to the obex. In comparison with the Sp5C, the Sp5I was slightly stronger labeled with dopamine fibers. The rostrocaudal gradient of dopamine staining in Sp5I was especially pronounced in the medial part. The dopamine labeling had almost completely disappeared at the level of the caudal pole of the facial nucleus (n7), except for some traversing fibers in the outer surface of Sp5I.

Similar to the dopamine pattern, the moderate DBH labeling in the Sp5I also showed a rostrocaudal gradient, such that more rostrally DBH labeling became less strong. A preferential orientation of the DBH fibers was not observed.

The oral subnucleus (Sp5O)

The Sp5O, which is the most rostral subnucleus of the Sp5, is located at the level of the facial nucleus. The Sp5O was sparsely labeled with dopamine fibers. The distribution of dopamine fibers was the same as in the caudal Sp5I, though the staining became less dense.

The Sp5O was moderately to strongly labeled with DBH fibers. The DBH fibers appeared continuous with the adjacent lateral reticular formation.

The principal trigeminal nucleus (Pr5)

The Pr5 extends caudally to the level of the rostral pole of the facial nucleus next to the Sp5O. Rostrally it ends at the same level as the rostral pole of the trigeminal motor nucleus (Mo5). In Pr5 the dopamine staining showed a dorsoventral gradient. The ventrolateral part of the Pr5 (Pr5VL) was sparsely labeled with dopamine fibers, while dopamine labeling was nearly absent in the dorsomedial part (Pr5DM).

In the alternate sections, Pr5 was moderately labeled with DBH fibers. Its distribution showed no dorsoventral gradient, both Pr5VL and Pr5DM were labeled equally strong with DBH.

The mesencephalic trigeminal nucleus (Me5)

The area that contains the large ganglion cells that form the mesencephalic trigeminal nucleus was moderately to strongly labeled with dopamine fibers and strongly labeled with DBH fibers.

Medulla oblongata

In addition to the trigeminal nucleus, dopamine immunoreactive fibers were present in many additional areas of medulla oblongata, like the nucleus of the solitary tract, the dorsal motor nucleus of the vagus, the hypoglossal nucleus, the nucleus prepositus hypoglossus, the inferior olive, the cochlear nuclei, and the cuneate and gracile nuclei and throughout the reticular formation.

The medial part of the dorsal medulla oblongata contains three important nuclei: the nucleus tractus solitarii (NTS), the dorsal motor nucleus of the vagus (DMNV) and the hypoglossal nucleus (n12), which is located just ventrally to the DMNV. The distribution of fibers and terminals immunoreactive for dopamine or DBH in these areas are shown in detail in figs. 3 and 4A. Dorsomedially within the nucleus tractus solitarius and DMNV, the medullary catecholamine group A2 is located. Densely stained dopaminergic or (nor)adrenergic neurons and dendrites (Armstrong *et al.*, 1982; Kalia *et al.*, 1985), belonging to the caudal A2 group, often appeared intermingled with the labeled fibers and terminals of the DMNV and nucleus tractus solitarius (NTS). Therefore, it was difficult to precisely identify the labeling as well as the underlying cytoarchitecture of these nuclei.

Hypoglossal area

The caudal hypoglossal nucleus (n12) showed moderate to strong dopamine staining in its ventromedial quadrant, while the remaining areas were sparsely to moderately labeled with dopamine fibers (fig. 3). At more rostral levels dopamine labeling became sparse both in the dorsal and ventral parts of n12. In the alternate sections, n12 was moderately labeled with DBH fibers (fig. 3). As with dopamine, the most intense DBH labeling also was found at the ventromedial part of the caudal n12. At its rostral pole, the DBH labeling appeared slightly stronger in the dorsal part.

The dopamine labeling of the nucleus prepositus hypoglossus (PrH), located rostrally to the n12, was sparse to moderate. The supragenual nucleus (SGe), located at the genu of the facial nerve (g7), showed no dopamine staining. Some dopamine fibers immediately surrounded the central canal (CC).The distribution of DBH fibers in the PrH was similar to that of dopamine, albeit slightly stronger.

The dorsal motor nucleus of the vagus (DMNV)

The caudal part of the DMNV was strongly stained with dopamine, resulting in a sharp and conspicuous transition between n12 and DMNV. Rostrally in the DMNV, at the level of the area postrema (AP), the dopamine labeling became even stronger.

Similar to the distribution of dopamine, the strongest DBH staining within the dorsomedial medulla also was present in the DMNV. Due to the massive DBH staining in its caudal part it was not possible to distinguish individual fibers or terminals.

The nucleus tractus solitarii (NTS)

At levels caudal to the obex, moderate dopamine staining was present in the commissural nucleus tractus solitarius (fig. 3). At the level of the obex the labeling became very strong and diminished again more rostrally. Strongest dopamine labeling was present in the medial nucleus tractus solitarius, especially in the region lining the dorsal motor nucleus of the vagus (fig. 3). Rostral to the obex (fig. 4), in the ventrolateral part of the medial subdivision, the central subnucleus was sparsely labeled. The ventrolateral nucleus tractus solitarius, which mainly constitutes the lateral

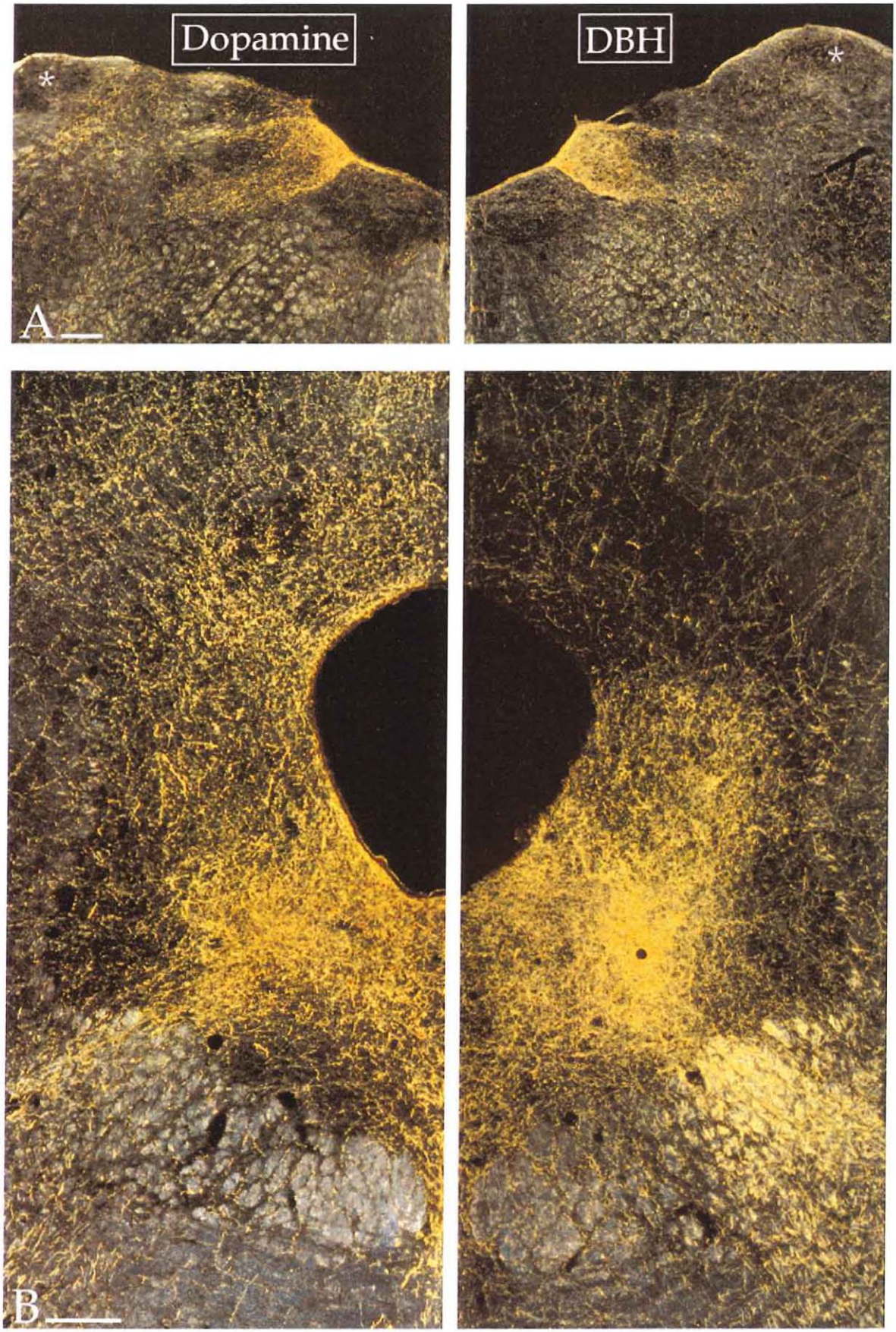


Figure 4: Photomicrographs with darkfield illumination of sections from the rat dorsomedial medulla oblongata (A) and the area of the periaqueductal gray (B) after processing for dopamine (left) or DBH (right) immunocytochemistry. The asterisk (A) indicates the external cuneate nucleus, which is labeled for dopamine, but not for DBH. See figs. 1, 3 and 8 for the underlying cytoarchitecture and additional notes. For details see text. Scale bars in A and B = 200 µm.

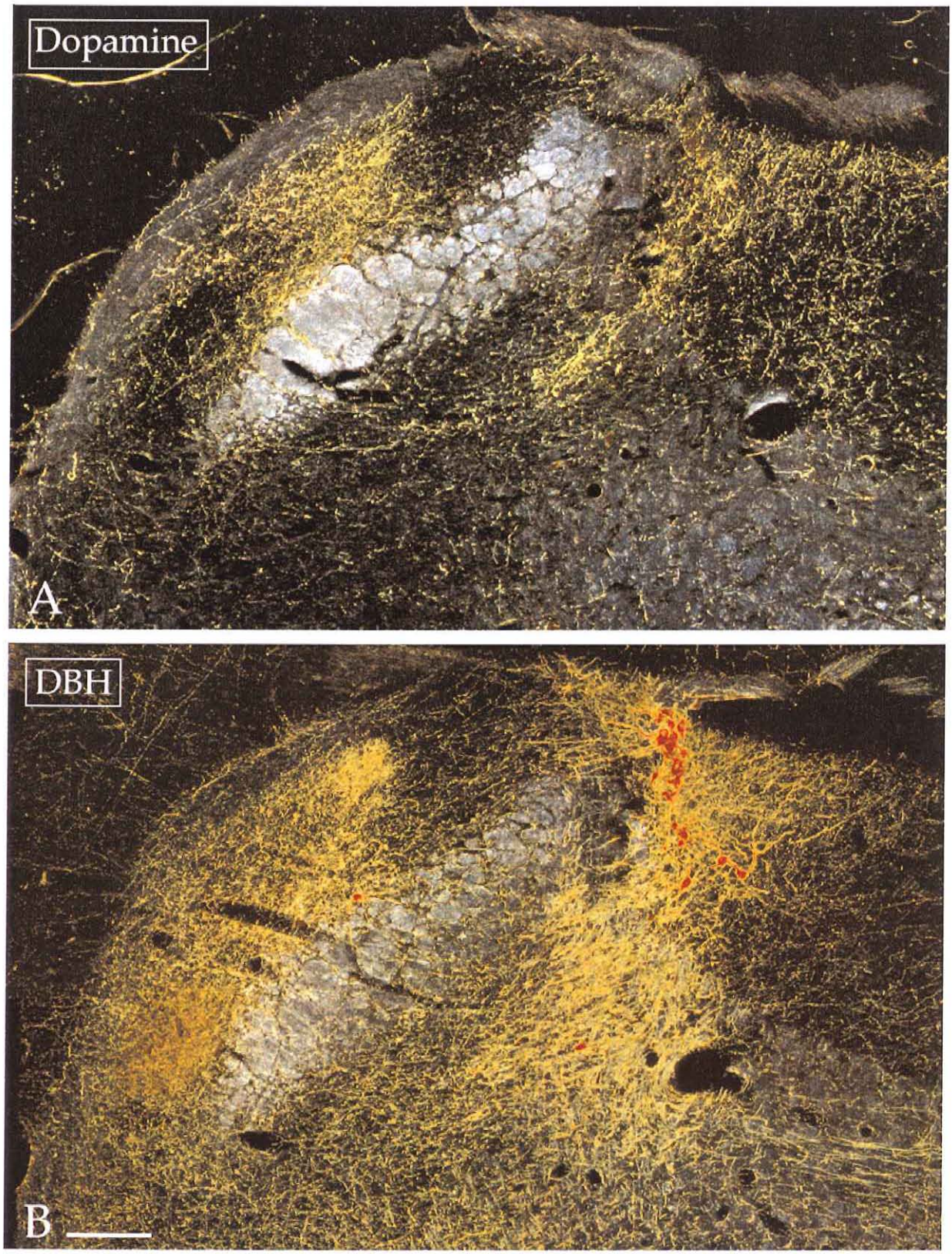


Figure 5: Photomicrograph with darkfield illumination of sections from the parabrachial nuclei after processing for dopamine (A) or DBH (B) immunocytochemistry. See figs. 1 and 7 for the underlying cytoarchitecture and additonal notes. For details see text. Scale bar = 200 µm.

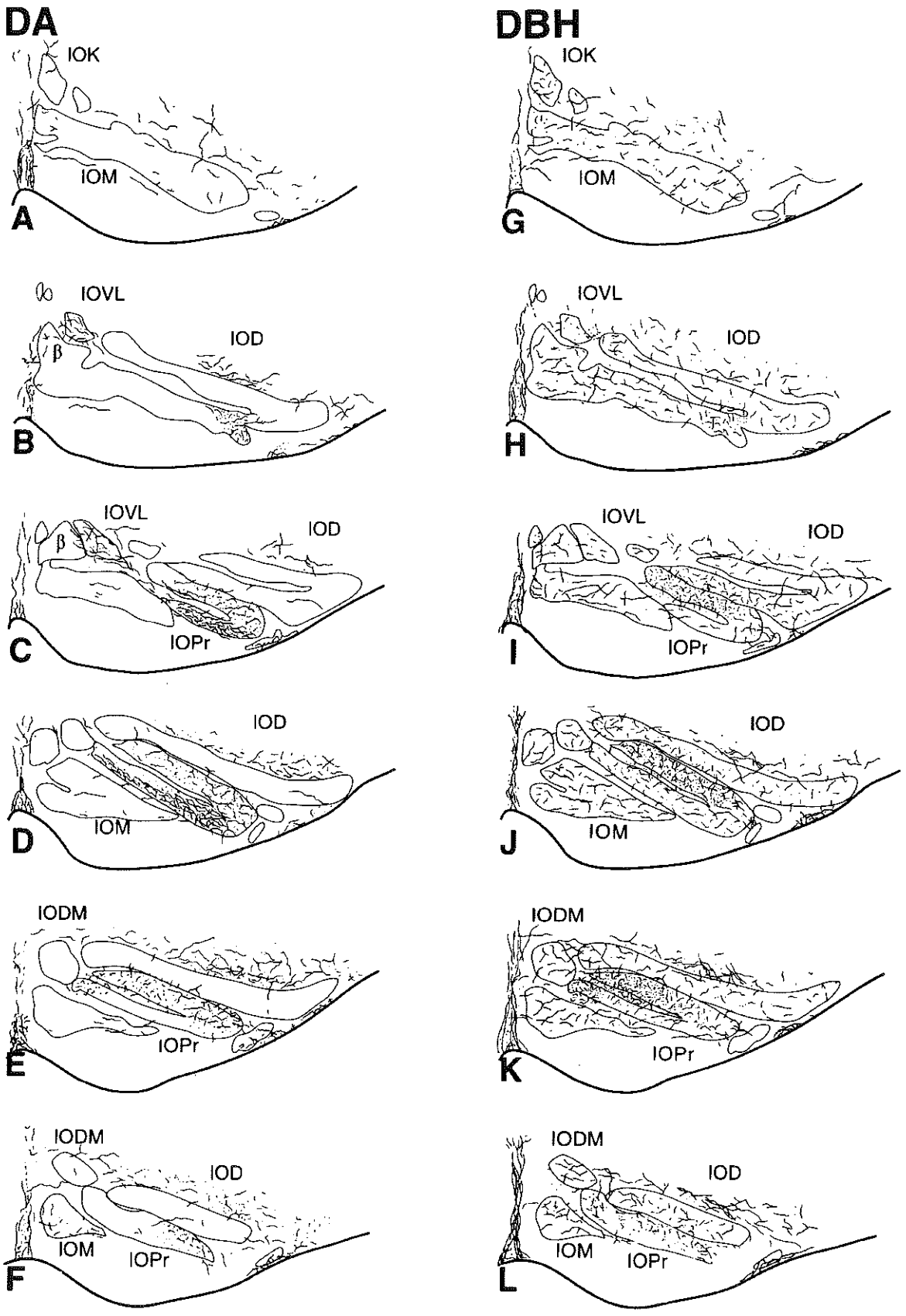


Figure 6: Schematic representation of the dopamine and DBH immunoreactive fibers and terminals in the rat inferior olive at different levels of the brain stem. Note that the strong labeling with coarse dopamine fibers of the ventral lamella of the principle olivary nucleus and the medially adjacent ventrolateral outgrowth is not matched by DBH, whereas DBH labeling present in all other olivary (sub)nuclei is not matched by dopamine. For details see text; for abbreviations see enclosed list of abbreviations.

subdivision of the nucleus tractus solitarius, was moderately labeled. The dopamine labeling became sparse at the most rostral levels. The interstitial nucleus, which is surrounded by solitary tract fibers, was also sparsely labeled with dopamine. The solitary tract itself was devoid of labeling.

The DBH pattern within the nucleus tractus solitarius was similar to the distribution of dopamine, but the staining densities were slightly stronger (Fig. 3).

The area postrema (AP), which is usually not considered as a portion of the nucleus tractus solitarius, was sparsely labeled with dopamine fibers. The periventricular gray (Pe), which is located rostrally to the area postrema just below the floor of the 4th ventricle, contained strong to very strong dopamine labeling. The labeling became very strong at the level of the adrenergic C2 group. DBH immunoreactive was strong in the AP and the Pe.

The inferior olivary complex

The inferior olive (IO) is located in the ventral part of the medulla oblongata and is composed of three main subdivisions, i.e. the principal olive and two accessory olives (Ruigrok and Cella, 1995). The principal olive (PO) consists of a folded sheet of gray matter. Rostrally a dorsal and a ventral lamella can be distinguished. Caudally the PO is continuous with the ventrolateral outgrowth (VLO). The medial accessory olive (MAO) is located medial from the PO and includes several subnuclei. The dorsal accessory olive (DAO) is located dorsal to the PO.

Dopamine immunoreactive fibers and presumptive terminals were primarily located in the principal olive (fig. 6). The labeling was not uniform but consisted of two very different types. The dorsal lamella and, more rostrally, also the dorsomedial parts of the ventral lamella of the principal olivary nucleus contained dense but extremely fine granular labeling. Coarse varicose fibers were occasionally observed. The labeling was already present at the most caudal part of the principal olivary nucleus, where it was almost continuous with the medial accessory olive. The medial accessory olive in turn was avoided by the dopamine labeling, thus clearly demarcating the borderline between the two nuclei (fig. 6B). In contrast to the very fine granular labeling of the dorsal lamella, the ventral lamella received coarse varicose fibers especially in its ventrolateral part at the middle level of the inferior olive, where it was very strongly labeled (figs. 6C and 6D). In a few sections these fibers were observed to form a narrow band, which spreads out dorsomedially to label the ventrolateral outgrowth (IOVL). The labeling of the medial and dorsal accessory olivary nuclei (and associated nuclei) was virtually absent.

The DBH labeling of the inferior olivary complex was also primarily aimed at the principal nucleus and at the same area as the fine grain dopamine labeling, i.e. mainly the dorsal lamella (fig. 6G-L). There were two major differences between the dopamine and the DBH labeling. In the first place there was a sparse to moderate labeling of all the other olivary (sub)nuclei with DBH, which were devoid of dopamine labeling. In the second place, the strong labeling with coarse dopamine fibers of the ventral lamella of the principle olivary nucleus and the medially adjacent ventrolateral outgrowth was not matched by DBH, although there were some DBH fibers in these nuclei, similar to all other subnuclei.

The vestibular complex

The vestibular complex consist of the superior (SuVe), lateral (LVe), medial (MVe), and spinal (SpVe) vestibular nuclei and some smaller cell groups. Only an occasional fiber was present in the LVe and SuVe. The MVe and SpVe were sparsely labeled. In the alternate sections, the vestibular complex showed a few prominent DBH fibers. The SuVe showed sparse to moderate DBH staining.

The dorsal column nuclei

The gracile nucleus (Gr) and cuneate nucleus (Cu) were moderately labeled with dopamine. At caudal levels the peripheral part of the Cu was labeled stronger than its central part, while more rostrally, at the level where the Gr had disappeared, dopamine staining in Cu was moderate to strong. The external cuneate nucleus (ECu) was virtually devoid of dopamine. The dorsal column nuclei, including the ECu, were moderately labeled with DBH.

The cochlear nuclei (CN)

The cochlear nuclei consist of a ventral and dorsal CN. Within the dorsal CN the layers containing giant cells received a sparse to moderate dopamine staining, whereas the outer layers contained sparse dopamine labeling. The ventral CN was sparsely labeled, except for a dopamine fiber plexus in the granule cell layers, especially more rostrally. The CN also received a prominent DBH staining. DBH fibers were present in both dorsal and ventral CN, with strong DBH staining within the anterior portion of the ventral CN.

Pons and mesencephalon

In the pons the most prominent labeling was found in the locus coeruleus, parabrachial nuclei, and pontine reticular nuclei (figs. 1, 5 and 7).

The locus coeruleus (LC)

Dopamine immunoreactive fibers and terminals were observed throughout the LC (figs. 1, 5 and 7). At caudal levels, the LC was moderately labeled with dopamine immunoreactive fibers and terminals. More rostrally, the LC proper (cell body area) was strongly labeled with dopamine immunoreactive fibers and terminals, especially its ventral part. In comparison with the LC proper, the dendritic area of the LC located more ventromedially, appeared to be stained somewhat stronger than the cell body area. In the area of Barrington's nucleus dopamine staining was sparse. In the alternate sections, DBH staining was very strong throughout the LC and in Barrington's nucleus.

The parabrachial complex (PB)

The parabrachial complex (PB) is made up of cytoarchitectonically distinct subnuclei surrounding the superior cerebellar peduncle in the rostral pons. It is divided into a lateral (LPB) and medial parabrachial nucleus (MPB). The LPB is located primarily dorsal to

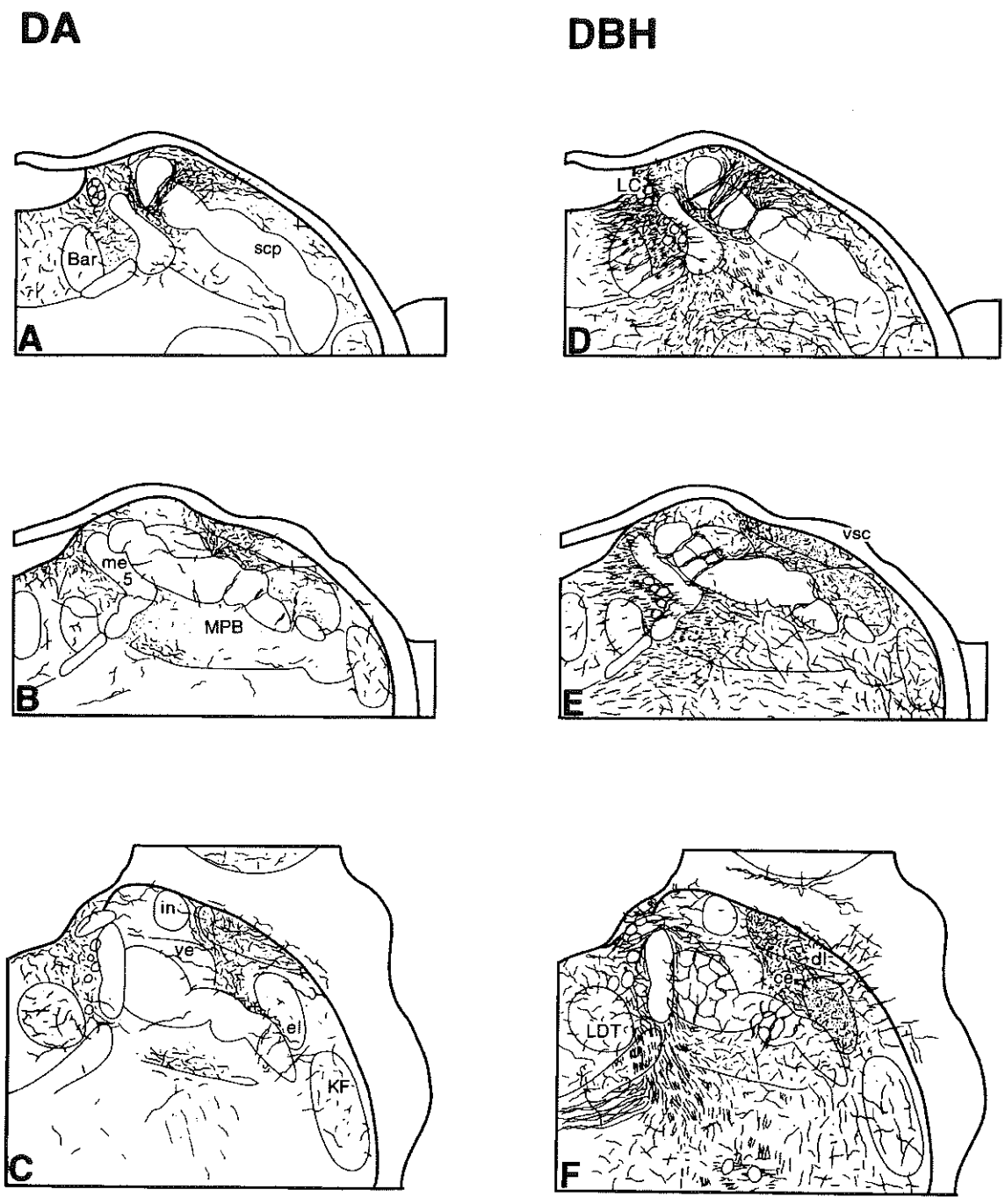


Figure 7: Schematic representation of the dopamine and DBH immunoreactive fibers and terminals in the rat parabrachial nucleus at different levels of the brain stem. Note that dopamine sparsely innervates the region of the external lateral parabrachial nucleus, whereas DBH is abundantly present. For details see text; for abbreviations see enclosed list of abbreviations.

the superior cerebellar peduncle and the MPB is located primarily ventral to the superior cerebellar peduncle. The MPB connects with the LPB via the so-called waist area. About 13 parabrachial subnuclei and regions have been identified (c.f. Herbert *et al.*, 1990; Saper, 1995). These include the central lateral, dorsal lateral, external lateral, extreme lateral, external medial, internal lateral, Kölliker-Fuse nucleus, superior lateral, and ventro-lateral subnuclei. The distributions of fibers and terminals immunoreactive for dopamine or DBH are given in figs. 5 and 7.

Both the LPB and the MPB received a very strong dopamine projection to specific subnuclei. Caudally, the MPB received a very strong innervation, which extended into the waist area where it connects with the central nucleus of the LPB. The labeling was confined

to the medial part of the MPB, while the lateral part was sparsely innervated (fig. 7A). At the level where the superior cerebellar peduncle traverses the mesencephalic tract, labeling in the MPB, including the external nucleus of the MPB and ventrolateral parabrachial nucleus, became sparse (fig. 7B). Very strong punctuate labeling was present in the ventral nucleus of the LPB at caudal levels, where it is continuous with the waist area (fig. 7A). More rostrally, labeling was confined to the central nucleus of the LPB, while the external and lateral nuclei of the LPB were labeled sparse to moderate (fig. 7B). This pattern continued in the rostral part of the LPB with strong labeling in the central and dorsal nucleus and sparse labeling in the internal and external nuclei of the LPB (fig. 7C). DBH staining in the parabrachial nuclei was moderate to strong (figs. 7D-F and 5).

The cerebellar nuclei and cerebellar cortex

The cerebellar nuclei are located within the base of the cerebellum. They are usually subdivided into a caudal medial cerebellar (Med) or fastigial nucleus, an interposed (Int) nucleus, and a lateral cerebellar (Lat) or dentate nucleus. The ventral part of the caudal Lat showed some dopamine labeled fibers, while the other cerebellar nuclei showed sparse dopamine staining. Although an extensive examination was not attempted, it was observed that the cerebellar cortex was almost devoid of dopamine staining, except for some occasional dopamine fibers distinctly present in the medial extension of the ventral paraflocculus. The cerebellar nuclei were all sparsely to moderately labeled with DBH. In the cerebellar cortex DBH fibers were widely distributed.

In the mesencephalon, at levels caudal to the substantia nigra, dopamine immunoreactive fibers and terminals were present in the periaqueductal gray, the superior and inferior colliculi, and deep mesencephalic nuclei (fig. 1).

The periaqueductal gray (PAG)

The PAG corresponds to the region of the midbrain that surrounds the cerebral aqueduct of Sylvius. The most caudal part of the PAG is located at the level of the dorsal tegmental nucleus (DTg), while the most rostral part of the PAG is at the level of the posterior commisure and the most rostral level of the oculomotor nucleus (n3). The PAG can be subdivided in several distinct regions based on cytoarchitectonic features (Beitz, 1985). Generally, the PAG is subdivided in dorsal, lateral, ventrolateral, and medial regions. Caudally, the ventral PAG contains the dorsal and dorsolateral tegmental nuclei. Rostrally, the ventrolateral subdivision is bordered ventrally by the supraoculomotor nucleus. At midlevels, the ventromedial part of the PAG is occupied by the dorsal raphe nucleus. The distributions of fibers and terminals immunoreactive for dopamine or DBH are given in fig. 4 and 8.

Strongest dopamine staining was present in the region of the dorsal raphe nucleus. The dopamine staining extended from the aqueduct towards the dorsal tegmental nucleus. Laterally, in the region medial to Me5, the staining in the ventrolateral PAG was sparse to moderate. The lateral PAG was less strongly labeled, with strongest labeling in its dorsal part. At the dorsal border of lateral PAG there was a small region that received less dopamine labeling than the surrounding region, especially at more rostral levels. Prominent dopamine staining was present in the dorsal PAG. In the medial parts of the PAG moderate dopamine staining was present in the region lining the aqueduct.

A dense DBH staining was present in the PAG. The distribution of DBH showed some similarities with the distribution of dopamine, but the regional DBH staining pattern differed (figs. 4 and 8E-F). Most striking was the rather sparse labeling of the dorsal subdivisions of the PAG.

The inferior colliculus

Dopamine immunoreactivity in the inferior colliculus showed a characteristic distribution, which may be best described as medial, middle and lateral areas (fig. 9). These areas do not seem to follow the cytoarchitectonical subdivisions that have been described previously for the inferior colliculus (Faye-Lund and Osen, 1985; Klepper and Herbert, 1985). The lateral area largely consists of the external cortex, while the middle area appears to include parts of the central nucleus as well as the dorsal and external cortex. The same holds true for the medial area. The middle area was sparsely labeled. Laterally there was a relatively abrupt transition to the lateral area, which showed sparse to moderate labeling. In the medial direction, there was a gradual transition from sparse to moderate dopamine labeling when going from the middle area to the medial area. Ventromedially, the strongest labeling was observed in a narrow strip at the most ventromedial part of the inferior colliculus. This area was not present at the most rostral and caudal parts.

DBH labeling, ranging from sparse to moderate, was distributed much more uniformly throughout the inferior colliculus. There was no indication of different areas as observed with the dopamine labeling.

The superior colliculus

In the typical layered cytoarchitecture of the superior colliculus (see e.g. Kanaseki and Sprague, 1974), the dopamine immunoreactivity showed a characteristic distribution (fig. 10A). In the most superficial layer, the stratum zonale, dopamine labeling was absent. In the second layer, the stratum griseum superficiale, labeling was sparse but increases ventrally at the transition with layer III, the stratum opticum, where it became sparse to moderate. This labeling was seen throughout layer III and also the dorsal part of layer IV, the stratum griseum intermedium. In the remainder of layer IV and in Layer V, the stratum album intermedium, labeling was sparse, with some traversing fibers. The area below layer V up to the border with the aqueduct and containing layers VI and VII, the stratum griseum profundum and the stratum album profundum respectively, showed moderate dopamine labeling, especially medially where the labeling blends in with that of the dorsal PAG.

The DBH labeling (fig. 10B) in the superior

colliculus was different from the dopamine labeling. It was strongest in the superficial layers and was still clearly present in layers III-V, where dopamine labeling was sparse. In layers VI and VII, up to the border with the PAG, DBH labeling was only sparse, whereas dopamine labeling is much stronger in these areas.

Motor nuclei of the cranial nerves

Moderate to strong dopamine staining was observed in somatomotor nuclei of the cranial nerves III, V (fig. 11), VII, and XII, whereas motor nuclei of the cranial nerve IV, VI and the ambiguus nucleus were only sparsely labeled. The dopamine staining in the motor nuclei had a very different appearance as compared to other areas of the brain stem. The large number of lightly labeled dopamine immunoreactive varicosities, with even less stained intervaricose segments, gave the labeling a fine granular (punctuate) appearance, especially at lower magnifications (fig. 11). Interestingly, the DBH labeling had a similar appearance.

Monoaminergic cell groups

Dopaminergic fibers and terminals were observed around cell bodies and dendrites of all the monoaminergic cell groups. The serotonergic raphe nuclei all received dopamine immunoreactive fibers and terminals, with very strong labeling in the dorsal raphe nucleus. The raphe pallidus (RPa) was strongly and the raphe obscurus (ROb) was moderately to strongly labeled with dopamine immunoreactive fibers, mostly showing a dorsoventral orientation. The labeling increased at more rostral levels. RPa appeared somewhat stronger labeled than ROb. The nucleus raphe magnus (RMg) was moderately labeled. The area between RMg and the noradrenergic A5 cell group received a moderate to strong dopamine labeling. The noradrenergic cell groups, most notably the locus coeruleus and subcoeruleus and the A5 group, but also the A1, A2 and the A7 noradrenergic cell groups of Dahlström and Fuxe (1964), all received a moderate to strong dopaminergic innervation. Similar dopamine labeling was observed in the adrenergic cell groups of the medulla.

DBH labeling in the monoaminergic cell groups was moderate to strong.

Discussion

Methodological considerations

In the present study dopamine fibers and terminals were identified in rat brain stem using an antiserum raised in rabbit against glutaraldehyde-conjugated dopamine. As a consequence, a relatively high concentration of glutaraldehyde is required for fixation, which is generally believed to be incompatible with the immunocytochemical detection of many other substances, including the catecholamine enzymes like DBH. However, the current fixation protocol appeared suitable not only for the detection of dopamine, but also for immunocytochemical detection of the noradrenaline biosynthetic enzyme DBH, which made it possible to use subsequent brain stem sections for comparing the distribution of dopamine with that of (nor)adrenaline.

The present immunocytochemical method for identifying dopaminergic structures, i.e. with the aid of an antibody raised against the neurotransmitter itself, has the distinct advantage that it offers a relatively simple and direct procedure for demonstrating dopaminergic fibers as well as terminals with a high specificity. No additional manipulations or pharmacological tools are required to distinguish the dopaminergic fibers and terminals in the brain stem from the (nor)adrenergic ones, which are also abundantly present.

Dopamine is present in the brain not only as a neurotransmitter, but also as a precursor in the biosynthesis of the catecholamines noradrenaline and adrenaline. Theoretically, the possibility exists that noradrenergic and adrenergic fibers and terminals contain levels of precursor dopamine that are detected by dopamine immunocytochemistry. As a consequence, these fibers would be falsely identified as dopaminergic, i.e. using dopamine as a transmitter. However, several lines of evidence suggest that the majority of dopamine immunoreactive fibers and terminals in the brain stem are indeed dopaminergic. Firstly, our control experiments with the neurotoxin DSP-4, that affects a specific subset of noradrenergic fibers and terminals (Fritschy and Grzanna, 1989), showed a selective loss of DBH staining. In the alternate sections the dopamine immunoreactivity remained unaffected by the drug (see also Holstege *et al.*, 1996). These results strongly suggest that in the affected areas the precursor dopamine in noradrenergic fibers and terminals does not reach detectable levels. Therefore, in the affected areas, which receive the noradrenergic input from the locus coeruleus, the present immunocytochemical method appears to visualize exclusively genuine dopaminergic fibers and terminals. The possibility exists, however, that in some of the areas which remained unaffected by DSP-4, precursor dopamine may have reached detectable levels as suggested by Alonso *et al.* (1995). They reported that metabolic dopamine could be detected, especially in areas receiving an extremely dense catecholaminergic innervation like the locus coeruleus, the dorsal motor nucleus of the vagus, the area postrema, the nucleus of the solitary tract and the hypoglossal nucleus. On the other hand, they found that dopamine immunoreactivity was not associated with the large majority of the noradrenaline immunoreactive fibers detected in several other regions of the brain stem, including vestibular, dorsal column and spinal trigeminal nuclei. Thus, only in areas receiving a very strong catecholaminergic innervation dopamine may reach detectable levels, whereas in the large majority of the brainstem nuclei precursor dopamine remains undetectable by immunocytochemistry. In accordance, biochemical studies (Westerink and de Vries, 1985; Verhage *et al.*, 1992) have shown that in areas with a rich noradrenergic innervation and very low dopamine levels, such as the cerebellum and lateral hippocampus, even these very low dopamine levels were not present as precursor dopamine in noradrenergic terminals, in agreement with the results of the present study. In some

areas of the brain stem, like the cerebellum, the area postrema, the anterior portion of the ventral cochlear nucleus, and the external cuneate nucleus, a relatively dense staining for DBH immunoreactive structures was present, whereas in the alternate sections, virtually no dopamine immunoreactive fibers and terminals were observed. This further emphasized that the dopamine antibody did not label (nor)adrenergic structures, identified by the presence of DBH staining. The few labeled fibers in these areas probably were true dopaminergic fibers, since some of these areas do contain a minor dopaminergic innervation. On the other hand, it should be mentioned that some of the presumed noradrenergic somata of locus coeruleus neurons showed a faint staining for dopamine, probably because more precursor dopamine is present in somata than in the fibers and terminals of (nor)adrenergic neurons.

It is concluded that the dopamine immunoreactive fibers and terminals, detected in the present study, virtually all contained dopamine, not as a precursor, but rather as a transmitter. Therefore, in the remainder of the discussion, we will consider the dopamine immunoreactive fibers and terminals as dopaminergic. Since noradrenaline and adrenaline can not be distinguished individually with the catecholamine biosynthetic enzyme DBH as a marker, they will be collectively termed (nor)adrenergic.

Anatomical distribution of dopaminergic and (nor)adrenergic fibers and presumptive terminals

The results of the present study show a characteristic distribution of dopaminergic fibers and terminals in various regions of the rat brain stem. (Nor)adrenergic fibers clearly outnumbered the dopaminergic ones and were observed in areas that were not or only scarcely innervated by dopamine. Dopaminergic and (nor)adrenergic fibers formed an complex pattern of fine varicose fibers and punctate labeling, the latter often appearing as terminals, although this can only be ascertained by electron microscopy. The distribution of dopaminergic and (nor)adrenergic fibers and terminals showed a considerable overlap in many brain stem regions indicating similarities in the innervation of the rat brain stem by different catecholamines. However, despite the overlap, the dopaminergic and the (nor)adrenergic fibers and terminals were not distributed identically in all brain stem regions.

Sensory trigeminal complex

The distribution pattern of dopamine immunoreactive fibers in the trigeminal complex showed a rostrocaudal, dorsoventral, and mediolateral gradient. Within the rostrocaudal gradient of the trigeminal complex the most prominent labeling was observed in its most caudal nucleus, the spinal trigeminal nucleus (Sp5), especially its caudal and ventral parts. The presence of dopamine fibers in Sp5 is in agreement with a previous immunocytochemical study on dopamine in the feline medulla (Maqbool *et al.*, 1993). The dorsoventral distribution in Sp5 implied that the ventrally situated ophthalmic division of the trigeminal nucleus (V1) received strongest dopamine innervation, followed by the maxillary (V2) and the mandibulary (V3) divisions. The functional meaning of these gradients is unclear, but the predominance of the dopaminergic input on the caudal Sp5 is of particular interest, since this area is specifically involved in pain transmission of the head and neck. In accordance, a dopamine distribution similar to that of the caudal Sp5 is also present in the cervical dorsal horn (for details see Holstege *et al.*, 1996). More detailed investigations on the effects of dopamine in the spinal cord have shown that D_2-receptors are present in the superficial and deep dorsal horn (van Dijken *et al.*, 1996) and exert an inhibitory role in the transmission of nociceptive stimuli (Clatworthy and Barasi, 1987; Fleetwood-Walker *et al.*, 1988; Liu *et al.*, 1992), whereas D_1-receptors are without effect (Barasi *et al.*, 1987; Liu *et al.*, 1992). These findings suggest that dopamine induces antinociceptive effects, which are mediated via spinal D_2-receptors located in the superficial and deeper dorsal horn (van Dijken *et al.*, 1996). The similarity in the distribution of dopamine in Sp5C and the spinal cord suggests that dopamine has a similar mode of action in these areas, an idea which is strengthened by the reported binding of D_2-receptor ligands in the Sp5 (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994; Lawrence *et al.*, 1995).

In contrast to the distribution of dopamine, the (nor)adrenergic innervation pattern of Sp5 showed no obvious gradients. The DBH immunoreactive fibers in Sp5 represent largely, if not exclusively, noradrenergic fibers originating in the locus coeruleus proper (Grzanna and Fritschy, 1991). The Sp5 also receives serotonergic input from all levels of the brain stem (Dahlström and Fuxe, 1964; Steinbusch, 1981; Beitz, 1982; Pearson and Jennes, 1988; Tallaksen-Greene *et al.*, 1993).

Taken together, the findings suggest that dopamine, together with other monoamines like serotonin and (nor)adrenaline, plays an important role in the processing of sensory information, including nociception, from the head and neck region.

Dopaminergic, (nor)adrenergic (Copray *et al.*, 1990a; Copray *et al.*, 1990b), and serotonergic (Kolta *et al.*, 1993; Liem *et al.*, 1993; Lazarov and Chouchkov, 1995) fibers in the mesencephalic trigeminal nucleus (Me5) have been reported to closely surround the large ganglion cells that form the Me5. Low levels of D_1-receptors and high levels of D_2-receptors were seen in Me5 neurons (Lazarov and Pilgrim, 1997). This suggests that the monoamines, and more particularly dopamine, are also involved in modulating proprioceptive information from the oro-facial region.

Solitary complex

The existence of a dopaminergic innervation of the nucleus tractus solitarius and the dorsal motor nucleus of the vagus is well established (Hökfelt *et al.*, 1984; Kalia *et al.*, 1985; Ruggiero *et al.*, 1994) and is in agreement with high densities of D_2 binding (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994) and D_2 mRNA (Bouthenet *et al.*, 1991). The present study confirms this strong innervation by dopaminergic as well as (nor)adrenergic fibers and presumptive terminals. Since the synthesizing enzyme of adrenaline (PNMT) is

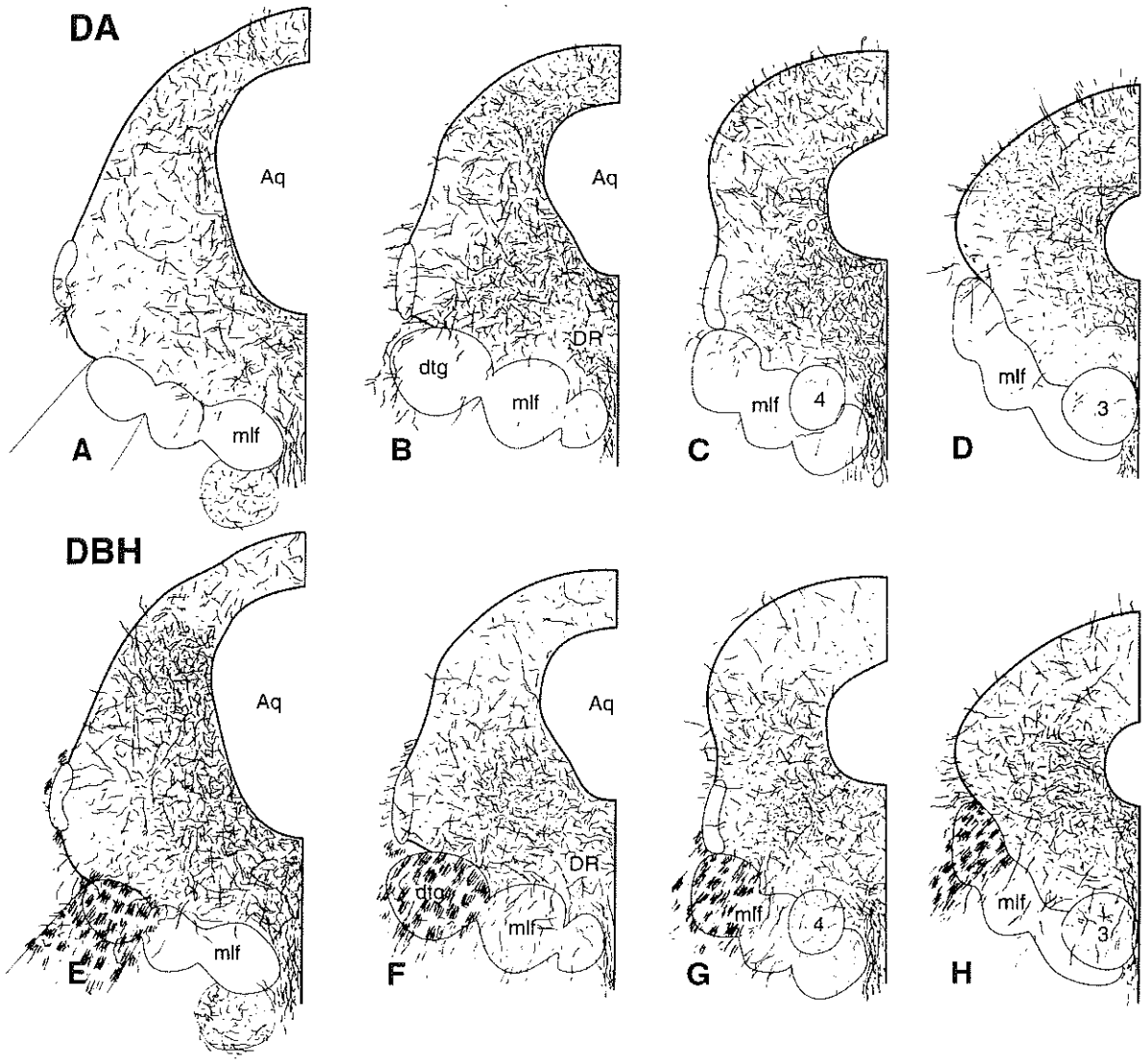


Figure 8: Schematic representation of the dopamine and DBH immunoreactive fibers and terminals in the rat periaqueductal gray (PAG) at different levels of the brain stem. Note the rather sparse DBH labeling in the dorsal subdivisions of the PAG, whereas dopamine labeling is abundantly present. For details see text; for abbreviations see enclosed list of abbreviations.

present in specific parts of the nucleus tractus solitarius (Ruggiero *et al.*, 1994), the adrenergic innervation appears to represent a specific input, separate from the noradrenergic one. The nucleus tractus solitarius also receives a very dense serotonergic innervation, especially in its caudal parts (Steinbusch, 1981), as well as many serotonin receptor binding sites (Thor *et al.*, 1992).

The nucleus tractus solitarius serves primarily as a sensory relay center for visceral, gustatory, respiratory, and cardiovascular afferent neural pathways (van Giersbergen *et al.*, 1992; Norgren, 1995). Several studies have focussed on the effects of dopamine on nucleus tractus solitarius functions. Application of dopamine, but also noradrenaline, into the lateral nucleus tractus solitarius resulted in inhibition of the swallowing reflex (Kessler and Jean, 1986). With respect to cardiovascular functions, microinjection of low doses of dopamine into the nucleus tractus solitarius elicited a dose dependent decrease in blood pressure and heart rate (Zandberg *et al.*, 1979), albeit less potent than noradrenaline or adrenaline. The depressor response of dopamine was mediated via α-adrenoceptors. Apparently, dopamine acted directly and not after conversion into noradrenaline (van Giersbergen *et al.*, 1992). In contrast, recent studies reported that microinjection of higher doses of dopamine (Granata and Woodruff, 1982) or a D_2-receptor agonist (quinpirole) (Yang *et al.*, 1990) into the posterior region of nucleus tractus solitarius caused a consistent increase in mean arterial pressure, suggesting that dopamine may produce an increase in blood pressure via its D_2-receptors. These dose dependent opposite hemodynamic effects were also found after

application of noradrenaline into the nucleus tractus solitarius (see De Jong, 1974; van Giersbergen *et al.*, 1992). Low doses of noradrenaline caused a fall in blood pressure and heart rate probably via ß-adrenoceptors, whereas relatively high doses caused an increase in arterial blood pressure probably via α_1-adrenoceptors (van Giersbergen *et al.*, 1992). In the case of serotonin, microinjection into the nucleus tractus solitarius evoked inhibitory effects (Feldman and Galiano, 1995), whereas $5HT_3$-receptor activation in the nucleus tractus solitarius increased mean arterial pressure (Nosjean *et al.*, 1995). Taken together, these findings may suggest that the monoamines, including dopamine, have a similar mode of action in the nucleus tractus solitarius such that the concentration of the monoamine will determine which receptors are activated and which hemodynamic effect are manifested.

Inferior olive

Dopaminergic and (nor)adrenergic fibers were observed to be densely distributed in the principal nucleus of the inferior olive and its ventrolateral outgrowth, which is in agreement with the original description of the catecholaminergic innervation the inferior olive by Dahlström and Fuxe (1964). The dopaminergic innervation of the dorsal lamella of the principle nucleus of the inferior olive appears to be characteristic for rat. In cat and monkey the catecholaminergic innervation, and more particularly the dopaminergic innervation, is distributed to other subnuclei of the inferior olive than in rat. In the cat, dopaminergic nerve terminals were reported to be most prominent in the dorsal accessory nucleus (Maqbool *et al.*, 1993), while in monkey the catecholaminergic fibers and terminals were detected either in the medial accessory nucleus (Kamei *et al.*, 1981) or in the lateral lamella of the principal nucleus (Sladek and Bowman, 1975).

The presence of a dopaminergic innervation of the inferior olive in rat is consistent with the existence of a dopaminergic projection from the subparafascicular thalamic nucleus, whose dopaminergic neurons might belong to the diencephalic A11 group (Takada, 1993). The distribution pattern of (nor)adrenaline is similar to that of dopamine, although DBH staining was not evident within the ventrolateral outgrowth. The (nor)adrenergic projection to the principal olive consists largely, if not exclusively, of noradrenergic fibers and terminals, which originate predominantly in the locus coeruleus proper (Grzanna and Fritschy, 1991). The presence of serotonergic fibers and terminals in the inferior olive has also been reported (Steinbusch, 1981), but the distribution of serotonergic fibers differs from the catecholaminergic innervation patterns (Paré *et al.*, 1987). The subnucleus most densely innervated by serotonergic fibers is the dorsal accessory olive, whereas only few punctuate serotonin profiles were present in the principal olive and ventrolateral outgrowth. These findings demonstrate that the innervation of the inferior olive is heterogeneous with respect to the different monoamines within a particular species, but also between different species for a particular monoamine like dopamine.

Weak D_2-receptor binding was reported for the inferior olivary nucleus of rat (Yokoyama *et al.*, 1994), especially in its central and medial part (Lawrence *et al.*, 1995). Since no further details were given, the exact distribution and the involvement of D_2-receptors in the inferior olivary nucleus presently remains unclear.

The dopamine innervation of the inferior olive in the rat is aimed exclusively at the principal inferior olivary nucleus, avoiding the medial and dorsal accessory olivary nuclei. The main differences between these nuclei may be that the medial and dorsal accessory nuclei receive direct sensory information from spinal cord, the trigeminal nucleus, the dorsal column nuclei and the vestibular nuclei, while the principal nucleus does not receive this type of direct sensory information, but rather receives integrated information from the cerebral cortex (Ruigrok and Cella, 1995). This indicates that the dopaminergic projection to the inferior olive is primarily involved in modulating integrative input from higher centers and is not involved in modulating direct sensory input to the inferior olive. This is, to some extent, in line with the avoidance by dopamine projections of spinal neurons projecting to the cerebellum (Holstege *et al.*, 1996). The lack of innervation of the cerebellar and vestibular nuclei and the limited innervation of the cerebellar cortex further suggest that the dopaminergic projection is aimed at modulating specific input to the inferior olive rather than the processing of this information in the projection area of the inferior olive in the cerebellum. The above may also hold true for the (nor)adrenergic innervation, which is similar to the dopaminergic one, except that the medial and dorsal accessory olivary nuclei also receive a limited projection. Surprisingly, the serotonergic projection in the rat appears to avoid the principal olive and mainly projects to the dorsal and medial accessory nuclei (Kojima *et al.*, 1983; Bishop and Ho, 1984; Compoint and Buisseret-Delmas, 1988). The heterogeneous distribution between species further complicates the issue. Therefore, functional aspects of the monoaminergic, and more particular dopaminergic, innervation of the inferior olive remain to be resolved.

Cerebellar nuclei and cerebellum

The cerebellar nuclei received few dopamine immunoreactive fibers and the same holds true for the cerebellar cortex (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992). In the present study, an extensive investigation of the cerebellum was not attempted. Nevertheless, it was noticed that the paraflocculus of the cerebellum received a small, but distinct dopaminergic innervation. A finding which is in good accord with previous studies (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992). Dopaminergic fibers in the cerebellar cortex were reported to be predominantly distributed to the lateral portions of the cerebellar hemisphere, like the crus I ansiform lobule and paraflocculus, and to a lesser extent in the crus II ansiform lobule (Ikai *et al.*, 1992). Retrograde tracing indicated that the dopaminergic innervation of the paraflocculus was derived from cell bodies in the ventral tegmental area (A10) (Ikai *et al.*, 1992).

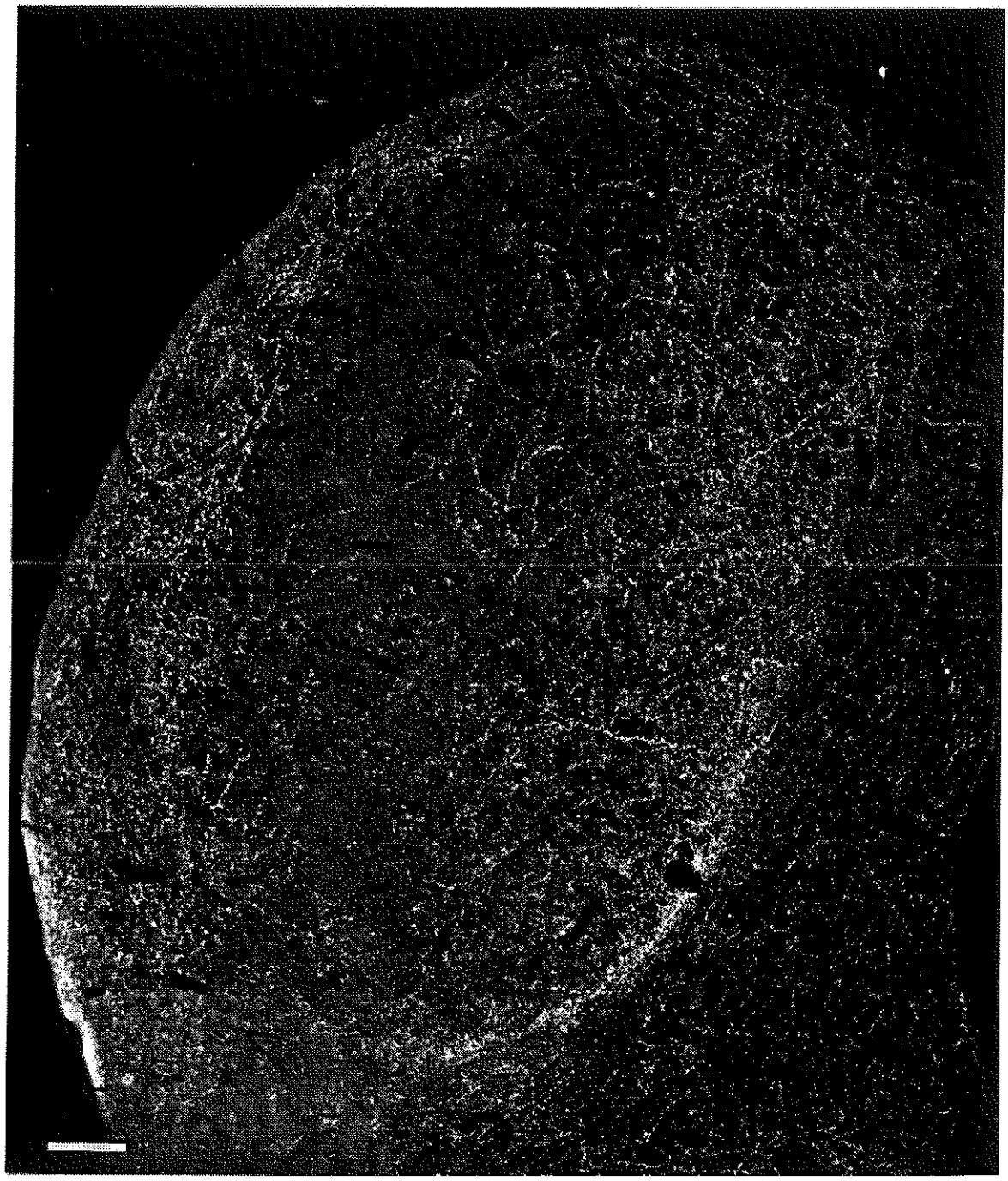


Figure 9: Photomicrograph with darkfield illumination of a section processed for dopamine immunocytochemistry, showing the inferior colliculus. Note that the dopamine immunoreactive fibers and terminals are not evenly distributed over the different parts of the inferior colliculus. See fig. 1 for the underlying cytoarchitecture. For details see text. Scale bar = 200 µm.

The cerebellar nuclei and cerebellar cortex received a prominent noradrenergic innervation, which, in the cat, originated in the locus coeruleus (Grzanna and Fritschy, 1991). In monkey, the noradrenergic innervation of the flocculus and paraflocculus of the cerebellum was found to originate in the noradrenergic A4 cell group (Demirjian *et al.*, 1976). Many cerebellar structures also receive a dense serotonergic projection, although the exact location of the cells of origin is uncertain (for review see Halliday *et al.*, 1995). Thus, the paucity of dopamine fibers in large parts of the cerebellar nuclei and cerebellum may suggest that monoamines, like noradrenaline and serotonin, play a more important role than dopamine.

Vestibular complex

A number of studies have indicated a possible interaction between dopamine and the vestibular system

(see e.g. Hozawa and Takasaka, 1993). In the present study, only weak dopamine staining was observed in the vestibular complex, mainly represented by a few dopaminergic fibers in the medial and spinal vestibular nuclei. The presence of dopaminergic fibers in the medial vestibular nucleus is in accordance with a study in guinea-pig brain slices (Vibert *et al.*, 1995) showing that medial vestibular nucleus neurons depolarized after application of dopamine as well as after application of selective D_2-agonists, while application of selective D_1 agonists had no effects on MVe neurons. The depolarizing responses to dopamine appeared to be dependent on the $GABA_A$ receptor, indicating that dopamine might exert a modulatory action on the vestibular system, either by a direct action on the vestibular neurons or by modulation of GABAergic transmission.

The vestibular system also receives noradrenergic (Steinbusch, 1991; Schuerger and Balaban, 1993) and serotonergic (Steinbusch, 1981; Steinbusch, 1991) input. The most prominent innervation arises from the serotonergic system, especially in the caudal part of the medial vestibular nucleus. The noradrenergic innervation of the vestibular complex is less prominent.

Parabrachial complex and area postrema

The parabrachial nucleus (PB) is the main relay for ascending visceral sensory information from the nucleus of the solitary tract (NTS) to the forebrain (see Herbert *et al.*, 1990). It has been proposed that part of the catecholaminergic innervation of the lateral parabrachial nucleus is dopaminergic (Hedreen, 1980). This idea has been further substantiated by findings with retrograde tracers combined with tyrosine hydroxylase immunocytochemistry (Sawchenko and Swanson, 1982). However, so far the detailed localization of dopaminergic fibers, separate from the other catecholamines, has not been investigated in the parabrachial nuclei. We found that the dopaminergic input is strongest in the lateral parabrachial nuclei, including the waist area. These parts receive a strong projection from the caudal part of the nucleus tractus solitarius, which also receives a strong dopamine input. With respect to the physiological effects of dopamine in the parabrachial nuclei, no data are available. The presence of dopaminergic fibers in the lateral parabrachial nucleus was suggested by the presence of scattered, presumed dopaminergic, cells in the ventral tegmental area after injection into the parabrachial area. Serotonergic fibers have also been reported in the parabrachial nucleus (Steinbusch, 1981; Halliday *et al.*, 1995; Zardetto-Smith and Johnson, 1995).

Periaqueductal gray

The periaqueductal gray (PAG) in the rat contained a dense network of dopaminergic and (nor)adrenergic fibers. Since DBH and PNMT are both present in the PAG, the catecholaminergic innervation of the PAG is generally considered to be largely noradrenergic and adrenergic. The results of the present study, however, indicate that a significant part of the catecholaminergic innervation consist of dopaminergic fibers, a finding which is in accord with the reported binding of D_2-receptor ligands in the PAG (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994).

The observed distribution of (nor)adrenergic fibers in the PAG is in general agreement with the report of Herbert and Saper (1992). In the present study, however, DBH staining was also observed in more dorsal subdivisions of the PAG, an area that they reported to be nearly devoid of DBH stained axons. A somewhat higher sensitivity of the DBH antibody or the different type of fixation used in the present study probably may explain this discrepancy in staining pattern. Interestingly, the dorsal subdivisions of the PAG were prominently innervated by dopamine fibers, indicating a clear difference between the dopaminergic and the (nor)adrenergic innervation.

The (nor)adrenergic innervation in the PAG largely represents projections from the noradrenergic cell groups (A1 and A2) and the adrenergic cell groups (C1 and C3) in the medulla oblongata (Herbert and Saper, 1992), with only a limited noradrenergic projection from the locus coeruleus (Fritschy *et al.*, 1990). The area most densely innervated with (nor)adrenergic fibers and terminals is the ventrolateral PAG. Injections into the ventrolateral subdivision reportedly result in retrogradely labeled neurons in medullary cell groups that contain catecholaminergic neurons, including the nucleus tractus solitarius, the dorsomedial medulla, and the ventrolateral medulla (Herbert and Saper, 1992). The dense noradrenergic fiber plexus in the ventral part of the ventrolateral PAG originates in the noradrenergic A2 neurons in the nucleus tractus solitarius, whereas the dorsal part of the ventrolateral PAG, bordering on the dorsolateral PAG, is more heavily innervated by the noradrenergic A1 neurons in the ventrolateral medulla. The origin of the dopamine projection to the PAG is at present unknown.

The involvement of the PAG in defensive reactions, analgesia and autonomic regulation, has been extensively reviewed (Carrive, 1993; Bandler and Shipley, 1994; Behbehani, 1995). Based on connections and anatomical representation of reactions evoked by electrical stimulation within the PAG in freely moving animals, it was suggested that the different functions of the PAG are integrated by longitudinal columns of neurons located dorsomedial, dorsolateral, lateral, and ventrolateral of the aqueduct. Attention has focused on the lateral and ventrolateral columns, activation of which results in different forms of defensive or protective reactions. Reactions evoked from the lateral PAG column are associated with somatomotor and autonomic activation, leading to active fight and flight reactions, and are related to superficial or cutaneous noxious stimuli. In contrast, reactions evoked from the ventrolateral PAG are associated with somatomotor and autonomic inhibition, leading to a quiescent reaction, and are related to deep and visceral noxious stimuli (Carrive, 1993). In the caudal ventrolateral PAG, stimulation produces immobility (Bandler *et al.*, 1991). Interestingly, the ventrolateral PAG receives the densest dopaminergic and (nor)adrenergic innervation within the PAG, suggesting that these catecholamines are especially involved in PAG functions related to a

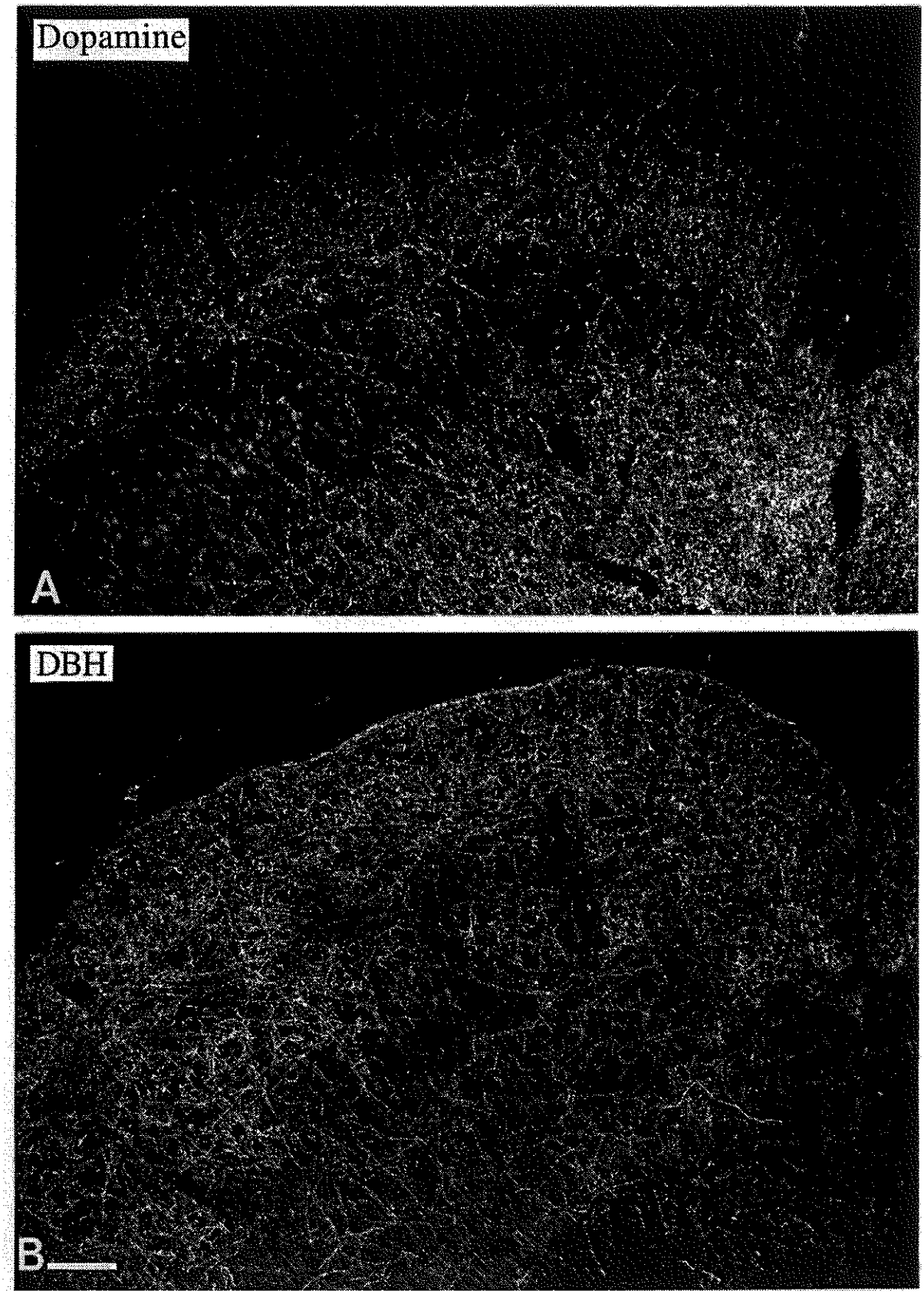


Figure 10: Photomicrograph with darkfield illumination of sections with the superior colliculus after processing for dopamine (A) or DBH (B) immunocytochemistry. Note the different distribution of the dopamine and DBH immunoreactive terminals over the different layers of the superior colliculus. See fig. 1 for the underlying cytoarchitecture. For details see text. Scale bar = 200 µm.

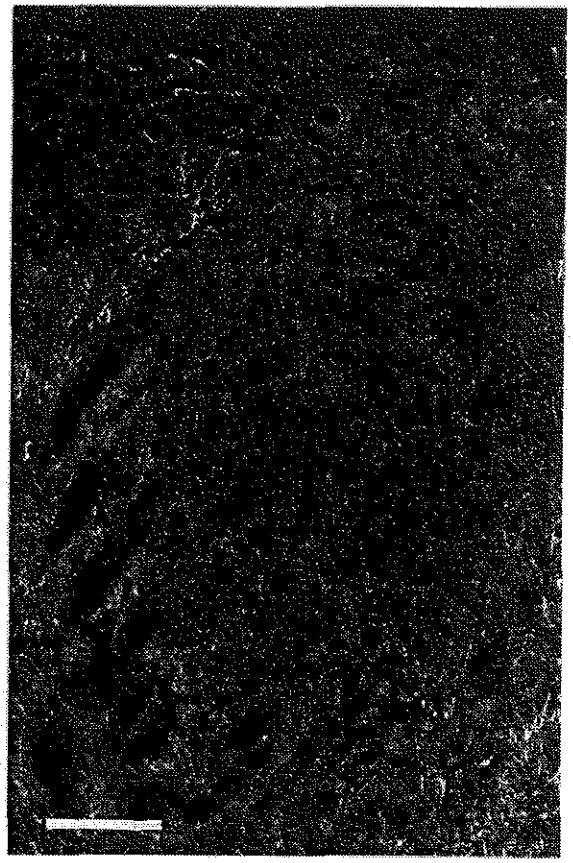


Figure 11: Photomicrograph with darkfield illumination of motor trigeminal nucleus after processing for dopamine immunocytochemistry. Note the very fine labeling in the motor nucleus, but not in the surrounding area. Scale bar = 200 µm.

quiescent reaction to deep visceral and muscular pain stimuli. The dorsal PAG is a major site for processing of fear and anxiety. In both cats and rats, stimulation of rostral dorsolateral PAG produced threat display associated with vocalization and strong flight responses (Behbehani, 1995). The predominant dopaminergic innervation of the dorsal PAG would suggest the involvement of dopamine in these functions. However, application of agonists or antagonists of dopaminergic, as well as adrenergic and serotonergic receptors had no effect on vocalization (Lu *et al.*, 1993).

The results of the present study show that dopamine, as well as (nor)adrenaline may play a role in some of the behaviors evoked from the PAG. Comparison of the distributions of dopaminergic fibers and terminals with the (nor)adrenergic ones, indicates that the dorsal PAG is predominantly innervated by dopamine fibers, whereas (nor)adrenergic fibers are less abundant in the dorsal part of the PAG. The role of serotonin in PAG function is complex, mainly because PAG contains several subtypes of serotonin receptors and activation of different types of receptors have different, sometimes opposite, effects (Behbehani, 1995).

Inferior Colliculus

In the inferior colliculus, the dopamine labeling showed a characteristic pattern in areas, which did not follow the cytoarchitectonical boundaries described previously (Faye-Lund and Osen, 1985; Klepper and Herbert, 1985), although it should be noted that these boundaries are difficult to identify in our material, which was not counterstained. The DBH labeling that we found was very similar to the DBH labeling described previously (Klepper and Herbert, 1991) and showed a much more homogeneous distribution than the dopamine labeling. The serotonergic input also showed a specific pattern (Klepper and Herbert, 1991) and is especially strong in the outer layers of the inferior colliculus. Although the distribution of dopamine in the inferior colliculus has not been described previously, the existence of a dopamine projection was suggested by the finding (Olazábel and Moore, 1989) that cells in the lateral part of the substantia nigra, that were retrogradely labeled from the inferior colliculus, showed a similar morphology as tyrosine hydroxylase immunoreactive cells observed in a separate experiment in the same area. The presence of D_2-receptors in the periphery of the inferior colliculus further suggests a role for dopamine. Studies on the effects of dopamine in the inferior colliculus have not been performed.

Superior Colliculus

The dopamine innervation of the superior colliculus showed a characteristic labeling pattern over the various layers, which is different from the DBH innervation. Strongest dopamine labeling is found in layer III and the immediate adjacent parts of layers II and IV and in layers VI and VII, while DBH labeling is present in all laminae, with an overall decreasing intensity when passing from layer I towards layer VII. The present study is the first to describe the localization of dopamine in the superior colliculus, but the DBH innervation has been described previously (Mooney *et al.*, 1990), in general agreement with our results. The serotonergic innervation is also present in all layers of the superior colliculus, showing the highest density in layers II and IV (Ueda *et al.*, 1985). Thus, dopamine, serotonin and (nor)adrenaline are all present in the superior colliculus. As in other parts of the brain, the numbers of serotonin as well as DBH immunoreactive fibers appear larger than the number of dopaminergic fibers, but the relative density of the monoamines over the various layers is very different.

With respect to the effects of dopamine in the superior colliculus no general data are available, but the presence of dopamine D_2-receptors in layers II and III suggests that dopamine does play a role in information processing in the superior colliculus. The effects of noradrenaline have been investigated: iontophoretic application of noradrenaline generally showed an inhibitory effect, reducing both spontaneous and stimulus evoked activity (Mooney *et al.*, 1990; Sato and Kayama, 1983). In addition it was found (Wichmann and Starke, 1988) in a slice preparation that the release of noradrenaline was inhibited by dopamine through presynaptic D_2-receptors, further underlining the complicated interplay that occurs between the different

monoamines throughout the brainstem.

Noradrenergic and serotonergic cell groups in the brain stem. Dopaminergic fibers and terminals were observed around cell bodies in many monoaminergic cell groups, including the serotonergic raphe nuclei (raphe pallidus, raphe obscurus, raphe magnus, raphe centralis and raphe dorsalis) and the noradrenergic locus coeruleus, a finding which confirmed previous reports (Geffard *et al.*, 1984; McRae-Deguerce *et al.*, 1988; Maqbool *et al.*, 1993; Maeda *et al.*, 1994). These monoaminergic cell groups have also been reported to contain D_2-receptors, as indicated by the presence of D_2 binding (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994) and D_2 mRNA (Bouthenet *et al.*, 1991). Dopaminergic afferents to the LC originate predominantly from the A13 cell group and partly from the rostral portion of the A11 group (Maeda *et al.*, 1994). The ventral tegmental area also projects to the locus coeruleus, but this projection is largely non-dopaminergic (Sawchenko and Swanson, 1982).

The existence of a dopaminergic innervation of monoaminergic cell groups located in the brain stem suggest that dopamine interacts with other monoaminergic cell groups, not only at the level of the terminal fields in the target areas, but also by directly affecting the activity of the parent cell bodies of the monoaminergic fibers. This strengthens the idea of a strong interaction between the different monoaminergic cell groups.

Functional considerations

The anatomical distribution of dopamine in the brainstem shows that the functions of dopamine are not limited to one system. In fact, dopamine is involved in sensory processing, which includes nociceptive and non-nociceptive sensory input, like proprioception (Me5 and the dorsal column nuclei), as well as in the processing of visceral afferent information (NTS and parabrachial), while dopamine is also involved in processing motor output, both somatic (hypoglossal) and branchiomotor (facial and trigeminal) as well as parasympathetic (DMNV) and, indirectly, sympathetic (e.g. RVL). In addition dopamine is present in a more integrative system like the PAG and in various monoaminergic cell groups (LC, raphe nuclei). In the spinal cord, a similar distribution, comprising motor, sensory and autonomic areas, is seen (Holstege *et al.*, 1996). It has been suggested that in the spinal cord, the dopaminergic system is specifically activated in fight and flight situations with inhibition of pain transmission and facilitation of motor neuron activity. In the brainstem, a similar situation may be applicable, in which blood pressure, heart rate and respiration are increased, while the activity in other, non-vital systems is diminished. At the same time it should be realized that there is a considerable degree of overlap in the distributions of the monoamines within the brain stem, suggesting that they are all involved in modulating the same brain stem functions. This notion strengthens the idea that the integrative effects of the monoamines together may determine the final effect at the level of a particular innervation area. The presence of a dopaminergic innervation in the noradrenergic and serotonergic cell groups further emphasizes the intricate relationship between the different monoamines.

Motor nuclei of the cranial nerves

The present study revealed the presence of dopamine immunoreactive processes in the motor nuclei of cranial nerves III, V, VII, and XII, whereas motor nuclei of the cranial nerve IV, VI and the ambiguus nucleus were only sparsely labeled. The presence of a dopaminergic innervation is in accordance with the reported D_2-receptor ligand binding in motoneuronal cell groups of the brain stem and spinal cord (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994). In spinal cord, the presence of dopamine in the motoneuronal cell groups was discovered much later than in other parts of the spinal cord (Yoshida and Tanaka, 1988; Shirouzu *et al.*, 1990; Ridet *et al.*, 1992). Under less optimal conditions dopamine labeling in the motoneuronal cell groups in the spinal cord is the first to disappear (Holstege *et al.*, 1996), which is in line with its lower dopamine content as compared to other areas of the spinal cord. Therefore, more sensitive detection methods are required to visualize dopaminergic fibers and terminals in the motoneuronal cell groups as compared to other parts of the brain stem and spinal cord.

Probably the most prominent feature of the dopaminergic, as well as noradrenergic, fibers located in motoneuronal cell groups was their fine granular aspect, which is probably caused by a large number of fibers with small varicosities and thin, or lightly stained, intervaricose segments. This dot-like innervation pattern of both catecholamines is also observed in the motoneuronal cell groups of the spinal cord (Grzanna *et al.*, 1987; Fritschy and Grzanna, 1989; Holstege *et al.*, 1996). Thus, it appears that, throughout the neuraxis, there is a consistent dopaminergic and noradrenergic innervation of motor nuclei by fibers with a similar morphology in immunocytochemical preparations.

Interestingly, Fritschy and Grzanna (1989) reported that predominantly noradrenaline axons of the dot-like type were spared after DSP-4 treatment, which was confirmed by our DSP-4 experiments. Indeed, the drug spared noradrenergic fibers in the motoneuronal cell groups. These noradrenergic fibers and terminals in the motoneuronal cell groups, including those of the spinal cord, appeared to have their parent cell bodies in the lateral and/or dorsal tegmental noradrenergic cell groups. In contrast, fibers in autonomic and somatosensory areas were reported to originate predominantly from the locus coeruleus proper (Grzanna *et al.*, 1987). This might suggest that there is a link between the morphology of the fibers and the origin of their parent cell bodies. However, in nuclei only partly depleted by the neurotoxin, such as the principal trigeminal sensory nucleus or the pontine nuclei, the remaining noradrenaline fibers were not different morphologically from fibers that were affected by the drug. Moreover, in the case of dopaminergic fibers and terminals in the spinal cord, the morphological differences can not be explained this way, since both areas receive their dopaminergic innervation solely from the diencephalic A11 cell group, which contains relatively few cells and give rise

to highly collateralized projections. Therefore, the fine granular aspect of the labeling appears to be dictated by the target area, i.e. motoneuronal cell groups, rather than the location of the parent cell bodies. A further explanation for these morphological differences remains to be determined.

Based on retrograde transport of fluorescent tracers combined with the comparison of biosynthetic marker enzymes, the catecholaminergic innervations of the brain stem nuclei like the (motor) hypoglossal (Aldes *et al.*, 1988) and (sensory) cochlear (Klepper and Herbert, 1991) nuclei are considered to be largely noradrenergic. Indeed, there is a noradrenergic projection from locus coeruleus to the cochlear nuclei (Kromer and Moore, 1980; Grzanna and Fritschy, 1991; Klepper and Herbert, 1991) and motor nuclei, like the trigeminal and facial motor nuclei, also receive a noradrenergic innervation (Grzanna *et al.*, 1987). The results of the present study, using a direct method for detection of dopamine, indicate that besides noradrenaline dopamine is also present in these nuclei, albeit less prominent. A serotonergic innervation also has been reported for the brain stem motor nuclei (Steinbusch, 1981; Lu *et al.*, 1993).

The presence of dopamine fibers in the hypoglossal nucleus is in agreement with findings in the cat (Maqbool *et al.*, 1993). Although colocalization of dopamine immunoreactive with noradrenaline immunoreactive has been reported within fibers innervating the hypoglossal nucleus (Alonso *et al.*, 1995), the presence of D_2-receptors (Yokoyama *et al.*, 1994) implicate the presence of a dopaminergic innervation. Within the hypoglossal nucleus, dopamine immunoreactive is greatest among protrusor motoneurons in the caudoventromedial quadrant of this nucleus, which also holds true for the noradrenergic innervation (Aldes *et al.*, 1988; Aldes *et al.*, 1990). The hypoglossal nucleus also receives a serotonergic innervation (Lu *et al.*, 1993). However, the functional role of the preferential innervation of this part of hypoglossal nucleus is at present still unknown.

Thus, motoneuronal cell groups in the brain stem are innervated by dopaminergic fibers and terminals, as well as by noradrenergic and serotonergic ones. The morphological differences observed in different brain stem and spinal cord areas may suggest that different subsets of the monoaminergic systems innervate sensory, autonomic and motor areas of the brain stem and spinal cord.

Conclusion

The present immunocytochemical study shows the existence of an extensive dopaminergic innervation of the brain stem, which constitutes a distinct neurotransmitter system separate from the other monoamines. Although prominent, the distribution of dopamine in the rat brain stem is not as extensive as that of (nor)adrenaline. The observed distribution of dopaminergic fibers and presumptive terminals in the brain stem demonstrates that dopamine is involved in modulating a large variety of brain stem functions. In many instances dopamine may exert its effects in complex interaction with other monoamines, which is indicated by their similar distribution and involvement within similar functional systems. Obviously, the effect of dopamine, and the other monoamines, depend on the receptors involved. Knowledge of their distribution in the brain stem is still incomplete.

Acknowledgments

The authors thank Prof. Dr. Jan Voogd for reading the manuscript. We thank Dr. B.M. Buijs for kindly providing the anti-dopamine antiserum and technical advice. We also thank Erika Sabel-Goedknegt and Richard Hawkins for their technical assistance. The assistance of Eddie Dalm in the preparation of the photomicrographs is appreciated.

Distribution Of Dopamine D_2 Receptor In The Rat Brain Stem Using Anti-Peptide Antibodies

Distribution Of Dopamine D_2 Receptor In The Rat Brain Stem Using Anti-Peptide Antibodies

Abstract

In the present study the distribution of dopamine D_2 receptors in the rat brain stem was determined immunocytochemically using a purified polyclonal antiserum directed against the putative 3rd intracellular loop of the dopamine D_2 receptor. Immunoreactivity, indicating the presence of D_2-receptor protein, was observed exclusively in neuronal cell bodies and their proximal dendrites. Neurons containing D_2-receptor protein showed a highly specific distribution throughout the brain stem. Strongest labeling was present in the dorsal motor nucleus of the vagus, the lateral and medial parabrachial nuclei, the substantia nigra pars compacta, the medial geniculate nucleus, and most monoaminergic cell groups including the raphe nuclei, the locus coeruleus and several other (nor)adrenergic cell groups. Areas that showed substantial labeling were the gigantocellular nucleus pars alpha, the nucleus of the solitary tract, the basal pontine nuclei, the periaqueductal gray, the inferior and superior colliculi and the ventral tegmental area. Furthermore, several brain stem nuclei contained weakly labeled neurons, including the medullary, pontine and mesencephalic reticular formation. D_2-receptor protein was not observed in many other brain stem areas, like the external cuneate nucleus, the inferior and superior olivary nuclei, the vestibular and cochlear nuclei, substantia nigra pars reticulata, the red nucleus and all motor nuclei of the cranial nerves.

The present anatomical data largely confirm and extend previous findings obtained with immunocytochemistry, ligand binding or *in situ* hybridization, with only a few discrepancies. It is concluded that the dopamine D_2-receptor plays a major role in the dopaminergic modulation of many brain stem functions. The high expression of D_2-receptors in most of the brain stem monoaminergic cell groups indicates that dopamine interacts with other monoamines not only in their mutual target areas, but also by modulating the activity of the brain stem monoaminergic neurons directly at the level of their somata and dendrites.

Introduction

Dopamine is one of the catecholaminergic neurotransmitters in the brain and is present throughout the central nervous system (CNS). Dahlström and Fuxe (1964) were the first to provide a detailed description of dopamine neurons and pathways in the brain, by visualizing them with the Falck-Hillarp formaldehyde histofluorescence technique. Since then, the anatomical organization of the major dopamine systems in the CNS has been unraveled in considerable detail (reviewed in Björklund and Lindvall, 1984; Hökfelt *et al.*, 1984), especially by using dopamine-specific antibodies for visualizing dopaminergic neurons, fibers as well as terminals (Geffard *et al.*, 1984; Smiley *et al.*, 1992; Maqbool *et al.*, 1993; Moons *et al.*, 1994). Using this technique, we recently provided a detailed description of the distribution of dopaminergic fibers and terminals in the brain stem (van Dijken and Holstege, this thesis) and spinal cord (Holstege *et al.*, 1996) of several species.

The actions of dopamine in the CNS are mediated by specific, high-affinity, G protein-coupled dopamine receptors (for review see Civelli *et al.*, 1991; Sibley and Monsma, 1992; Gingrich and Caron, 1993; O'Dowd, 1993). Five subtypes of dopamine receptors have now been identified and termed D_1-D_5. Based on their pharmacology and sequence homology, D_1 and D_5-receptors are classified as D_1-like receptors while the D_2, D_3, and D_4-receptors are classified as D_2-like receptors. In this classification scheme, D_1-like receptors are responsible for the stimulation of adenylate cyclase, whereas D_2-like receptors mediate the inhibition of adenylate cyclase, or have no effect on it. The dopamine D_2-receptor has been found to exist in two isoforms (D_{2short} and D_{2long}), related by alternative splicing of a common gene (Strange, 1991; Sibley and Monsma, 1992). As a result, a splice insert of 29 amino acids in the putative 3rd intracellular loop of the D_2-receptor is present in the long isoform, but is lacking in the short isoform.

In comparison with the other dopamine receptor subtypes, the distribution of dopamine D_2-receptors in the CNS has been studied most extensively, using receptor ligand binding (Boyson *et al.*, 1986; Dubois *et al.*, 1986; Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994), *in situ* hybridization (Meador-Woodruff *et al.*, 1989; Bouthenet *et al.*, 1991) and, more recently, immunocytochemistry (Brock *et al.*, 1992; Ariano *et al.*, 1993; Levey *et al.*, 1993; van Dijken *et al.*, 1996). The distribution of dopamine D_1-receptors in the CNS has also been studied using receptor ligand binding (Boyson *et al.*, 1986; Dubois *et al.*, 1986; Fremeau *et al.*, 1991; Mansour *et al.*, 1992), *in situ* hybridization (Fremeau *et al.*, 1991; Mansour *et al.*, 1992), and immunocytochemistry (Levey *et al.*, 1993; Ariano and Sibley, 1994). Some of these studies have also included the distribution of dopamine D_3-receptors in the CNS detected with *in situ* hybridization (Bouthenet *et al.*, 1991) or immunocytochemistry (Ariano and Sibley, 1994).

The use of antibodies to visualize dopamine receptor protein has a number of distinct advantages over other methods, like ligand binding and *in situ* hybridization. First, the anatomical resolution of conventional autoradiographic ligand binding studies

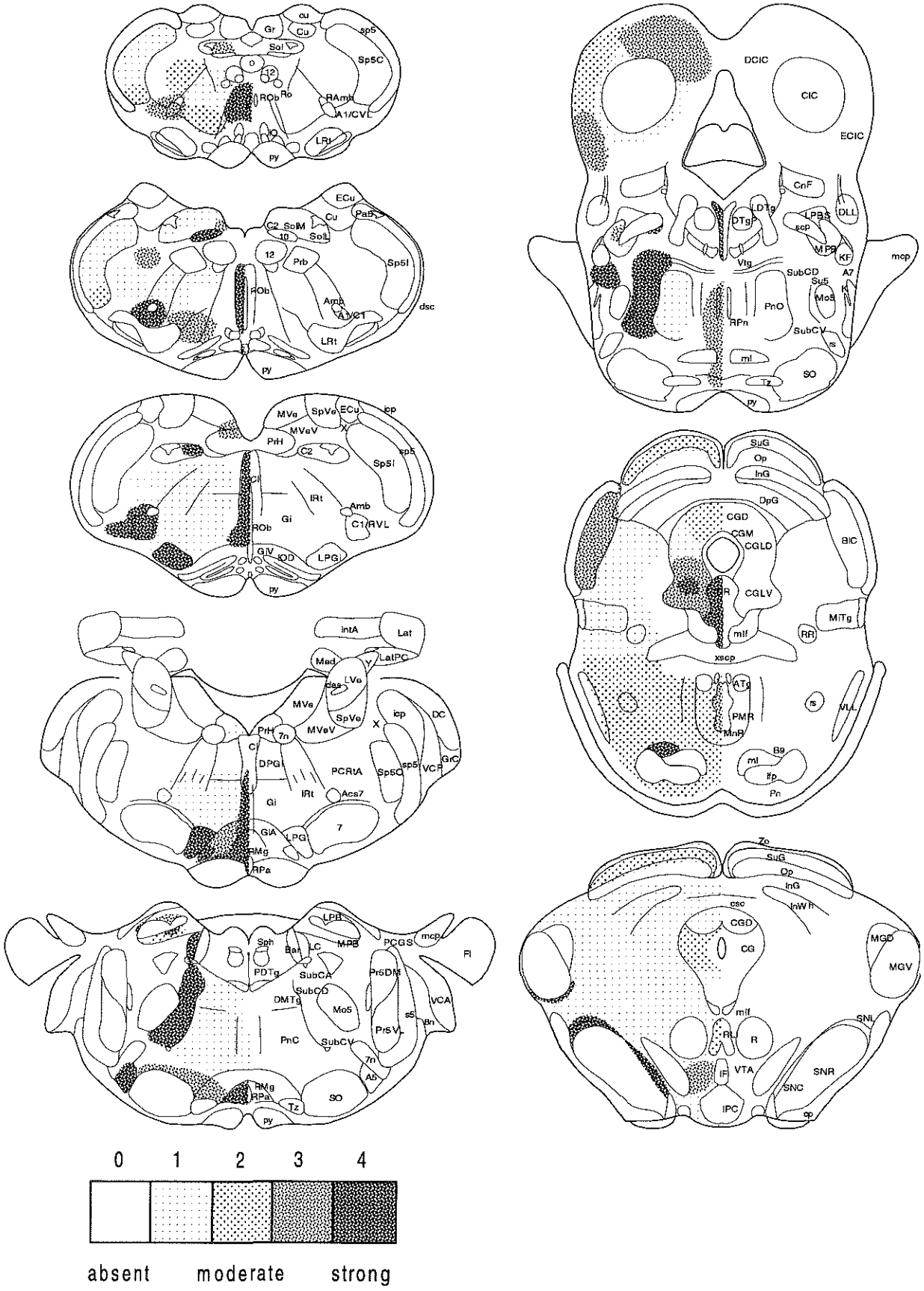


Figure 1: Schematic representation showing the distribution of neurons immunoreactive for dopamine D_2 receptor protein in the rat brain stem, including the medulla oblongata, the pons, and the mesencephalon caudal to the substantia nigra. For details see text; for abbreviations see enclosed list of abbreviations and table 1.

for localization of dopamine receptors is rather limited. Second, such methods lack the precision necessary to distinguish between receptor subtypes, mostly because of differences in the affinity and specificity of the ligands used (Boyson *et al.*, 1986; Dubois *et al.*, 1986; Bouthenet *et al.*, 1987; Meador-Woodruff *et al.*, 1991). As a result, the reported distributions of D_2-receptor ligand binding in the CNS were often inconsistent. On the other hand, recent *in situ* hybridization studies detected dopamine D_2-receptor mRNA with considerable reliability and reproducibility (Meador-Woodruff *et al.*, 1989; Savasta *et al.*, 1989; Bouthenet *et al.*, 1991; Meador-Woodruff *et al.*, 1991; Weiner *et al.*, 1991; van Dijken *et al.*, 1996), but provided little information about the distribution of the encoded receptor proteins. Moreover, the distribution and levels of protein may not match that of their mRNA's (Mansour *et al.*, 1990; Mansour *et al.*, 1992). Therefore, immunocytochemical methods may provide a better tool for the direct identification and localization of dopamine receptors in the brain. After discovery of the sequence of the D_2-receptor (Bunzow *et al.*, 1988) it became possible to prepare specific antibodies against the D_2-receptor, using fusion proteins or synthetic peptides corresponding to a specific part of the D_2-receptor (McVittie *et al.*, 1991; Plug *et al.*, 1992; Ariano *et al.*, 1993; Boundy *et al.*, 1993b; Boundy *et al.*, 1993a; Chazot *et al.*, 1993; Sesack *et al.*, 1994; Smiley *et al.*, 1994). In a similar manner antibodies against other dopamine receptors, including the D_1-, D_3- and D_4-receptors were prepared (Ariano and Sibley, 1994; Smiley *et al.*, 1994; Defagot *et al.*, 1997).

The distribution of D_2-receptors in the brain stem was examined in only a few studies, using receptor ligand binding (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994; Lawrence *et al.*, 1995) and/or *in situ* hybridization (Bouthenet *et al.*, 1991). These studies showed several brain stem areas that expressed D_2-receptors. In contrast, a previous immunocytochemical study (Levey *et al.*, 1993), using specific antibodies directed against dopamine D_2-receptor protein, disclosed only few regions in the brain stem with D_2-receptors, suggesting rather low levels of dopamine D_2-receptor protein in the hind brain as compared to forebrain. Similarly, in our previous study on the D_2-receptor in rat spinal cord it was noticed that the levels of D_2-receptors in spinal cord were low, both at the protein and mRNA level (van Dijken *et al.*, 1996). Owing to these low expression levels in hindbrain and spinal cord the total number of cells expressing dopamine D_2-receptors might be underestimated when using immunocytochemistry. This shortcoming may be (partly) overcome by refinement of the methods used to detect these low receptor protein levels. The recent introduction of powerful signal amplification methods based on deposition of biotinylated tyramine on the tissue through the enzymatic action of horseradish peroxidase (Adams, 1992), enabled investigators to substantially increase the sensitivity of their staining procedures. In the present study we have used this signal amplification method in order to obtain a higher yield of dopamine D_2-receptor staining in the rat brain stem.

Materials and methods

Immunocytochemistry

Adult, male Wistar rats were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 50 ml saline, followed by 500-750 ml of 4% (wt/vol) freshly prepared paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After perfusion, brain stems were removed immediately and stored overnight at 4 °C in phosphate buffer containing 25-30% (wt/vol) sucrose. Frozen sections (40 µm) were cut and collected in 0.1 M Tris buffered saline (pH 8.6). All subsequent procedures were performed on free-floating sections at room temperature, except where specified otherwise. Between steps, sections were thoroughly rinsed with Tris buffered saline. Sections were first incubated with a solution of 5% (vol/vol) normal goat serum (Gibco) in Tris buffered saline containing 0.3% (vol/vol) Triton X-100 for 90 minutes to reduce background staining. Next, sections were incubated for 24-48 hours at 4 °C with purified polyclonal D_2-receptor anti-peptide antibody from rabbit (Plug *et al.*, 1992; van Dijken *et al.*, 1996) diluted 1:1000-1:4000 in a solution of 1% (vol/vol) normal goat serum (Gibco) in Tris buffered saline containing 0.1% (vol/vol) Triton X-100. Then the sections were incubated for 120 minutes in biotinylated goat-anti-rabbit (Vector) diluted 1:200, followed by the ABC procedure (ABC Elite kit, Vector). After completion of the ABC procedure a tyramide signal amplification step (TSA-Indirect, NEN Dupont) was performed: sections were placed in biotinylated tyramide (1:200) for 10 minutes, thoroughly rinsed with Tris buffered saline, and incubated in streptavidin labeled conjugate (SA-HRP, 1:400) for 30 minutes at room temperature. In all sections, the horseradish peroxidase complex was visualized with 0.05% (wt/vol) 3,3'-diaminobenzidine (Sigma) in 0.05 M Tris-HCl solution (pH 7.6) in the presence of 0.01% (vol/vol) hydrogen peroxide, which yields a brown precipitate. After completion of the immunocytochemical procedure, sections were mounted onto glass slides with gelatin-chrome alum adhesive and allowed to dry at room temperature. Next, sections were dehydrated in graded ethanol baths, transferred to xylene, and cover slipped with Permount. In a few cases, slides were counter stained with cresyl violet. Sections were examined light microscopically with bright-field illumination.

Immunocytochemical controls

The controls that were used for the immunocytochemical procedure included (i) substitution of the anti-peptide antiserum with pre-immune serum in the same concentration, (ii) omission of the primary antiserum, (iii) adding an excess of antigen (1 mg/ml) to the antiserum prior to incubation, and (iv) omission of the tyramide amplification step. Omission of the primary antibody or substitution of pre-immune serum for the anti-peptide antibody resulted in clean sections without staining. Preadsorption of the antiserum with an excess of its peptide antigen prior to incubation prevented

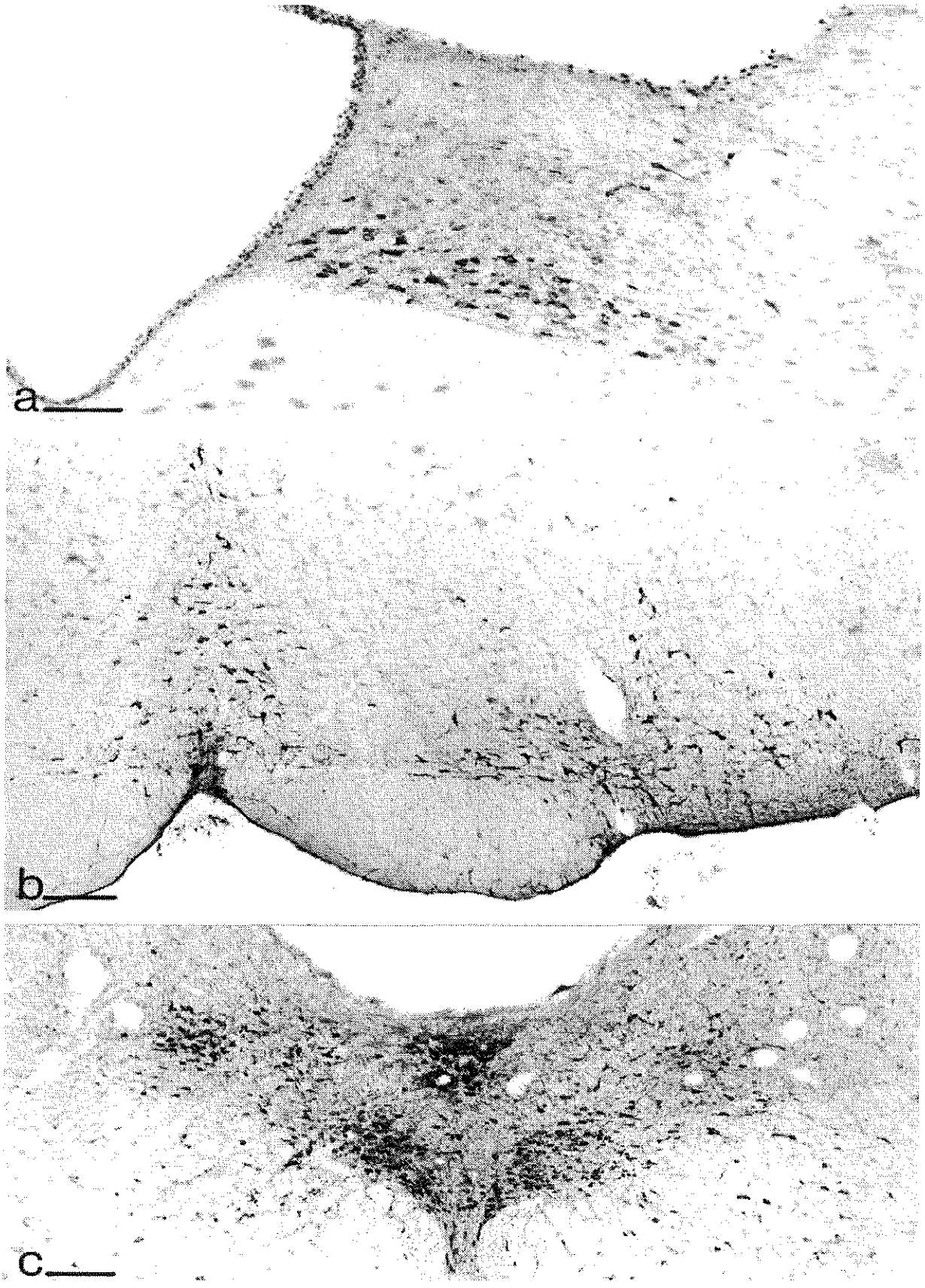


Figure 2: Light micrographs of dopamine D_2 receptor immunoreactivity in the rat brain stem. **A.** A large number of strongly labeled cells are present in the dorsal motor nucleus of the vagus with (at this level) only a few cells in the nucleus of the solitary tract. Scale bar = 100 µm. **B.** Labeled cells are present in the nucleus raphe magnus and pallidus. Laterally labeled cells are present in the lateral paragigantocellular nucleus. Scale bar = 200 µm. **C.** Strongly labeled cells are present in the dorsal raphe nucleus and adjacent parts of the ventrolateral central gray. Scale bar = 200 µm.

neuronal staining, except for some white matter glia, which is, therefore, considered as background staining. Omission of the signal amplification step resulted in a staining pattern which was nearly identical in regional distribution and cellular localization when compared to the staining after signal amplification, except that fewer cells were stained and most of them were weakly stained. We therefore concluded that the amplified immunocytochemical labeling of neuronal elements obtained in the present study represented D_2-receptor protein.

Results

General Observations

In the present study the anatomical localization of dopamine D_2-receptors in the rat brain stem was investigated by means of signal amplified immunocytochemistry. D_2-receptor labeling was associated primarily with neuronal cell bodies and their proximal dendrites. Occasionally dendritic processes could be followed for some distance within a section. The pattern of the immunocytochemical labeling was not suggestive for the presence of D_2-receptor in axons or terminals, although ultrastructural studies are needed to confirm this.

In several brain stem nuclei numerous strongly labeled cells were observed, but in most cases neuronal labeling was weak to moderate. Signal amplification resulted in darker staining of labeled neurons and disclosed additional neurons that stained weakly. In order to categorize the brain stem (sub)nuclei that showed neurons that were immunoreactive for the D_2-receptor, five relative densities were distinguished: level 0, labeling absent or uncertain; level 1, some moderately to weakly labeled neurons; level 2, many moderately to weakly labeled neurons; level 3, some strongly labeled and many moderately to weakly labeled neurons and level 4, many strongly labeled neurons. The results are schematically represented in fig. 1.

Medulla Oblongata

In the medial medulla oblongata, the most prominent labeling was observed in the dorsal motor nucleus of the vagus, where numerous strongly labeled neurons were observed (fig. 2a). The nucleus of the solitary tract, especially its caudal part, contained several strongly labeled cells (level 3), located mainly dorsomedially. At the "head" of the nucleus of the solitary tract strongly labeled cells were present in the presumed catecholaminergic C2 cell group (level 4). In the area postrema some moderately to weakly labeled cells were observed (level 2). Labeled cells were also observed in the periventricular gray (level 3, not shown in fig. 1). The hypoglossal nucleus was devoid of labeled cells, whereas some moderate to weakly labeled cells were present within the rostrally situated nucleus prepositus hypoglossus (level 1). Medially within the medulla oblongata, many moderately to weakly labeled cells were lining the 4th ventricle (level 3, not shown in fig. 1).

In the inferior olivary nucleus no labeled cells were observed. The vestibular complex was also devoid of D_2-labeled neurons, except for a few labeled neurons in the medial vestibular nucleus (level 1). The dorsal column nuclei (gracile, cuneate and external cuneate) were devoid of D_2-receptor labeling. Within the caudal part of the spinal trigeminal nucleus, a few prominently labeled cells (level 1-2) were observed, especially in the ventral part. Labeled cells in more rostral parts of the trigeminal nucleus were rare.

In the reticular formation, most of the strongly labeled cells were located ventrally. In the midline the serotonergic nuclei raphe obscurus, pallidus and magnus and the adjacent gigantocellular nucleus pars alpha (fig. 2b), contained many strongly labeled neurons (level 3-4). More laterally there were strongly labeled neurons in the lateral paragigantocellular nucleus and the adjacent ventral gigantocellular nucleus (level 4). In addition, the area of the (retro)ambiguus, contained many labeled neurons, which were observed close to, but not within, the nucleus ambiguus (level 4). The majority of these cells presumably belonged to the catecholaminergic A1 and C1 groups. The lateral reticular nucleus, which is also present in this area appeared devoid of D_2 labeling. Throughout the reticular formation labeled cells were seen, which occasionally formed small clusters (level 2), which were too small to be associated with a particular nucleus. In fig. 1 the reticular area containing these scattered labeled neurons is indicated as level 1.

Pons and mesencephalon

In this area, strongest labeling (level 3 - 4) was found in the serotonergic and noradrenergic cell groups. The serotonergic cell groups included the raphe nuclei (the raphe pontis, the median and paramedian raphe nuclei, and the dorsal raphe nucleus (fig. 2c) as well as the serotonergic cell group B9, as defined by Dahlström and Fuxe (1964). The labeled noradrenergic cell groups included the locus coeruleus and subcoeruleus (fig. 4), the A5 (fig. 3) and A7 cell groups, which all contained numerous strongly labeled cells (level 4).

In the pons, strongly labeled neurons were also present in the medial part of the medial parabrachial nucleus (level 4), whereas many weakly labeled neurons were observed within the rest of the medial parabrachial nucleus (level 2). The lateral parabrachial nucleus almost completely stained level 3. The oral part of the pontine reticular nucleus showed few labeled neurons (level 1). No labeled cells were observed in the area of the Kölliker-Fuse nucleus. The basal pons and the cerebellar nuclei were devoid of D_2 labeling.

In the mesencephalon, at levels caudal from the substantia nigra, strongest labeling was found in neurons located in specific regions of the periaqueductal gray, most prominently in its posterior (level 3) and dorsal (level 2) parts. Other areas of the mesencephalon containing labeled neurons included the inferior and superior colliculus and the nucleus of the brachium of the inferior colliculus. The superficial gray layer and the presumed optic nerve layer of the superior colliculus displayed level 1-2 staining, whereas the lateral parts of the superior colliculus were somewhat stronger labeled. Strongly labeled neurons were present

in the ventral tegmental area (level 3) and substantia nigra pars compacta (level 4; fig. 5), while no labeling was present within the substantia nigra pars reticulata. Strong labeling was also observed in the marginal zone of the medial geniculate nucleus (level 4; fig. 5). No labeled neurons were observed in the red nucleus.

Discussion

In the present study, the distribution of dopamine D_2-receptors has been examined in the rat brain stem using a purified polyclonal antiserum generated against the putative 3rd intracellular loop of the native receptor protein.

Methodological considerations

The observation that D_2-receptor immunoreactivity was located mainly within the somata and proximal dendrites of labeled neurons is in agreement with previous immunocytochemical studies (Brock *et al.*, 1992; Ariano *et al.*, 1993). At the electron microscopical level D_2-receptor immunoreactivity in neostriatal neurons was reported to be uniformly present throughout the cytoplasm (Fisher *et al.*, 1994; Hersch *et al.*, 1995) and did not appear to be associated with a particular cellular organelle. Only a small part of the labeling was associated with postsynaptic densities suggesting that the antibody preferentially recognized D_2-receptor protein before its incorporation as a functional receptor into the neuronal membrane or that the amount of cytoplasmic D_2-receptor protein is much higher than the amount of membrane incorporated protein. If this also holds true for the antibody used in the present study, it would explain the diffuse staining of cell somata and proximal dendrites.

It was noticed that, compared to the forebrain, the brain stem contained relatively few cells that were immunoreactive for the D_2-receptor and that most of them were not strongly labeled, suggesting that the level of D_2-receptors in the brain stem is low. Owing to these low expression levels the total number of cells expressing D_2-receptors may have been underestimated.

Anatomical distribution

The results of the present study showed that the dopamine D_2-receptor is expressed in several areas of the rat brain stem. Previous immunocytochemical studies on D_2-receptors in brain, did either not include the brain stem, or it was concluded that most hindbrain regions displayed no or little D_2-immunoreactivity (Levey *et al.*, 1993). The distribution of D_2-receptors in the brain stem was given attention only in one study (Brock *et al.*, 1992), which did not include the lower brain stem. This study described moderate densities of D_2-receptor protein in several areas of the upper brain stem, including the substantia nigra pars compacta, central gray area, medial geniculate nucleus, superior colliculus (superficial layers), inferior colliculus and periaqueductal gray area. Other studies on the regional distribution of D_2-receptors in brain, which have included the brain stem, mostly used receptor ligand binding (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994; Qian *et al.*, 1997) or *in situ* hybridization (Bouthenet *et al.*, 1991).

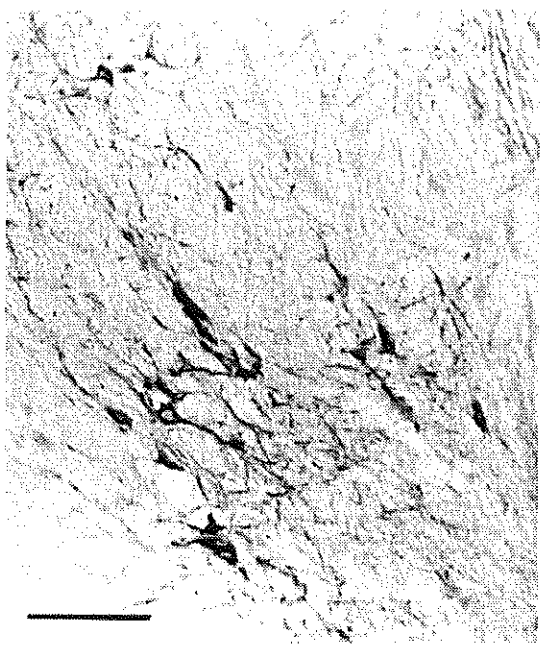


Figure 3: Light micrograph showing cells in the A5 noradrenergic cell group that are strongly immunoreactive for the dopamine D_2 receptor. Scale bar = 100 µm.

In table 1 the available data on the distribution of dopamine D_2-receptor protein and its mRNA as well as ligand binding data are compared with the results of the present study. In most areas of the brain stem there is a high degree of overlap between the distribution of D_2-receptors obtained with immunocytochemistry and the distributions obtained with ligand binding and *in situ* hybridization. Brain stem areas containing D_2-receptor protein, D_2-receptor mRNA as well as functional binding sites are the dorsal motor nucleus of the vagus, the gigantocellular nucleus pars alpha, the nucleus of the solitary tract, the locus coeruleus, the lateral and medial parabrachial nuclei, the basal pontine nuclei, the periaqueductal gray, the deep mesencephalic nucleus, the inferior and superior colliculi, the substantia nigra pars compacta, and the ventral tegmental area. Since the presence of D_2-receptors in the aforementioned brain stem nuclei was suggested by several different detection techniques, it is highly probable that D_2-receptors are actually present in these areas and play an important functional role.

In some areas there were obvious discrepancies between the distribution of dopamine D_2-receptor protein, obtained with immunocytochemistry and the distribution obtained with ligand binding or *in situ* hybridization. In these cases it must be kept in mind that the binding pattern may differ from the cellular distribution obtained with immunocytochemistry, because the functional D_2-receptors, i.e. the binding sites, may be located on dendrites or the terminal axons of neurons which have their cell somata elsewhere in the brain stem. On the other hand, the ligands may also bind to other D_2-like receptors, due to a lack of pharmacological selectivity that is inherent to the

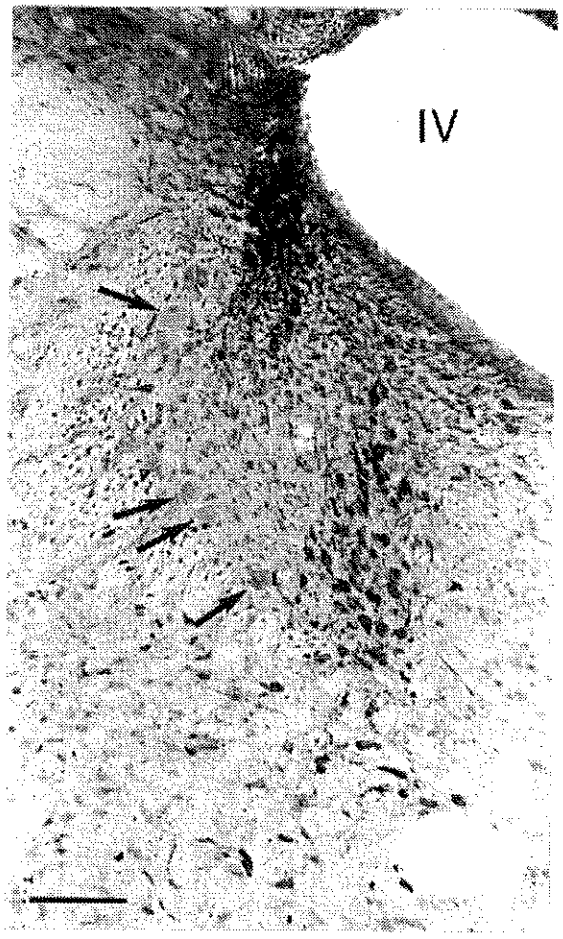


Figure 4: Light micrograph showing neurons strongly immunoreactive for the dopamine D_2 receptor. Many densely packed neurons are present in the locus coeruleus, close to the aqueduct (IV). More ventrally, labeled neurons are present in the nucleus subcoeruleus. Arrows point at neurons of the trigeminal mesencephalic tract that are unlabeled. Scale bar = 100 µm.

receptor ligand binding technique. Comparison of our immunocytochemical results with the available data on D_2-receptor ligand binding reveals that the distribution of D_2-protein mostly resembles that of the highly selective D_2-antagonist ligand YM-09151-2 (Yokoyama *et al.*, 1994; see also table 1), extending the results of earlier studies on the distribution of D_2-receptor in the rat brain stem (table 1). The additional areas in which D_2-receptor protein and YM-09151-2 binding was observed include the reticular formation, the raphe magnus, the raphe pallidus, the dorsal and the median raphe nuclei. In other areas, like the cuneate and the inferior olivary nuclei, D_2-receptor protein is absent and YM-09151-2 binding is either weak or not described. In the latter areas these results are in contrast with the results of other ligand binding (Bouthenet *et al.*, 1987; Qian *et al.*, 1997) and *in situ* hybridization (Bouthenet *et al.*, 1991) studies, which describe weak to high signals. Although there is a possibility that, due to a lack of pharmacological selectivity, D_2-receptor ligands also bind to dopamine D_3-receptors, which are present in the inferior olive (Bouthenet *et al.*, 1991), it is also possible that in this region the concentration of D_2-receptor protein is very low and, therefore, undetectable with immunocytochemistry. Finally, it should be mentioned that the existence of strain differences in the distribution of dopamine D_2-receptor distribution in rat cannot be excluded, since different strains of rats were used in the studies that were compared.

It is concluded that most of the brain stem areas described contain functional D_2-receptors, as indicated by the presence of mRNA, protein, and ligand binding. There are discrepancies between the results of different techniques in a few brain stem areas, which can be partially explained by the nature of the different detection methods used.

Functional implications

The existence of a dopaminergic projection to the nucleus tractus solitarius and dorsal motor nucleus of the vagus is now well established (see e.g. van Dijken and Holstege, this thesis). Studies that focused on the functional role of dopamine in the nucleus tractus solitarius (Zandberg *et al.*, 1979; Granata and Woodruff, 1982; Kessler and Jean, 1986; van Giersbergen *et al.*, 1992) have reported opposite effects of dopamine on heart rate and blood pressure. The presence of high densities of D_2-receptors in the nucleus tractus solitarius indicates that the effects of dopamine are, in part, mediated by D_2-receptors. In accordance, microinjection of the D_2-receptor agonist (quinpirole) into the posterior region of nucleus tractus solitarius caused a consistent increase in mean arterial pressure (Yang *et al.*, 1990), suggesting that D_2-receptors are especially involved in producing an increase in blood pressure. On the other hand, microinjection of dopamine into the (right) nucleus ambiguus elicits a dose-dependent decrease in heart rate, which is mediated by excitation of vagal preganglionic cardioinhibitory neurons. This effect was blocked by a D_2-receptor specific antagonist (sulpiride) and not by a D_1-receptor antagonist (SCH-23390) (Chitravanshi and Calaresu, 1992). The presence of D_2-receptor containing neurons in the area of the nucleus ambiguus, as found in the present study, may explain these effects. The above data show that the effects of dopamine on cardiovascular functions are complex and occur at different brainstem sites. The exact role of D_2-receptors in producing cardiovascular effects are still unclear.

Within the trigeminal complex, cells containing D_2-receptor protein were few and located exclusively in the caudal part of the spinal trigeminal nucleus. The cytoarchitectonic organization of this area is very similar to that of the spinal dorsal horn, with which it is continuous. Both areas are involved in the processing sensory information, in particular pain and temperature. In the spinal dorsal horn D_2-receptors are present in the dorsal horn (van Dijken *et al.*, 1996) and their agonists exert an inhibitory effect on the transmission of nociceptive inputs (Barasi *et al.*, 1987; Fleetwood-Walker *et al.*, 1988; Liu *et al.*, 1992), whereas D_1-

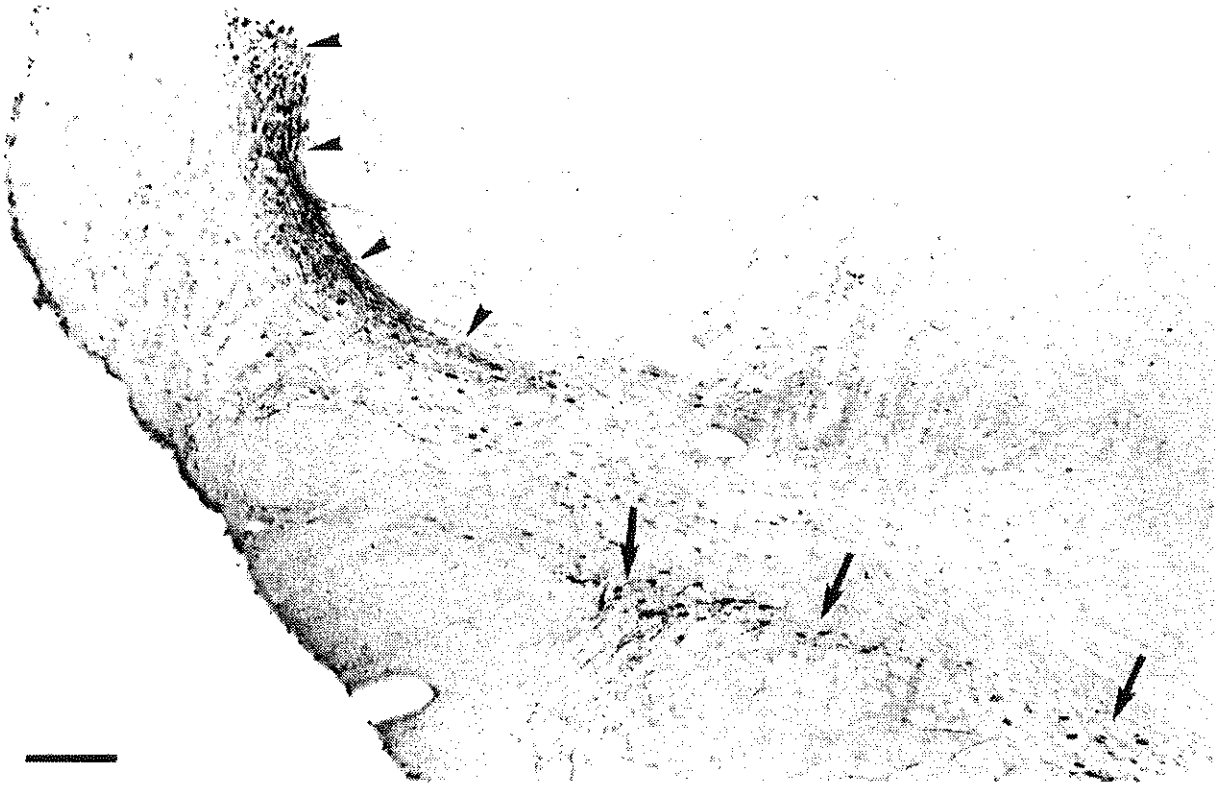


Figure 5: Light micrograph showing neurons strongly immunoreactive for the dopamine D_2 receptor. Arrows point at strongly labeled neurons in the substantia nigra, pars compacta. The marginal zone of the medial geniculate nucleus is also strongly labeled (arrowheads). Scale bar = 200 µm.

receptor agonists are without effect (Barasi *et al.*, 1987; Liu *et al.*, 1992). The distribution of dopamine (van Dijken and Holstege, this thesis) and its D_2-receptor (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994; Lawrence *et al.*, 1995) in the caudal spinal trigeminal nucleus is similar to that of the dorsal horn. Therefore it seems likely that the D_2-receptors in the caudal spinal trigeminal nucleus are involved especially in modulating the transmission of pain and temperature impulses derived from the head and neck region.

Dopaminergic fibers are densely distributed in the principal nucleus of the inferior olivary nucleus and its ventrolateral outgrowth (van Dijken and Holstege, this thesis). In the present study D_2-receptor protein was undetectable in the inferior olive, suggesting a more prominent role for dopamine D_1-receptors. However, D_1-receptor (SCH-23982) binding appears not to be present in the inferior olive (Qian *et al.*, 1997). Furthermore, weak to high D_2-receptor binding (Bouthenet *et al.*, 1987; Yokoyama *et al.*, 1994; Qian *et al.*, 1997) and *in situ* hybridization signals (Bouthenet *et al.*, 1991) were found in the inferior olivary nucleus of rat, especially in its central and medial part (Lawrence *et al.*, 1995). Since no further details were given and the possibility of strain differences cannot be excluded, the presence of D_2-receptors in the inferior olivary nucleus remains unsettled. A number of studies have indicated a possible interaction between dopamine and the vestibular system (see e.g. Hozawa and Takasaka, 1993). In the present study, only weak D_2-receptor protein staining was observed in the vestibular complex, mainly represented by a few stained cells in the medial vestibular nucleus. This is in accordance with a study in guinea-pig brain slices (Vibert *et al.*, 1995) showing that medial vestibular nucleus neurons depolarized after application of dopamine as well as after application of selective D_2-receptor agonists, while application of selective D_1-receptor agonists had no effects on neurons located within the medial vestibular nucleus.

In the pons, the most prominent labeling was found in the noradrenergic locus coeruleus and subcoeruleus (see below) and the parabrachial nuclei. It has been proposed that part of the catecholaminergic innervation of the lateral parabrachial nucleus is dopaminergic (Hedreen, 1980), which we have recently confirmed (van Dijken and Holstege, this thesis). The presence of D_2-receptors in the parabrachial nuclei further substantiates the involvement of dopamine in the processing of information in this area.

In the mesencephalon, D_2-receptor was found in the substantia nigra pars compacta, periaqueductal gray, inferior and superior colliculi and dorsal raphe. In the dorsal raphe nucleus (B6 and B7 areas) ligand binding density is fairly high for D_2-receptors, but not for D_1-receptors (Bouthenet *et al.*, 1987). Microdialysis showed an inhibitory dopaminergic control of ascending serotonergic pathways, mediated by D_2-receptors at the somatodendritic level in the dorsal

raphe nucleus (Ferré *et al.*, 1994). The results of the present study further emphasize the role of D_2-receptors, which are abundantly present within the dorsal raphe nucleus.

Monoaminergic cell groups in the brain stem

Noradrenergic and serotonergic cell groups in the brain stem usually show a prominent network of dopaminergic fibers and terminals around cell bodies and dendrites (van Dijken and Holstege, this thesis). In the present study neurons containing dopamine D_2-receptors were observed in many monoaminergic cell groups, including the serotonergic raphe nuclei (raphe pallidus, raphe obscurus, raphe magnus, raphe centralis, and raphe dorsalis) and the noradrenergic locus coeruleus and subcoeruleus, as well as the A1, A5 and A7 cell groups. These findings are in agreement with reports showing D_2-receptor binding (Bouthenet *et al.*, 1987; Bouthenet *et al.*, 1991; Yokoyama *et al.*, 1994) and D_2-receptor mRNA (Bouthenet *et al.*, 1991) in several of these areas.

The existence of a substantial dopaminergic innervation of the monoaminergic cell groups in the brain stem, and more particularly the presence of D_2-receptors, suggests that dopamine interacts with other monoaminergic cell groups, not only at the level of their terminal fields, but also by directly affecting the activity of the parent cell bodies of the monoaminergic fibers.

Conclusion

The present study, together with previous studies using different techniques, further establishes the presence of D_2-receptors in specific regions of the rat brain stem. Although prominent, the distribution of D_2-receptors in the brain stem is not as extensive as in other parts of the brain, more particularly the forebrain. The specific distribution of D_2-receptors supports the involvement of dopamine in the modulation of many brain stem functions, including the interaction with other monoaminergic systems. In view of the widespread dopaminergic innervation of the brain stem, it seems likely that the effects of dopamine on brain stem functions depend not only on D_2-receptors but also on other types of dopamine receptors. Detailed knowledge about their distribution in the brain stem is still incomplete.

Acknowledgments

The authors thank Prof. Dr. Jan Voogd for reading the manuscript. We thank Erika Sabel-Goedknegt and Richard Hawkins for their technical assistance and Eddie Dalm for his help with the (digital) photography.

Table 1: A comparison of the relative densities of dopamine D_2-receptor labeling in the brain stem, obtained with different labeling techniques. Data from present study (D_2 protein); Qian *et al.*, 1997 ([^{125}I]NCQ-298, fmol/mg); Yokoyama *et al.*, 1994 ([^{3}H]YM-09151-2, fmol/mg); Brock *et al.*, 1992 (D_2 protein); Bouthenet *et al.*, 1991 (D_2 mRNA); Bouthenet *et al.*, 1991 ([^{125}I]iodosulpiride), respectively.

I. Medulla Oblongata		D_2 protein	[^{125}I]NCQ-298		[^{3}H]YM-09151-2		D_2 protein	D_2 mRNA	[^{125}I]iodo sulpiride
10	Dorsal motor nucleus of the vagus	4+	3+	(7.1)	3+	(36)		3+	1+
12	Hypoglossal nucleus	0	0	(0)	4+	(51)			
Cu	Cuneate nucleus	0	2-4+	(5.0-8.9)				4+	3+
DC	Dorsal cochlear nu	0	0-1+	(2.2-3.4)				1+	2+
ECu	External cuneate nu	0	0	(0)				1+	1+
GiA	Gigantocellular reticular nu, alpha	3+			2+	(21)		2+	1+
IO	Inferior olive	0	4+	(9.5)	1+	(16)		4+	2-4+
MdD	Medullary reticular nu, dorsal part	1+	2+	(4.2)				3+	3+
MdV	Medullary reticular nu, ventral part	1+	1+	(1.9)				3+	3+
MVe	Medial vestibular nu	0						2+	0
MVPO	Medioventral preolivary nu							1+	0
RF	Reticular formation of medulla oblongata	1-2+			1+	(12)			
RMg	Raphe magnus nu	4+			2+	(19)			
RPa	Raphe pallidus nu	4+			2+	(28)			
Sol	Nucleus of the solitary tract	2-3+	1-3+	(3.1-7.1)	2+	(27)		2+	3+
SPO	Superior paraolivary nu							2+	0
SO	Superior olive	0			1+	(10)			
Sp5C	Spinal trigeminal, caudal part	1-2+	2-3+	(3.6-6.7)					
Sp5	Spinal trigeminal, medial/lateral part		3-4+	(7.4-8.8)					
Sp5I	Spinal trigeminal, interpolar part	0	3+	(6.1)					
AP	Area postrema	2+	2+	(4.0)					

II. Pons		D_2 protein	[^{125}I]NCQ-298	[^{3}H]YM-09151-2		D_2 protein	D_2 mRNA	[^{125}I]iodo sulpiride
n7	Facial nu	0		2+	(31)			
Amb	Nucleus ambiguus	0		2+	(28)			
Co	Cochlear nu	0		1+	(10)			
LC	Locus coeruleus	4+		2+	(31)		3+	4+
LPB	Lateral parabrachial nu	3+					3+	3+
LVe	Lateral vestibular nu	0		1+	(16)			
Mo5	Motor trigeminal nu	0		2+	(29)			
Me5	Mesencephalic trigeminal nu	0					3+	2+
MPB	Medial parabrachial nu	3-4+					2+	3+
MVe	Medial vestibular nu	0		1+	(17)			
PnC	Pontine reticular nu, caudal part	1+					2+	2+
PnO	Pontine reticular nu, oral part	1+					2+	2+
Pr5	Principal sensory nu	0		1+	(11)			
Sp5	Spinal trigeminal nu	1-2+		1+	(20)			

Table 1: *(continued)*

III. Mesencephalon		D_2 protein	[^{125}I]NCQ-298	[^{3}H]YM-09151-2		D_2 protein	D_2 mRNA	[^{125}I]iodo sulpiride
n3	Oculomotor nu	0		3+	(37)			
CG	Central gray	2-3+		2+	(19)	Moderate	3+	3+
Cli	Caudal linear nu of raphe			2+	(23)			
DpMe	Deep mesencephalic nu	1+					3+	3+
DR	Dorsal raphe nucleus	4+		4+	(51)			
IC	Inferior colliculus (external cortex)	2-3+		2+	(24)	Moderate	3+	2+
IF	Interfascicular nu (A10)	0		2+	(31)			
IPR	Interpeduncular nu rostral			4+	(46)			
IPC	Interpeduncular nu caudal			1+	(18)			
MG	Medial geniculate nu (ventral border)	4+				Moderate		
MnR	Median raphe nu	3+		1+	(13)			
OT	Nu of the optic tract						4+	3+
Rli	Rostral linear nu of raphe	2+		1+	(11)			
RN	Red nu	0		1+	(14)			
RRF	Retrorubral field (A8)			1+	(14)		4+	2+
SNC	Substantia nigra compacta (A9)	4+		3+	(40)	Dense	4+	4+
SNL	Substantia nigra compacta, lateral part	4+					0	4+
SNR	Substantia nigra reticulata	0		1+	(9)			
Su	Superior colliculus (superficial grey layer)	2+		2+	(25)	Moderate	3+	3+
VTA	Ventral tegmental area (A10)	3+		1+	(12)	Moderate	4+	4+

Summary and General Discussion

Summary and General Discussion

Summary

This thesis, which describes an anatomical study of the dopamine innervation of the brain stem and spinal cord and one of the receptors involved, may be regarded as an anatomical study of a chemically identified system, i.e. characterized by using the transmitter dopamine.

In *Chapter I* these aspects are described separately: first a short description of the anatomy of the spinal cord and brain stem is given, followed by a description of the chemical anatomy of the nervous system, with the emphasis on the dopaminergic system and its receptors.

Chapter II describes the distribution of dopaminergic fibers and (presumed) terminals in all segments of the rat, cat and monkey spinal cord as identified by light microscopy immunocytochemistry using antibodies directed against dopamine.

Strongest dopamine labeling was present in the sympathetic intermedio-lateral cell column (IML). Strong dopamine labeling, consisting of many varicose fibers, was found in all laminae of the dorsal horn, including the central canal area (region X), but with the exception of the substantia gelatinosa, which was only sparsely labeled, especially in rat and monkey. In the motoneuronal cell groups dopamine labeling was also strong, showing a fine granular appearance. The sexually dimorphic cremaster nucleus and Onuf's nucleus (or its homologue) showed a much stronger labeling than the surrounding somatic motoneurons. In the parasympathetic area at sacral levels, labeling was moderate. The remaining areas, like the intermediate zone (lamina VI-VIII), were only sparsely innervated. The dorsal nucleus (column of Clarke) showed the fewest dopamine fibers as did the central cervical nucleus, suggesting that cerebellar projecting cells were avoided by the dopamine projection. In all species, the descending fibers were located mostly in the dorsolateral funiculus, but laminae I and III also contained many rostro-caudally oriented fibers.

It is concluded that dopamine is widely distributed within the spinal cord, with few differences between species, emphasizing that dopamine plays an important role as one of the monoamines that influences sensory input as well as autonomic and motor output at the spinal level

Chapter III describes the distribution of dopamine D_2-receptors in rat spinal cord as determined by means of immunocytochemistry using an anti-peptide antibody, directed against the putative 3rd intracellular loop of the D_2-receptor, and by means of *in situ* hybridization (ISH) using a [^{35}S]UTP labeled anti-sense riboprobe.

With the immunocytochemical technique, labeling was confined to neuronal cell bodies and their proximal dendrites. Strongest labeling was present in the parasympathetic area of the sacral cord and in two sexually dimorphic motor nuclei of the lumbosacral cord, the spinal nucleus of the bulbocavernosus, and the dorsolateral nucleus. Moderately labeled cells were present in the intermediolateral cell column, the area around the central canal and lamina I of the dorsal horn. Weak labeling was present in the lateral spinal nucleus and laminae VII and VIII of the ventral horn. Except for the two sexually dimorphic motor nuclei of the lumbosacral cord labeled motoneurons were not encountered.

With the ISH technique radioactive labeling was present in many neurons, indicating that they contained D_2-receptor mRNA. The distribution of these neurons was very similar to the distribution obtained with immunocytochemistry, but with ISH additional labeled cells were detected in laminae III and IV of the dorsal horn, which were never labeled with immunocytochemistry.

Thus, the expression of D_2-receptor in specific areas of the rat spinal cord provides anatomical support for the involvement of D_2-receptors in modulating nociceptive transmission and autonomic control. Our data further indicate that D_2-receptors are not directly involved in modulating motor neurons with the exception of those located in the sexually dimorphic motor nuclei.

Chapter IV describes the distribution of dopamine immunoreactive fibers and (presumptive) terminals as identified at the light microscopical level in the rat brain stem. The distribution of dopamine immunoreactivity is compared with that of dopamine-β-hydroxylase (DBH), the (nor)adrenaline synthesizing enzyme.

The dopaminergic fibers and terminals showed a specific distribution within the brain stem. They were present in many brain stem nuclei with various labeling densities. Strongest dopamine labeling was found in the dorsal motor nucleus of the vagus, principal nucleus of the inferior olive and dorsal raphe nucleus. Other areas, like the nucleus of the solitary tract, raphe pallidus and obscurus, lateral parabrachial nuclei, and the periaqueductal gray, were strongly labeled, whereas the sensory trigeminal complex, the medial parabrachial nucleus, the locus coeruleus and the noradrenergic A5 cell group were labeled moderate to strong. Furthermore, dopamine immunoreactive fibers and terminals were present in many other areas of the brain stem, including the inferior and superior colliculi, deep mesencephalic nuclei, pontine reticular nuclei, prepositus hypoglossal nuclei, cochlear nuclei, cuneate and gracile nuclei. In most nuclei, dopamine was not distributed randomly, but aimed at specific subnuclei. DBH containing fibers and terminals were distributed more ubiquitously throughout the brain stem. In most areas the distribution of DBH labeling overlapped with the dopamine labeling, but the regional innervation pattern differed. In other areas like the area postrema, cerebellum, anterior portion of the ventral cochlear nucleus and external cuneate nucleus, which were all

sparsely innervated by dopaminergic fibers, the (nor)adrenaline synthesizing enzyme was abundantly present.

These results demonstrated that there exist an extensive dopaminergic fibers system in the rat brain stem, which is independent from the (nor)adrenergic fiber system. As a consequence, the influence of dopamine in the brain stem should be considered of equal importance to other monoamines like (nor)adrenaline and serotonin.

Chapter V describes the distribution of dopamine D_2-receptors in rat brain stem as determined by signal amplified immunocytochemistry using a polyclonal antiserum with antibodies directed against the putative 3rd intracellular loop of the dopamine D_2-receptor. The labeling was mostly associated with neuronal cell bodies and their proximal dendrites, regardless whether amplification was used or not. Neurons containing D_2-receptor protein were observed throughout the rat brain stem in specific regions, like the dorsal motor nucleus of the vagus, the gigantocellular nucleus pars alpha, the nucleus of the solitary tract, the locus coeruleus, the lateral and medial parabrachial nuclei, the basal pontine nuclei, the central gray, the deep mesencephalic nucleus, the inferior and superior colliculi, substantia nigra pars compacta, and ventral tegmental area. Furthermore, additional brain stem nuclei were observed that contained D_2-receptor protein, including the reticular formation and all raphe nuclei. In other brain stem areas, like the cuneate and the inferior olivary nuclei, D_2-receptor protein was undetectable, indicating that in these regions of the brain stem the concentration of D_2-receptor protein may be very low and, therefore undetectable with immunocytochemistry. These results are in general agreement and further extend the results of previous studies on D_2-receptor in rat brain stem, using the ligand binding or *in situ* hybridization technique.

The widespread distribution of D_2-receptors in the rat brain stem provides anatomical support for the involvement of D_2-receptors in modulating a large variety of brain stem functions, further emphasizing the important role of the dopamine system in the brain stem.

Chapter VI discusses the anatomical and functional significance of the dopaminergic system in brain stem and spinal cord. It is concluded that the present thesis definitively establishes the existence of a widespread dopaminergic neurotransmission in the brain stem and spinal cord, independent from the other monoaminergic neurotransmitters in which dopamine acts as a precursor. Dopamine projections are found in nearly all the sensory information processing areas, motoneuronal cell groups and many areas that subserve various autonomic functions, suggesting that dopamine is involved in various brain stem and spinal cord functions. Its distribution further suggests that dopamine interacts with the other monoaminergic cell groups, not only at the level of the terminal fields in the target areas, but also by directly affecting the activity of the parent cell bodies of the monoaminergic fibers. The high degree of collateralization in the dopamine diencephalo-spinal projection implies that dopamine is released simultaneously in many different areas of the brain stem and spinal cord, which suggest that individual A11 neurons subserve integrative functions. The role of dopamine in different behavioral states like fight and flight and sexual behavior are discussed. At present, there is no evidence that A11 plays a role in Parkinson's disease.

Taken together the present thesis emphasizes that besides noradrenaline and serotonin, dopamine should also be included when considering the monoaminergic effects on brain stem and spinal processing. In fact, the interaction between the various monoamines may be of even greater importance than the effect of the respective transmitters alone. Investigations along this line may prove worthwhile.

General Discussion

Introduction

The studies presented in this thesis provide a comprehensive description of the localization of the neurotransmitter dopamine and one of its major receptors, the dopamine D_2-receptor, in the brainstem and spinal cord. Chapter II and IV describe the existence of a distinct and extensive dopaminergic innervation of specific areas of the brainstem and spinal cord. In chapter III and V the localization of D_2-receptors in the spinal cord and brainstem is described in detail. Together these studies establish dopamine as a distinct and extensive neurotransmitter system in the brainstem and spinal cord and provide the anatomical basis for detailed studies on the involvement of dopamine in the modulation of the many functions that are organized in brain stem and spinal cord. Below, the various aspects of the dopaminergic innervation as described in this thesis will be discussed, including the methodological aspects, the interaction of dopamine with other transmitters and its possible roles in the various functions of the spinal cord and brainstem.

Methodological aspects

Dopamine

Like most of the identified neurotransmitter systems in the brain (see e.g. Nieuwenhuys, 1985) dopamine is widely distributed throughout the brainstem and spinal cord, although the density of the dopamine innervation is generally less than that of (nor)adrenaline and serotonin, the distribution is generally similar (table 1). For a long time it has been difficult to demonstrate that dopamine in the brain stem and spinal cord functions as a neurotransmitter in its own right and not only as a precursor for the biosynthesis of the catecholamines noradrenaline and adrenaline. Theoretically, the possibility exists that noradrenergic and adrenergic fibers and terminals contain levels of precursor dopamine that are detected by dopamine immunocytochemistry. As a consequence, these fibers would be falsely identified as dopaminergic, i.e. using

dopamine as a transmitter. However, several lines of evidence suggest that the large majority of the dopamine immunoreactive fibers and terminals identified in the brain stem and spinal cord are indeed dopaminergic.

Firstly, it was shown in biochemical studies of areas with a high noradrenaline and a low dopamine innervation, like the cerebellum and lateral hippocampus, that the dopamine concentration was only 1-3% of the noradrenaline concentration (Commissiong *et al.*, 1978; Westerink and Mulder, 1981; Westerink and de Vries, 1985; Verhage *et al.*, 1992). Destruction of the locus coeruleus, and consequently the noradrenaline innervation of several brain areas, including the cerebellum and hippocampus, had no effect on dopamine levels in those areas (Westerink and De Vries, 1985). Thus, even in areas with a relatively high noradrenaline and a low dopamine concentration, dopamine represents a "transmitter pool" rather than a "precursor pool" of noradrenaline, as confirmed by additional biochemical and/or anatomical studies in cerebellum (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992) and hippocampus (Verney *et al.*, 1985; Verhage *et al.*, 1992; Gasbarri *et al.*, 1994). In this thesis areas of the brain stem with a relatively dense DBH immunoreactive innervation (like the cerebellum, the area postrema, the anterior portion of the ventral cochlear nucleus, and the external cuneate nucleus, see also table 1) showed virtually no dopamine immunoreactive fibers and terminals. This finding showed that a dopamine "precursor pool" in (nor)adrenergic terminals could not be identified by the dopamine antibody. The most probable explanation for this observation is the very high turnover rate of dopamine in (nor)adrenergic terminals, leading to very low "free" dopamine levels in these terminals.

Secondly, in our control experiments with the neurotoxin DSP-4, which affects a specific subset of noradrenergic fibers and terminals, a selective loss of DBH staining was observed in brain stem and spinal cord, due to the degeneration of noradrenergic fibers (Fritschy and Grzanna, 1989). In alternate sections, however, the dopamine immunoreactivity remained unaffected by the drug, showing that the immunoreactive dopamine was not present in noradrenergic fibers. Similarly, the use of the neurotoxin 6-OHDA, which causes a specific degeneration of noradrenergic terminals, resulted in the disappearance of noradrenaline immunoreactivity from the spinal cord of rats, sparing the dopamine immunoreactive fibers (Mouchet *et al.*, 1992). These selective lesion studies using the neurotoxins DSP-4 and 6-OHDA thus indicate that precursor dopamine in noradrenergic fibers and terminals is not detected with the immunocytochemical method used in the studies of this thesis. Alonso *et al.* (1995), using a IgG monoclonal antibody against dopamine, confirmed that in most brainstem nuclei precursor dopamine is undetectable by dopamine immunocytochemistry. However they also reported that precursor dopamine can actually be detected by immunohistochemistry in areas that receive a very dense catecholaminergic innervation and are not, or only partially, affected in lesion studies using a catecholamine-selective neurotoxin (regions like the locus coeruleus, the dorsal motor nucleus of the vagus, the area postrema, the nucleus of the solitary tract and the hypoglossal nucleus). Thus, in some areas with a dense noradrenergic and dopaminergic innervation, some dopamine immunoreactivity may have been produced by noradrenergic terminals. The existence of a dopaminergic transmitter system in areas that contain dopamine fibers is supported by the presence of dopamine D_1 and/or D_2 dopamine receptors (Bouteneth *et al*, 1987; Bouteneth *et al*, 1991; Yokoyama, 1994) in the large majority of those areas. Although mismatches between the distribution of a transmitter and its receptor occur (see below), it seems most likely that areas that express dopamine receptors also receive a dopaminergic innervation. In addition, the presence of dopamine transporters, i.e. molecules that are involved in the transmembrane transport of dopamine, may also be used to indicate the presence of a dopaminergic neurotransmission. There are two basic transporters that are essential for normal dopamine neurotransmission: the vesicular monoamine transporter (in rat: VMAT2) and the plasma membrane dopamine transporter (DAT) (see Hoffman *et al.*, 1998 for review). VMAT2 loads cytoplasmic dopamine into vesicles for storage and subsequent release. However VMAT2 is not specific for dopamine but also transports other monoamines. Thus in dopaminergic terminals VMAT2 is used to load vesicles with dopamine, while in noradrenergic terminals VMAT2 is used to load vesicles with noradrenaline. Therefore VMAT2 cannot be used to differentiate between monoaminergic fibers and is therefore not suitable to specifically identify dopaminergic fibers. In contrast, DAT, which takes up dopamine from the intercellular space by transporting it into the neuronal cytoplasm, is specific for dopamine and does not transport any of the other monoamines. DAT is considered to play an important role in the termination of the actions of dopamine after its release by rapidly removing dopamine from the intercellular space, including the synaptic cleft. Thus, DAT may be used to specifically identify dopaminergic fibers. However, studies using *in situ* hybridization combined with tyrosine hydroxylase immunocytochemistry have shown that, in contrast to the majority of the dopaminergic cell groups, A11 dopaminergic neurons do not express DAT mRNA (Lorang *et al.*, 1994) nor DAT protein (Ciliax *et al.*, 1995; 1999). Since the dopaminergic projection to the brain stem and spinal cord largely originates from the A11 group, DAT immunoreactive fibers are not encountered in these brain regions. As a consequence, the localization of VMAT2 or DAT cannot be used to verify the presence of dopaminergic innervation in brainstem and spinal cord systems, as studied in this thesis.

It may thus be concluded that virtually all dopamine immunoreactive fibers and terminals, detected in the present study, contained dopamine, not as a precursor, but as a neurotransmitter. However, it can not be completely excluded that in a few areas dopamine

immunoreactivity was produced by dopamine in noradrenergic terminals. Although our control experiments with DSP-4 have shown that it is unlikely that this methodological problem has in any way compromised our results, it cannot be completely excluded that in a minority of the fibers in a few areas immunoreactivity was produced by dopamine in noradrenergic terminals.

Dopamine D_2 receptor

The amount of dopamine in the brain stem and spinal cord is generally low, especially in comparison with dopamine levels in the forebrain (Westerink and de Vries, 1985; Batten *et al.*, 1990). Similarly, a comparison of the available data on dopamine receptor levels (Boyson *et al.*, 1986; Dubois *et al.*, 1986; Yokoyama *et al.*, 1994) indicates that the levels of dopamine D_1- and D_2-receptors are 5-20 times lower than those measured in the forebrain. The amount of dopamine D_3-receptors in the central nervous system appears to be several orders of magnitude lower than that of D_2-receptors and they are not found in spinal cord (Bouthenet *et al.*, 1991). The distribution of dopamine D_4-receptors in the central nervous system has been studied by immunocytochemistry (Khan *et al.*, 1998); according to biochemical assays it was found to be concentrated in cerebral cortex, hippocampus, caudate-putamen and, in the mesencephalon, the inferior colliculus and to a much lesser extent in the colliculus inferior. Some labeling was also found in the cerebellum. However, in other areas of the brain stem D_4 labeling was very low or absent. Dopamine D_5-receptors are expressed at very low levels in the central nervous system (Grandy and Civelli, 1992) and, therefore, probably are not detectable in brain stem or spinal cord. Thus, in brain stem and spinal cord dopamine exerts its effects primarily via dopamine D_1 and D_2-receptors.

Few data are available on the detailed localization of D_1 and D_2-receptors in brainstem and spinal cord. The studies presented in this thesis have focussed on the distribution of D_2 dopamine receptors and are the first to identify a consistent regional distribution of the D_2-receptors in spinal cord and brain stem. It was noticed that, compared to the forebrain, the brain stem and the spinal cord contained relatively few cells that were immunoreactive for the D_2-receptor and that most of them were not strongly labeled. Furthermore, the relatively long exposure times that were needed to visualize the radioactively labeled riboprobe in spinal cord indicated that the hybridization signal was relatively low. These findings indicate that, in comparison with other brain areas, the level of D_2-receptors in rat brain stem and spinal cord is low, both with respect to the protein and its mRNA. If the expression of D_2-receptors is constitutively low and difficult to detect, the number of cells expressing D_2-receptors may have been underestimated.

The observation that D_2-receptor immunoreactivity was located mainly within the somata and proximal dendrites of labeled neurons, rather than in terminals, is in agreement with other studies using different D_2-receptor (Brock *et al.*, 1992; Ariano *et al.*, 1993), D_{1A}-receptor or D_3-receptor antibodies (Ariano, 1994). In neostriatal neurons, the D_2-receptor immunoreactivity was uniformly present throughout the somatic cytoplasm, became less strong towards the periphery and appeared not to be associated with a particular cellular organelle (Fisher *et al.*, 1994; Hersch *et al.*, 1995). Furthermore, only a small part of the labeling was associated with postsynaptic densities. The typical localization of D_2-receptor immunoreactivity in somata and proximal dendrites, as we also observed, may indicate that the antibody preferentially recognized D_2-receptor protein before its incorporation as a functional receptor into the neuronal membrane. On the other hand, if the antibody would recognize both functional and nonfunctional receptors, our results would indicate that the highest proportion of D_2-receptor protein is located within the cell body and proximal dendrites whereas only minute amounts of functional receptors are present in more distal dendrites. The amount of cytoplasmic D_2-receptor protein appears to be much higher than the amount of membrane incorporated protein. Antibodies against other receptors or their subunits also primarily stain neuronal somata and dendrites at the light microscopical level (see e.g. glutamate receptor subunits; Kennis and Holstege 1997), although at the ultrastructural level incorporation into membranes has also been identified (Baude *et al.*, 1995). The antibody that we have used is able to shift the affinity of the D_2-receptor for dopamine from high to low (Plug *et al.*, 1992), suggesting that the antibody does react with functional receptors.

It may be concluded that several nuclei in spinal cord and brain stem express D_2-receptors, usually at a relatively low level. Physiological studies in a few areas, both in the brain stem and spinal cord (see below), have shown that D_2-receptor activation has substantial functional effects. The present description of the distribution of D_2-receptors supports an important role of dopamine and its receptors in the brainstem and spinal cord and provides a firm anatomical basis for future functional studies.

The anatomical distribution of dopamine and its receptors: the mismatch problem

A comparison of the distribution of dopamine with that of its D_2-receptor (see table 2) shows that generally the distribution of dopamine is more widespread. This anatomical discrepancy (or mismatch) between the distribution of dopamine and its D_2-receptor can be explained by a complementary distribution of the D_1-receptor. Analysis of the available data on the localization of dopamine receptors in spinal cord (Dubois *et al.*, 1986; Yokoyama *et al*, 1994) indicates that D_2-receptors are more prominent in the superficial dorsal horn, whereas D_1-receptors are more prominent in central and ventral regions of the spinal cord. The few data that are available on the distribution of D_1-receptors indicate that this explanation is also valid for a number of dopamine-innervated areas in the brainstem, like motoneurons in the brain stem, which lack D_2-receptors (see table 2), but do express D_1-receptors (Dubois *et al.*, 1986; Levey *et al.*, 1993).

Two types of mismatch may occur. In one the corresponding receptor is found to be lacking in a region where the receptor is present. In the other the receptor is present, but the corresponding neurotransmitter cannot be identified. In areas where the localization of a transmitter does not correspond with that of its receptors, a number of explanations have been proposed (see e.g. Herkenham, 1987). However, the precise reasons for such a mismatch in a particular region usually remain difficult to establish. Explanations include (1) methodological problems and (2) points of interpretation. (1) Mismatches caused by various methodological problems (see methodological aspects) may be subdivided in two categories: selectivity problems and sensitivity problems. A selectivity problem may occur when a probe is used which only recognizes a subset of the structures it is supposed to identify or, instead, recognizes additional structures. With respect to receptors, selectivity problems may occur with the identification of occupied receptors, spare receptors, low-affinity receptors, non-functional receptors etc. Cross reactivity of an antibody or the identification of dopamine in noradrenergic terminals, as discussed above, may also be regarded as a selectivity problem. Sensitivity problems mainly occur when a probe does not identify low levels of a receptor. The finding in the present receptor studies, that cell somata and dendrites are labeled rather than axons and terminals, may be due to a selectivity problem, i.e. that D_2-receptors incorporated in the (terminal) membrane may not be recognized. On the other hand, the number of dopamine's incorporated in the membrane may be too low to detect, in which case there would be a sensitivity problem. The latter possibility may play a role in the brain stem and spinal cord since expression levels of D_2-receptors are generally low. (2) Mismatches may be caused by problems of interpretation. These mismatches are not due to technical problems, but to problems in the interpretation of the morphological data from immunocytochemical studies. Some neurons which only present immunoreactivity of their cell body, where the receptor is synthesized, may express their functional receptors only at their terminals in distant projection areas. If a dopaminergic projection is lacking in the region containing receptor-immunoreactive neurons, this may be interpreted as a mismatch (presence of receptors but not the appropriate transmitter). The reverse situation, i.e. the presence of dopamine but not (one of) its receptors may occur if transmitter molecules can "travel" through the intercellular space after their release and act on target receptors located at a distance of their release site. This phenomenon has been termed non-synaptic or volume-transmission (see Zoli *et al.*, 1999 for review). In the spinal dopamine system there is evidence that dopamine terminals in the dorsal horn show very few synapses, and may therefore communicate through volume transmission, while the dopamine terminals in the ventral horn show classical synaptic structures and may therefore communicate through conventional synaptic release (Ridet *et al.*, 1992). Since D_2-receptors are predominantly localized in the dorsal horn and D_1-receptors in the ventral horn, this would suggest that in spinal cord especially D_2-receptors are involved in volume transmission. Thus the phenomenon of volume transmission may explain a mismatch in some cases.

In the present studies, we found that nearly all areas expressing D_2-receptors also received a dopaminergic projection. On the other hand there are several areas that receive a dopamine projection, but do not contain D_2-receptors (table 2). In some of these areas the D_1-receptor is expressed, e.g. in motoneuronal cell groups. However, the present data on the distribution of D_1-receptors are incomplete, and a detailed mapping of the D_1-receptor in brain stem and spinal cord is needed to determine whether or not there is a mismatch between dopamine and its receptors. The present data (summarized in table 2) on the localization of dopamine and its receptors in brain stem and spinal cord indicate that, if a mismatch exists, it is only limited.

The origin of the dopaminergic fibers in spinal cord and brainstem (the A11 cell group)

Retrograde tracing studies have shown that the dopaminergic fibers in brain stem (Takada, 1993; Maeda *et al.*, 1994) and spinal cord (Björklund and Skagerberg, 1979; Skagerberg and Lindvall, 1985; Hökfelt *et al.*, 1979; Skagerberg *et al.*, 1988) largely originate in the A11 cell group located in the caudal diencephalon. The spinal-cord projecting dopamine neurons are found in dorsal and caudal hypothalamic areas scattered along the dorsal and medial aspects of the fasciculus mammillothalamicus and, more caudally, in between the fasciculus retroflexus and the third ventricle.

Although the A11 group should be considered as a major diencephalic cell system it is very small in comparison with the mesencephalic dopamine cell groups. The number of dopamine cells in the A11 group (about 130 cells on each side) only amounts to about 1-2% of that in the substantia nigra or A10 (Skagerberg and Lindvall, 1985). In contrast to the total population of A11 dopamine cells, most of which are found rostrally, the dopamine cells projecting to the spinal cord are more evenly distributed along the antero-posterior axis of the A11 group (Skagerberg and Lindvall, 1985). They thus seem to constitute a relatively greater proportion of the population of A11 cells at caudal levels. Due to the small number of dopamine cells and their scattered distribution, physiological and tracing experiments are difficult to perform in the A11 group.

The route of the descending dopamine fibers is not completely known. There is some evidence that at least part of the axons may descend within the dorsal longitudinal fasciculus of Schütz in the periaqueductal and periventricular gray of the mesencephalon, pons, and medulla oblongata (Mouchet *et al.*, 1981; Lindvall *et al.*, 1983). Accordingly, in longitudinal sections we have observed fibers running in the rostro-caudal direction in this area, a finding that is also reported by others (Björklund and Skagerberg, 1982). Retrograde

tracing studies have shown that the descending diencephalospinal dopamine system is totally uncrossed (Björklund and Skagerberg, 1979; Hökfelt *et al.*, 1979; Björklund and Skagerberg, 1982; Skagerberg *et al.*, 1982; Lindvall *et al.*, 1983). In the spinal cord, we have shown that the axons descend partly within lamina I of the dorsal horn and the adjoining parts of the dorsolateral funiculus, and partly along the central canal (see also Skagerberg *et al.*, 1982; Lindvall *et al.*, 1983). The former pathways probably give rise to the dopamine terminals in the dorsal horn and possibly the ventral horns at all levels of the cord, while the latter may contribute to the innervation of the intermediolateral cell column and associated parts of the intermediate zone in the thoracic and upper lumbar segments. The presence of radiating fibers in the white matter destined for the intermediolateral column also suggests the possibility of dopamine fibers descending within the lateral funiculus.

Since the dopamine projections to the brainstem and spinal cord originate from a relatively small group of cells that project to a very wide area, they show a high degree of collateralization leading to a diffuse mode of projection. This feature is shared with the other biogenic amines, which also show a high degree of collateralization, but contrasts with the projection mode of many other spinal projecting systems, e.g. those originating in the cortex, the red nucleus, parts of the pontine and medullary reticular formation and the vestibular nuclei (Kuypers, 1981), which are often more focussed. The functional significance of a diffuse projection from the A11 nucleus depends on collateralization profile and the activity pattern of the individual A11 neurons. It is possible that different A11 neurons project to different target areas and thus are involved in specific functions. In analogy with the serotonergic system, a subset of A11 neurons may influence sensory transmission by projecting specifically to the trigeminal nucleus and the spinal dorsal horn, while another subset may project to the motoneurons and pre-motor areas in the brainstem and spinal cord (Tallaksen-Greene *et al.*, 1993; Halliday *et al.*, 1995). However, the few data available favor the existence of a diffuse, aspecific collateralization of the axons from the A11 neurons. Indeed, single dopaminergic neurons in the subparafascicular fasciculus belonging to the diencephalic A11 dopamine cell group, give rise to collateral projections to the neocortex and spinal cord in the rat (Takada *et al.*, 1988). Similarly, spinal projecting A11 cells send their axons through most of the length of the cord and the long main axons of the A11 cells give off collateral terminal branches at most or all segmental levels (Skagerberg and Lindvall, 1985).

The available evidence on collateralization suggests that individual A11 neurons modulate a wide range of different functions. Functions influenced by the A11 group constitute behaviorally relevant combinations. For example, in threatening situations or under conditions when pain is perceived or is anticipated, cognitive functions like attention to specific environmental aspects and the planning of motor actions as well as more basic functions like respiration, heart rate, blood pressure, motor activity and the threshold for pain may all increase simultaneously. Under these circumstances a collateralized projection system may be the most suitable organization for modulating simultaneously the diverse functions that constitute a complex behavioral response to specific external stimuli. In order to gain more insight in the functional role of the A11 neurons, experimental data on the activity pattern of (individual) A11 dopamine neurons in different environmental conditions and identification and activity of the afferent projections they receive, are essential. Presently these data area lacking, probably due to technical difficulties associated with the small number of A11 dopamine neurons and their scattered distribution.

The relation of dopamine with other monoamines and glutamate

Dopamine and the other monoamines

Many structures in the brain stem and spinal cord receive a dopaminergic (Yoshida and Tanaka, 1988; Mouchet *et al.*, 1992; Ridet *et al.*, 1992; Maqbool *et al.*, 1993) as well as a noradrenergic (Westlund *et al.*, 1983; Fritschy and Grzanna, 1990; Hagihira *et al.*, 1990; Rajaofetra *et al.*, 1992b; Doyle and Maxwell, 1991; Westlund *et al.*, 1984; Grzanna and Fritschy, 1991) and a serotonergic (Marlier *et al.*, 1991; Ruda *et al.*, 1982; Kojima and Sano, 1983; LaMotte and Lanerolle, 1983; Dahlström and Fuxe, 1964; Steinbusch, 1981; Tallaksen-Greene *et al.*, 1993; Halliday *et al.*, 1995) innervation. In table 1 a summary is made of areas in the brainstem and spinal cord that receive a dopaminergic innervation. The noradrenergic and serotonergic innervations in these areas are also shown for comparison. The anatomical data show that in most areas of the brainstem the three monoamines are all present. Exceptions are the different subnuclei of the inferior olivary complex, vestibular complex, area postrema, dorsal periaqueductal gray, superior colliculus, cerebellar nuclei and cerebellum. The similarity in the distribution of the three monoamines indicate that they are all involved in regulating neural activity in the innervated areas. It is not known, however, whether the different monoaminergic projections are also simultaneously active, or whether they have opposite or synergistic effects.

A complex interaction between dopamine and the other monoamines, noradrenaline and serotonin has been suggested by a number of studies, most of which have focussed on the effect of dopamine on the release of other monoamines. In the frontal cortex of freely-moving rats a complex interaction between different monoamines was found (Gobert *et al.*, 1998). Most notably, activation of presynaptic α_2-adrenergic receptors inhibited the release of noradrenaline, dopamine and serotonin. Stimulation of $5HT_{1A}$ receptors suppressed serotonin, yet facilitated noradrenaline and dopamine release. In addition, dopamine D_2/D_3 autoreceptors restrained dopamine release, while (terminal-localized) $5HT_{1B}$ receptors

reduced serotonin release. In a similar fashion, dopamine inhibited the activity of serotonergic dorsal raphe neurons projecting to the striatum via D_2-receptors (Ferré *et al.*, 1992; Ferré *et al.*, 1994) and D_2-receptors were also involved in the apomorphine-induced facilitation of forebrain serotonin output (Mendlin *et al.*, 1998). Also, studies in the superior colliculus showed that the release of noradrenaline was inhibited by dopamine through presynaptic D_2-receptors (Wichmann and Starke, 1988).

The dopaminergic innervation of the mono-aminergic cell groups located in the brain stem further suggest that dopamine interacts with the other monoaminergic cell groups, not only at the level of their terminal fields in the various target areas, but also by directly affecting the activity of the parent cell bodies of the monoaminergic fibers. Dopamine fibers are present in all the raphe nuclei, which contain the serotonergic cells, in the locus coeruleus and the other noradrenergic cell groups, including the A5 and A7 with their extensive spinal projections, and the adrenergic cell groups in the lower brainstem. This emphasizes that the interaction between the various monoamines is extensive and complex. If these complex interactions occur throughout the brainstem and spinal cord, it may be difficult to determine the effect of e.g. dopamine alone without considering the effects of the other monoamines and vice versa. In fact, the effect of a monoamine in a particular area may vary, depending on other monoamines present in that area at that time. This highly interactive behavior of the monoamines is important and should be kept in mind when considering the effect of a single monoamine.

Dopamine and glutamate

Since the original observation of Kaneko *et al.* (1990) that immunoreactivity for glutamate or glutaminase (a glutamate synthesizing enzyme) is present in catecholaminergic and serotonergic neurons subsequent studies have confirmed the presence of glutamate in serotonergic neurons of the raphe nuclei (Nicholas *et al.*, 1992) and noradrenergic neurons in the locus coeruleus area (Fung *et al.*, 1994; Liu *et al.*, 1995). More recently the presence of glutamate in dopaminergic neurons was confirmed (Sulzer *et al.*, 1998), indicating that there may indeed exist an extensive co-localization of glutamate with dopamine, serotonin and noradrenaline. The presence of glutaminase in dopaminergic neurons of the A11 cell group (Kaneko *et al.*, 1990) suggests that glutamate is present in the dopaminergic terminals in the spinal cord and brain stem. If true, dopamine neurons may exert rapid synaptic actions via the release of glutamate and slower modulatory actions via the release of dopamine, as suggested for the dopaminergic neurons in substantia nigra pars compacta which exert their excitatory and inhibitory synaptic effects upon caudate nucleus neurons by using glutamate and dopamine, respectively (Kaneko *et al.*, 1990). This suggests that glutamatergic cotransmission may be the rule for central monoaminergic neurons, and, if carried one step further, the monoaminergic projections may be considered as a glutamatergic system with monoamines as co-released modulators. Obviously, more detailed investigations are needed to determine if this is indeed the case.

The functional role of dopamine in spinal cord and brainstem

General remarks

There are several distinct dopamine systems in the central nervous system. Those that have been studied most extensively are the nigrostriatal and the so-called mesocorticolimbic projections which originate primarily in the substantia nigra pars compacta and the ventral tegmental area, respectively (for review see Björklund and Lindvall, 1984). The primary projection of the nigrostriatal system is to the striatum. The mesocorticolimbic system projects to much of the limbic system, including the frontal and cingulate cortex, septum, accumbens nucleus and olfactory tubercle. In addition there are projections to the pituitary and median eminence from dopamine neurons in the hypothalamus. Most studies on the effects of dopamine on behavior have focussed on the nigrostriatal and mesocorticolimbic dopamine systems, while the contributions of other brain dopamine systems, like the diencephalospinal dopamine system (A11), have been the subject of less systematic investigation. It is likely that the effects of systemically injected drugs that act on the dopamine system, like *L*-DOPA, will influence all the central dopamine systems, including those in the brainstem and spinal cord. Similarly, the different dopamine receptor subtypes are pharmacologically similar, making it difficult to selectively stimulate or block a specific receptor subtype *in vivo*. In view of this, the use of highly selective genetic approaches to alter the expression of individual dopamine receptor subtypes has produced new insights into dopaminergic receptor function, which would have been difficult to obtain through other approaches (for review see Sibley, 1999). Obviously it is difficult to determine the contribution of the brainstem and spinal cord to changes that are observed after general applications of drugs or use of receptor gene alteration that influence the dopamine system. To determine the role of the dopamine system in the brainstem and spinal cord in normal brain functioning, two experimental approaches can be used: 1) monitoring normal activity or using selective activation of A11 neurons that project to the brainstem and spinal cord and 2) studying the effects of dopamine in the caudal projection areas of the A11 neurons. The most direct way to answer the first question can be obtained by monitoring the activity of the A11 dopaminergic cells in conscious, freely moving animals for a prolonged period of time and in different circumstances. This experimental approach has been used for noradrenergic cells in the locus coeruleus (Singewald and Philippu, 1998; Vankov *et al.*, 1995) and for serotonergic cells in the raphe nuclei (Jacobs and Fornal, 1999; Rueter *et al.*, 1997), but similar experiments have not yet been done for the A11 dopaminergic cells. The second experimental approach has been used in several studies on the effects of

dopamine in the spinal cord and brainstem. The results of some of these studies will be summarized below.

Dopamine effects on sensory systems

There are extensive dopaminergic projections to spinal cord and brainstem areas that are involved in sensory processing. These areas include the caudal spinal trigeminal nucleus and the spinal superficial dorsal horn which are involved in nociception, and the principal, mesencephalic and spinal trigeminal nuclei, the dorsal column nuclei and the nucleus proprius of the dorsal horn, which are mainly involved in the processing of non-nociceptive sensory input. Many other brainstem areas, like the cochlear nuclei and the superior and inferior colliculus, involved in audio-visual processing, also receive a dopamine input. Dopamine D_2-receptors are mainly expressed in the areas processing nociceptive information and in the colliculi, but are much less prominent in the other areas. Studies investigating the functional role of dopamine in sensory transmission are scarce. Most studies have been performed in the spinal cord, where dopamine exerts an inhibitory effect on nociceptive transmission, mediated by D_2-receptors (Barasi *et al.*, 1987; Fleetwood-Walker *et al.*, 1988; Liu *et al.*, 1992), while the effects on non-nociceptive transmission and the involvement of D_1-receptors appears to be less prominent (Barasi *et al.*, 1987; Liu *et al.*, 1992). It can be concluded that there are extensive dopamine projections to nearly all primary areas involved in the processing of sensory information. The effects of dopamine in these areas have not been studied yet, with the exception of the inhibitory effect on nociceptive transmission.

Dopamine effects on motor systems

Dopamine is also involved in processing motor output at the motoneuronal level. Probably the most prominent anatomical feature of the dopaminergic, as well as noradrenergic, fibers located in motoneuronal cell groups is their fine granular aspect, caused by a large number of fibers with small varicosities and thin, or lightly stained, intervaricose segments (Grzanna *et al.*, 1987; Fritschy and Grzanna, 1989, this thesis). Thus, it appears that, throughout the neuraxis, there is a consistent dopaminergic and noradrenergic innervation of motor nuclei by fibers with a similar morphology in immunocytochemical preparations. Dopamine D_2-receptors were not found in motoneurons, which is consistent with the finding that they express D_1-receptors (Dubois *et al.*, 1986; Levey *et al.*, 1993).

The reported effects of dopamine on somatic motor functions at the spinal level are contradictory (Carp and Anderson, 1982; Ono and Fukuda, 1984; Kamijo *et al.*, 1993). In some cases facilitation was found (Dupelj and Geber, 1981), while others reported an inhibitory effect (Carp and Anderson, 1982; Ono and Fukuda, 1984; Pehek *et al.*, 1989). It has been suggested that D_1 and D_2-receptors are functionally coupled at the spinal level in modulating spinal motor output (Gajendiran *et al.*, 1996). Since D_1-receptors couple to stimulation of adenylate cyclase activity and D_2 either inhibit enzyme activity or are not coupled to this enzyme, the presence of D_1-receptors in the absence of D_2-receptors indicates that the direct effect of dopamine on motoneurons is facilitatory.

Dopamine effects on autonomic systems

In brain stem and spinal cord the strongest dopaminergic innervation is found in nuclei known to be involved in autonomic control. Most studies on the role of dopamine in central autonomic systems have focussed on the regulation of heart rate and blood pressure. These studies have reported substantial hemodynamic effects of dopamine both at the level of the nucleus tractus solitarius (Zandberg *et al.*, 1979; Granata and Woodruff, 1982; Kessler and Jean, 1986; van Giersbergen *et al.*, 1992), the nucleus ambiguus (Chitravanshi and Calaresu, 1992), and the intermedio-lateral cell column, containing the spinal preganglionic sympathetic neurons (Petitjean *et al.*, 1984; Clatworthy and Barasi, 1987; Pellissier and Demenge, 1991; Lahlou and Demenge, 1993). However, the reported effects of dopamine on blood pressure and heart rate are not straightforward. Low doses of dopamine elicit a dose dependent decrease in blood pressure and heart rate (Zandberg *et al.*, 1979) and higher doses of dopamine (Granata and Woodruff, 1982) or a D_2-receptor agonist (quinpirole) (Yang *et al.*, 1990) cause a consistent increase in mean arterial pressure, suggesting that dopamine may produce an increase in blood pressure via its D_2-receptors. These findings suggest that the concentration of dopamine determines which receptors are activated and which hemodynamic effect emerges at the level of the brainstem and spinal cord. Thus, the effects of dopamine may depend not only on the types of receptors involved, but also on the concentration of the transmitter at the receptor and on the interaction with many other transmitters that are present in these cardiovascular brain stem and spinal cord regions.

The presence of a dopaminergic innervation of areas known to be involved in respiratory control, like the nucleus of the solitary tract, lateral parabrachial nucleus and the phrenic nucleus, suggest that dopamine may be involved in respiratory control. The phrenic nucleus of the spinal cord (C4-C6), which contains the motor neurons innervating the diaphragm, receives strong descending connections from the ventrolateral nucleus of the solitary tract (called the dorsal respiratory group). The nucleus of the solitary tract is reciprocally connected with the far lateral parts of the dorsal and central lateral parabrachial nuclei (Herbert *et al.*, 1990). It receives pulmonary and tracheal afferents through the vagus nerve (van Giersbergen *et al.*, 1992). Since these nuclei are tightly interconnected and all receive a dopaminergic innervation suggest that dopamine may play an important role in respiratory control at the level of the brain stem and spinal cord, which is also indicated by a few studies. Dopamine accelerated the respiratory frequency when applied by perfusion to the in vitro brain stem (Murakoshi *et al.*, 1985), which may be dependent on co-activation of alpha adrenergic receptors (Johnson *et al.*, 1998). Local perfusion of D_1-receptor blocker into the region of the nucleus of the solitary tract had no effect (Srinivasan *et al.*, 1991), suggesting that D_2-receptors may play a more important

role in this brain stem area.

To date, research on the central control of micturition has concentrated on noradrenergic influences at the level of the brain stem and spinal cord. Besides noradrenaline (de Groat *et al.*, 1999) there is some evidence that serotonin (Espey *et al.*, 1998) and dopamine (Kontani *et al.*, 1990) may be inhibitory to micturition at the spinal level. Interestingly, sacral parasympathetic preganglionic neurons receive a strong dopaminergic innervation and express dopamine D_2-receptors. These findings suggest that it may be promising to include dopamine in future research on the central control of micturition.

In conclusion, dopamine plays an important role in diverse autonomic reflexes, among which hemodynamic control, respiration and micturition. Further investigations are needed get more insight in the effects of dopamine on other autonomic functions that are organized in the brain stem and spinal cord .

Dopamine and the cerebellum

Although an extensive examination of the cerebellum was not attempted in this thesis, a limited dopaminergic innervation was observed in the cerebellar cortex, more particularly the medial extension of the ventral paraflocculus. The functional role of the limited dopaminergic innervation still is obscure (Panagopoulos *et al.*, 1991; Ikai *et al.*, 1992). The dopaminergic fibers projecting to the cerebellar cortex terminate mainly in the granular layer, additionally in the Purkinje cell layer, but not at all in the molecular layer (Ikai *et al.*, 1992). Dopamine receptor binding, especially for the D_1 subtype, occurs in both the molecular and granular layers (Dubois *et al.*, 1986; Mansour *et al.*, 1992), while dopamine receptor mRNA's for both the D_1 and D_2 subtypes are present in the granular layer only (Bouthenet *et al.*, 1991; Mansour *et al.*, 1992).

Thus the function of the limited dopaminergic projection to the cerebellum remains obscure. The paucity of dopamine suggests that in the cerebellum other monoamines, like noradrenaline and serotonin, play a more important role. In this respect it is interesting to note that many cerebellar-projecting neurons, like the dorsal nucleus (column of Clarke) and central cervical nucleus, appear to be avoided by the dopamine system. The reason for the avoidance by dopamine of spinal nuclei projecting to the cerebellum is still unclear.

The role of dopamine in different behavioral states

The dense and specific regional distribution of dopamine in the PAG suggests that dopamine is involved in regulating various PAG functions. The strong dopamine projection to the ventrolateral PAG may be implied in modulating quiescent behavior, i.e. a strong decrease in spontaneous activity and responsiveness to the environment, together with hypotension, bradycardia and pain inhibition, which occurs after deep visceral and muscular pain stimuli (like after a fight or internal pains). This type of behavior appears to be useful in circumstances of wound healing or recuperating from disease and is organized in the ventrolateral part of the PAG (Bandler and Shipley, 1994). The predominant dopaminergic innervation of the dorsal PAG, on the other hand, would suggest the involvement of dopamine in organizing behavior associated with fear and anxiety, like threat display associated with vocalization and strong fight or flight responses (Behbehani, 1995). However, application of agonists or antagonists of dopaminergic, as well as adrenergic and serotonergic receptors had no effect on vocalization per se (Lu *et al.*, 1993). Nevertheless, it may be concluded that dopamine is involved not only in modulating basic autonomic functions in the lower brainstem and spinal cord as described earlier, but is also involved in modulating more complex behavior in more rostral areas of the brainstem, like the PAG and the parabrachial nuclei, where these basic autonomic functions are regulated depending on the environmental situation.

Dopamine is among the central neurotransmitters involved in the control of sexual behavior, which may be conceptualized as consisting of both motivational and performance components (for review see Sachs, 1995). At the level of the brain stem and spinal cord, the strong dopaminergic innervation of the sexually dimorphic nuclei in the lumbosacral spinal cord is most conspicuous and suggests that dopamine receptors are directly involved in regulating male sexual performance, as also indicated by studies (Stefanick *et al.*, 1982; Pehek *et al.*, 1989) showing that dopamine agonists inhibit specific sexual motor functions like penile reflexes. Our finding that D_2-receptors are present in (the homologue of) Onuf's nucleus in the rat would strengthen this idea, although the specific function of dopamine in this case is unclear.

Relation with Parkinson's disease

Parkinson's disease is one of the major neurodegenerative diseases of the nervous system. It leads to a variety of motor disturbances and is caused by selective degeneration of dopaminergic neurons in the substantia nigra. Although the dopaminergic projections of the substantia nigra are aimed rostrally at the striatum, rather than caudally, the presence of dopamine in the motoneuronal cell groups of the brainstem and spinal cord may suggest that part of the disturbed motor performance in Parkinson's disease is due to changes in dopamine neurotransmission at the level of the brain stem and spinal cord (Lindvall *et al.*, 1983). However, there is no evidence that A11 dopaminergic neurons are affected in Parkinson's disease and in addition it was shown that the dopamine concentration in the spinal cord of Parkinsonian patients (Scatton *et al.*, 1986; Sofic *et al.*, 1991) was the same as in healthy subjects.

Therefore, it seems unlikely that changes in brain stem or spinal dopamine levels are involved in producing the motor deficits associated with Parkinson's disease.

Conclusions

In conclusion, the present thesis definitively establishes the existence of an extensive dopaminergic innervation in the brain stem and spinal cord, which constitutes a distinct neurotransmitter system separate from the other

monoamines. The distribution of dopamine is more widespread than that of its D_2-receptor. This anatomical discrepancy may be largely explained by a complementary distribution of the D_1-receptor. The levels of D_3, D_4, and D_5-receptors in the brain appear to be several orders of magnitude lower than that of D_1 and D_2-receptors. Although D_3 and D_4-receptors are reported in a few brain stem areas, they do not exist in the spinal cord.

The dopaminergic projection to the monoaminergic cell groups located in the brain stem suggest that dopamine interacts with the other monoaminergic cell groups, not only at the level of the terminal fields in the target areas, but also by directly affecting the activity of the parent cell bodies of the monoaminergic fibers. The high degree of collateralization in the dopamine diencephalo-spinal projection would imply that dopamine is released simultaneously in many different areas of the brain stem and spinal cord, which suggest that individual A11 neurons subserve integrative functions. Dopamine neurons may also use glutamate as a transmitter and thus exerts rapid synaptic actions via the release of glutamate and slower modulatory actions via the release of dopamine.

There are extensive dopamine projections to nearly all the sensory information processing areas, but the effects of dopamine in these areas is largely unclear, except for an inhibitory effect on nociceptive transmission in which D_2-receptors are involved. Motoneuronal cell groups receive an extensive dopamine projection and express D_1 rather than D_2-receptors, indicating that the effect of dopamine on motoneurons is facilitatory.

The cerebellum receives only a very limited dopaminergic projection, the functional role of which is still obscure. Dopamine is also observed in many areas that subserve various autonomic functions, including hemodynamic, respiratory and micturition control at the brain stem and spinal level. More rostral areas, like the periaqueductal gray and the parabrachial nuclei, which are involved in organizing the different autonomic control centers, also receive a strong dopaminergic projection. The role of dopamine in different behavioral states like fight and flight and sexual behavior are discussed. At present, there is no evidence that A11 plays a role in Parkinson's disease.

Taken together the present thesis emphasizes that besides noradrenaline and serotonin, dopamine should also be included when considering the monoaminergic effects on brain stem and spinal processing. In fact, the interaction between the various monoamines may be of even greater importance than the effect of the respective transmitters alone. Investigations along this line may prove worthwhile. Hopefully, the studies presented in this thesis will contribute to these future studies on the role of dopamine and the other monoamines in the nervous system by providing a firm basis in the anatomy, which is always needed when searching to understand the brain

Table 1: Overview of the distribution of dopamine (this thesis), noradrenaline (Mouchet *et al.*, 1992; Rajaofetra *et al.*, 1992; this thesis) and serotonin (Steinbusch, 1981; Tallaksen-Greene *et al.*, 1993; Halliday *et al.*, 1995) in brain stem and spinal cord. + = present, - = absent.

I. Spinal cord	Dopamine	Noradrenaline	Serotonin
Dorsal Horn	+	+	+
Intermediate zone	+	+	+
Ventral Horn	+	+	+
Specialized areas			
Region around the central canal	+	+	+
Sympathetic preganglionic neurons	+	+	+
Dorsal nucleus (column of Clarke)	-		-
Lumbosacral motoneurons	+	+	+

II. Brain stem	Dopamine	Noradrenaline	Serotonin
Sensory Trigeminal Complex	+	+	+
Dorsal motor nucleus of the vagus	+	+	+
Hypoglossal nucleus	+	+	+
Solitary Complex	+	+	+
Dorsal column nuclei	+	+	+
Inferior olive, Principal nu	+	+	-/+
Inferior olive, VLO	+	+	-
Inferior olive, DAO	-	-/+	+
Inferior olive, MAO	-	-/+	+
Cerebellar nuclei	-	+	+
Cerebellum	-	+	+
Motor nuclei of the cranial nerves	+	+	+
Locus coeruleus	+	+	+
Raphe nuclei	+	+	+
Vestibular complex	-/+	+	+
Area postrema	-	+	+
Parabrachial complex, LPB	+	+	+
Periaqueductal gray	+	+	+
Periaqueductal gray, dorsal	+	-	+
Inferior Colliculus	+	+	+
Superior Colliculus, superficial layers	-	+	+
Superior Colliculus, inner layers	+	-	+

Table 2: A comparison of dopamine (this thesis), dopamine D_1-receptor (Dubois *et al*, 1986), dopamine D_2-receptor (Bouthenet *et al.*, 1987; Bouthenet *et al.*, 1991; this thesis) and dopamine D_3-receptor (Bouthenet *et al.*, 1991) in the brain stem and spinal cord. + = present, - = absent.

I. Spinal cord	Dopamine	D_1-receptor	D_2-receptor	D_3-receptor
Dorsal Horn				
Lamina I	2+		3+	-
Lamina II (substantia gelatinosa)	1+	present	-	-
Lamina III	3+		-	-
Lamina IV	3+		1+	-
Lamina V	3+		1+	-
Lamina VI	3+		1+	-
Intermediate zone				
Lamina VII	1+		2+	-
Lamina VIII	1+		2+	-
Ventral Horn				
Lamina IX	3+	present	-	-
Specialized areas				
Central cervical nucleus	+/-		-	
Lateral Cervical nucleus	2+		-	
Region around the central canal	3+	present	3+	
Phrenic nucleus	3+			
Sympathetic preganglionic neurons	4+		3+	
Dorsal nucleus (column of Clarke)	-			
Cremaster nucleus	4+			
Lumbosacral motoneurons	3+		3+	
Sacral parasympathetic area	3+		4+	

II. Cerebellum	Dopamine	D_1-receptor	D_2-receptor	D_3-receptor
Granular layer	-	present	1+	1+*
Purkinje cells in lobule 10	-		-	4+
Molecular layer	-	present	-	

*, Possibly non-specific

III. Medulla Oblongata		Dopamine	D_1-receptor	D_2-receptor	D_3-receptor
10	Dorsal motor nucleus of the vagus	3+		4+	-
12	Hypoglossal nucleus	2-3+		-	
Cu	Cuneate nucleus	2+		0-4+	-
DC	Dorsal cochlear nu	1-2+		0-1+	3+
Ecu	External cuneate nu	-		0-1+	-
GiA	Gigantocellular reticular nu, alpha	1+		3+	-
IO	Inferior olive	0-4+		0-4+	2+
MdD	Medullary reticular nu, dorsal part	1+			-
MdV	Medullary reticular nu, ventral part	1+			-
Mve	Medial vestibular nu	1+		0-2+	-

Table 2: *(continued)*

III. Medulla Oblongata		Dopamine	D_1-receptor	D_2-receptor	D_3-receptor
RF	Reticular formation of medulla oblongata	1+		1-2+	
RMg	Raphe magnus nu	2+		4+	
RPa	Raphe pallidus nu	3+		4+	
Sol	Nucleus of the solitary tract	2-4+		2-3+	1+
SO	Superior olive	-		-	
Sp5C	Spinal trigeminal, caudal part	1-2+		2+	
Sp5	Spinal trigeminal, medial/lateral part	1+			
Sp5I	Spinal trigeminal, interpolar part	3+		-	
AP	Area postrema	-		2+	

IV. Pons		Dopamine	D_1-receptor	D_2-receptor	D_3-receptor
n7	Facial nu	2-3+		-	
Amb	Nucleus ambiguous	1+		-	
Co	Cochlear nu	1+		-	
LC	Locus coeruleus	2-3+		4+	-
LPB	Lateral parabrachial nu	4+		3+	-
LVe	Lateral vestibular nu	+/-		-	
Mo5	Motor trigeminal nu	2-3+		-	
Me5	Mesencephalic trigeminal nu	2-3+		0-3+	-
MPB	Medial parabrachial nu	4+		3-4+	-
MVe	Medial vestibular nu	1+		-	
PnC	Pontine reticular nu, caudal part	1+		1-2+	-
PnO	Pontine reticular nu, oral part	1+		1-2+	-
Pr5	Principal sensory nu	0-1+		0-2+	
Sp5	Spinal trigeminal nu	1+		1-2+	

V. Mesencephalon		Dopamine	D_1-receptor	D_2-receptor	D_3-receptor
N3	Oculomotor nu	2-3+		0-4+	1+
CG	Central gray	1-2+	present	2-3+	-
DpMe	Deep mesencephalic nu	1+		1-2+	-
DR	Dorsal raphe nucleus	4+		4+	
IC	Inferior colliculus (external cortex)	1-2+		2-3+	
IF	Interfascicular nu (A10)	1-2+		0-2+	
MG	Medial geniculate nu(ventral border)	1-2+		4+	
MnR	Median raphe nu	1+		3+	
RLi	Rostral linear nu of raphe	1-2+		2+	
RN	Red nu	1+		0-2+	
SNC	Substantia nigra compacta (A9)	1+	present	4+	1+
SNL	Substantia nigra compacta, lateral part	1+		0-4+	3+
SNR	Substantia nigra reticulata	4+	present	0-4+	
Su	Superior colliculus(superficial grey layer)	1-2+	present	2+	2+
VTA	Ventral tegmental area (A10)	1+	present	3+	1+

Samenvatting

Samenvatting

Dit proefschrift omvat verschillende studies naar de dopaminerge innervatie van de hersenstam en het ruggenmerg en één van de daarbij betrokken receptoren, de D_2-receptor. Het resultaat geeft een gedetailleerd inzicht in een chemisch geïdentificeerd systeem, gekarakteriseerd door het gebruik van de neurotransmitter dopamine.

In *Hoofdstuk I* wordt een korte beschrijving van de anatomie van het ruggenmerg en de hersenstam gegeven. Dit wordt gevolgd door een kort overzicht van de chemische anatomie van het centraal zenuwstelsel, waarbij de nadruk ligt op het dopaminerge systeem en haar receptoren.

Hoofdstuk II beschrijft de verdeling van dopaminerge vezels en (veronderstelde) eindigingen in alle segmenten van het ruggenmerg van de rat, kat en aap. De vezels en eindigingen zijn immunocytochemisch geïdentificeerd op lichtmicroscopisch niveau, gebruik makend van antilichamen gericht tegen dopamine.

De sterkste dopaminekleuring werd gevonden in de sympathische zijhoorn (IML). Sterke dopaminekleuring, bestaande uit een groot aantal varicose vezels, werd gevonden in alle laminae van de dorsale hoorn, inclusief het gebied rond het centrale kanaal (lamina X). Een uitzondering vormde de substantia gelatinosa, die met name in rat en aap slechts beperkt gekleurd was. Vezels in de motoneuronale celgroepen vertoonden eveneens een sterke dopaminekleuring en lieten een fijn granulair aspect zien. De seksueel dimorfe cremaster kern en Onuf's nucleus (of zijn homoloog) waren veel sterker gekleurd dan de omliggende somatische motoneuronen. In het parasympathische gebied op sacraal niveau was de kleuring van gemiddelde sterkte. De rest van de gebieden, waaronder de intermediaire zone (lamina VI-VIII), werden slechts in beperkte mate geïnnerveerd. Het kleinste aantal dopamine vezels werd gevonden in de dorsale kern (kolom van Clarke) en de centrale cervicale kern, hetgeen aangeeft dat cellen die projecteren naar het cerebellum werden gemeden door de dopaminerge projectie. In zowel rat, kat als aap waren de meeste afdalende vezels aanwezig in de dorsolaterale funiculus, alhoewel laminae I en II ook veel rostrocaudaal georiënteerde vezels bevatten.

Uit de resultaten van deze studie kan worden geconcludeerd dat de verdeling van dopamine in het ruggenmerg wijdverspreid is, met slechts kleine verschillen tussen de onderzochte diersoorten. Dit benadrukt de belangrijke rol die dopamine speelt als een van de monoaminen die, zowel sensorische input, als autonome en motor output op het niveau van het ruggenmerg beïnvloeden.

Hoofdstuk III beschrijft de lokalisatie van dopamine D_2-receptoren in het ruggenmerg van de rat. Hierbij werd gebruik gemaakt van immunocytochemie met een anti-peptide antilichaam gericht tegen de veronderstelde 3^e lus van de D_2-receptor, en *in situ* hybridisatie (ISH) met een [^{35}S]UTP gelabelde anti-sense riboprobe.

Na gebruik van de immunocytochemie techniek bleek dat de kleuring beperkt bleef tot de neuronale cellichamen en hun proximale dendrieten. De sterkst gekleurde neuronen werden gevonden in het parasympathische gebied van het sacrale ruggenmerg en in de twee seksueel dimorfe motorische kernen van het lumbosacrale ruggenmerg (de spinale kern van de bulbocavernosus en de dorsolaterale kern). Cellen met een kleuring van gemiddelde sterkte waren aanwezig in de sympathische zijhoorn, het gebied rond het centrale kanaal en in lamina I van de dorsale hoorn. Een zwakke kleuring werd gevonden in de laterale spinale kern en in laminae VII en VIII van de ventrale hoorn. Naast de twee seksueel dimorfe motorische kernen van het lumbosacrale gebied werden verder geen gekleurde motoneuronen aangetroffen.

Wanneer de ISH techniek gebruikt wordt, wordt een groot aantal radioactief gelabelde neuronen aangetroffen. Dit geeft aan dat deze neuronen D_2-receptor mRNA bevatten. De verdeling van deze neuronen was vergelijkbaar met de gevonden verdeling bij immunocytochemie. Een verschil tussen beide technieken betrof de laminae III en IV. Gebruik makend van de ISH techniek werden ook cellen gelabeld in deze gebieden, terwijl een vergelijkbare kleuring in deze gebieden gebaseerd op immunocytochemie niet aangetroffen werd.

De lokalisatie van de neuronen die D_2-receptoren tot expressie brengen in het ruggenmerg suggereert dat D_2-receptoren met name betrokken zijn bij de modulatie van nociceptieve transmissie en het autonome systeem. De resultaten geven verder aan dat D_2-receptoren niet direct betrokken zijn bij de modulatie van motoneuronen, met uitzondering van motoneuronen in de seksueel dimorfe kernen.

Hoofdstuk IV beschrijft de verdeling van dopamine immunoreactieve vezels en hun (veronderstelde) eindigingen op licht microscopisch niveau in de hersenstam van de rat. De verdeling van dopamine immunoreactiviteit wordt vergeleken met die van dopamine-β-hydroxylase (DBH), het noradrenaline synthetiserend enzym.

De dopaminerge vezels en eindigingen vertoonden een specifieke verdeling binnen de hersenstam. Ze waren aanwezig in een groot aantal hersenstam kernen en lieten verschillende niveaus van van kleuringsintensiteit zien . De sterkste dopaminekleuring werd aangetroffen in de dorsale motor kern van de nervus vagus, de hoofdkern van de oliva inferior en de dorsale raphe kern. Andere gebieden, zoals de kern van de tractus solitarius, de raphe pallidus en obscurus, de laterale parabrachiale kern en het periaqueductale grijs vertoonden eveneens een sterke kleuring, terwijl het sensorische trigeminus complex, de locus coeruleus en de noradrenerge A5 groep gemiddeld tot sterk gekleurd waren. Verder waren ook in vele andere gebieden van de hersenstam dopamine immunoreactieve vezels en eindigingen aanwezig, waaronder de colliculi inferior en superior, de diepe mesencephalische kern, de

reticulaire pons kern, de kern van de prepositus hypoglossus, de cochleaire kernen en de cuneatus en gracilis kernen. Binnen een kern is de dopamine niet willekeurig verspreid, maar vaak gericht op specifieke sub-kernen. Hoewel de verspreiding van vezels en eindigingen die met DBH waren gekleurd over het algemeen uitgebreider was dan de dopaminerge kleuring, bleek in vele hersenstam gebieden een duidelijke overlap tussen de DBH en dopamine gekleurde vezels en eindigingen. Er was echter wel een verschil in het regionale innervatie patroon. In andere gebieden, zoals de area postrema, cerebellum, het anterior gedeelte van de ventrale cochleaire kern en de externe cuneatus kern is DBH overvloedig aanwezig, terwijl dopaminerge vezels slechts in beperkte mate aangetoond konden worden.

Deze resultaten tonen aan dat er een uitgebreid dopamine vezelsysteem aanwezig is in de hersenstam van de rat, dat, in tegenstelling tot hetgeen lange tijd werd aangenomen, onafhankelijk is van het (nor)adrenerge vezelsysteem. Dit betekent dat de invloed van dopamine in de hersenstam te vergelijken is met dat van de andere monoaminen, zoals (nor) adrenaline en serotonine.

In *Hoofdstuk V* wordt de verdeling van dopamine D_2 receptoren in de hersenstam van de rat beschreven, zoals bepaald met signaalversterkte immuno-cytochemie. Hierbij werd gebruik gemaakt van een polyklonaal antiserum met antilichamen gericht tegen de veronderstelde 3^e lus van de dopamine D_2 receptor. De resulterende kleuring was in de meeste gevallen geassocieerd met neuronale cellichamen en hun proximale dendrieten. Dit resultaat was onafhankelijk van het al dan niet toepassen van signaalversterking. In de hersenstam van de rat werden neuronen met het D_2-receptor-eiwit waargenomen in specifieke gebieden, zoals de dorsale motor kern van de nervus vagus, de gigantocellulaire kern (pars alpha), de kern van de tractus solitarius, de locus coeruleus, de laterale en mediale parabrachiale kern, de basale pons kern, het centrale grijs, de diepe mesencephalische kern, de colliculi inferior en superior, de substantia nigra pas compacta en het ventraal tegmentum. Daarnaast werd ook in andere hersenstam kernen het D_2-receptor-eiwit waargenomen, waaronder de reticulaire formatie en alle raphe kernen. In andere hersenstamgebieden, zoals de cuneatus kern en de oliva inferior, kon het D_2-recepter-eiwit niet worden aangetoond met de gebruikte immunocytochemie techniek, wat aangeeft dat in deze gebieden de D_2-receptor-eiwit niet, of nagenoeg niet, aanwezig was.

De gevonden resultaten zijn in overeenstemming met, en zijn een uitbreiding op, de resultaten van eerdere studies naar de D_2-receptor in de hersenstam van de rat, waarbij gebruik gemaakt werd van ligand binding of *in situ* hybridisatie. De wijdverspreide verdeling van D_2-receptoren in de hersenstam van de rat ondersteunt de hypothese dat D_2-receptoren betrokken zijn bij de modulatie van een groot aantal verschillende hersenstamfuncties. Dit resultaat benadrukt nogmaals de belangrijke rol van het dopaminerge systeem in de hersenstam.

In *Hoofdstuk VI* wordt de anatomische en functionele betekenis van het dopaminerge systeem in de hersenstam en het ruggenmerg besproken met als belangrijkste conclusie dat in dit proefschrift het bestaan van een wijdverspreide dopaminerge neurotransmissie systeem in de hersenstam en het ruggenmerg onomstotelijk wordt aangetoond. Deze neurotransmissie is onafhankelijk van de andere monoaminerge neurotransmitters waar dopamine als precursor aanwezig is. Dopaminerge vezels worden gevonden in vrijwel alle gebieden, die sensorische informatie verwerken, in de motoneuronale celgroepen en in vele gebieden die verschillende autonome functies verzorgen. Dit suggereert dat dopamine is betrokken bij verschillende hersenstam- en ruggenmergfuncties. De verdeling geeft tevens aan dat dopamine een interactie aangaat met de andere monoaminerge celgroepen, niet alleen op het niveau van de eindigingen maar ook door direct de activiteit van de moeder cellichamen van de monoaminerge vezels te beïnvloeden. Een relatief laag aantal dopaminerge neuronen in de A11 groep in het meso-diëncefale overgangsgebied vormen de oorsprong van de dopaminerge projecties naar de hersenstam en het ruggenmerg. De hoge graad van collateralisatie in deze projecties suggereert dat dopamine tegelijkertijd wordt afgegeven in veel verschillende gebieden in de hersenstam en het ruggenmerg. Dit suggereert een rol voor individuele A11 neuronen in de integratie van diverse functies, bijvoorbeeld in gedragingen zoals gedurende 'flight and fight' en seksueel gedrag. Er is tot op heden geen bewijs dat de A11 een rol speelt in de ziekte van Parkinson.

Dit proefschrift benadrukt dat dopamine, naast noradrenaline en serotonine, beschouwt dient te worden als een belangrijke neurotransmitter binnen het monoaminerge systeem in de hersenstam en het ruggenmerg. Het is zelfs aannemelijk dat de interactie tussen de verschillende monoaminen in de hersenstam en het ruggenmerg van groter belang is dan het effect van de afzonderlijke monoaminen. Dit proefschrift vormt een belangrijke anatomische basis voor toekomstig onderzoek in deze richting.

Abbreviations

AADC	aromatic *L*-amino acid decarboxylase
ABC	avidin biotin complex
DA	dopamine(ergic)
DBH	dopamine-ß-hydroxylase
DSP-4	N-(2-chloroethyl)-N-ethyl-2-bromo-benzylamine
NA	noradrenaline
PAP	peroxidase-antiperoxidase
PNMT	phenylethanol-N-methyltransferase
SA-HRP	streptavidin-horseradish peroxidase
TBS	Tris buffered saline
TH	tyrosine hydroxylase
TSA	tyramide signal amplification
DLN	dorsolateral nucleus
RNA	messenger ribonucleic acid
UTP	uridine-5'-triphosphate

Spinal cord areas:

IML	intermediolateral cell column
SNB	spinal nucleus of the bulbocavernosus

Brainstem areas:

3	principal oculomotor nucleus
4n	trochlear nerve
6	abducens nucleus
7	facial nucleus
7n	facial nerve
7n	facial nerve
8cn	cochlear root vestibulocochlear nerve
8n	cochlear nerve
8vn	vestibulocochlear nerve
10	dorsal motor nucleus of vagus
12	hypoglossal nucleus
A1	A1 noradrenaline cells
A5	A5 noradrenaline cells
A7	A7 noradrenaline cells
Amb	ambiguus nucleus
AP	area postrema
APT	anterior pretectal nucleus
Aq	aqueduct
Atg	anterior tegmental nucleus
ATg	anterior tegmental nucleus
B9	B9 serotonergic cells
Bar	Barrington's nucleus
BIC	nucleus brachium inferior colliculus
C1	C1 adrenaline cells
C2	C2 adrenaline cells
CG	central gray
CGD	central gray, dorsal
CGLD	central gray, lateral dorsal
CGLV	central gray, lateral ventral
CGM	central gray, medial
CIC	central nucleus inferior colliculus
CLI	caudal linear nucleus raphe
Cll	commissure lateral lemniscus
CnF	cuneiform nucleus
Cp	cerebral peduncle, basal
Cp	cerebral peduncle, basal
Csc	commissure superior colliculus
Cu	cuneate nucleus
cu	cuneate fasciculus
CVL	caudoventrolateral reticular nucleus
das	dorsal acoustic stria
DC	dorsal cochlear nucleus
DCIC	dorsal cortex of inferior colliculus
DLL	dorsal nucleus lateral lemniscus
DMTg	dorsomedial tegmental area
DpG	deep gray layer superior colliculus
DPGi	dorsal paragigantocellular nucleus
DpWh	deep white layer superior colliculus
DR	dorsal raphe nucleus
Dsc	dorsal spinocerebellar tract
DTg	dorsal tegmental nucleus
ECIC	external cortex inferior colliculus
Ecu	external cuneate nucleus
EW	Edinger-Westphal nucleus
Fl	flocculus
Gi	gigantocellular reticular nucleus
GiA	gigantocellular reticular nucleus, alpha
GiV	gigantocellular reticular nucleus, ventral
Gr	gracile nucleus
GrC	granular layer cochlear nucleus
Icp	inferior cerebellar peduncle
IF	interfascicular nucleus
ILL	intermediate nucleus lateral lemniscus
In	intercalated nucleus
InG	intermediate gray layer superior colliculus
IntA	interposed cerebellar nucleus, anterior
InWh	intermediate white layer superior colliculus
IO	inferior olive
IOA	subnucleus a of medial nucleus
IOB	subnucleus b of medial nucleus
IOC	subnucleus c of medial nucleus
IOD	dorsal nucleus
IODM	dorsomedial nucleus
IOK	cap of Kooy of medial nucleus
IOM	medial nucleus
IOPr	principal nucleus
IOVL	ventrolateral outgrowth
IPC	interpeduncular nucleus, caudal subnucleus
Irt	intermediate reticular nucleus
K	nucleus K
KF	Kölliker-Fuse nucleus
Lat	lateral cerebellar nucleus
LatPC	lateral cerebellar nucleus, parvocellular
LC	locus coeruleus
LDTg	laterodorsal tegmental nucleus
Lfp	longitudinal fasciculus pons
lfp	longitudinal fasciculus pons
Li	linear nucleus medulla
ll	lateral lemniscus

LPB	lateral parabrachial nucleus
LPBC	lateral parabrachial nucleus, central
LPBD	lateral parabrachial nucleus, dorsal
LPBE	lateral parabrachial nucleus, external
LPBI	lateral parabrachial nucleus, internal
LPBS	lateral parabrachial nucleus, superior
LPGi	lateral paragigantocellular reticular nucleus
LRt	lateral reticular nucleus
Lve	lateral vestibular nucleus
mcp	middle cerebellar peduncle
MdD	reticular nucleus medulla, dorsal
Me5	nucleus mesencephalic tract trigeminal nerve
Med	medial cerebellar nucleus
MGD	medial geniculate nucleus, dorsal
MGV	medial geniculate nucleus, ventral
MiTg	Microcellular tegmental nucleus
Ml	medial lemniscus
ml	medial lemniscus
mlf	medial longitudinal fasciculus
MnR	Median raphe nucleus
Mo5	motor trigeminal nucleus
MPB	medial parabrachial nucleus
Mve	medial vestibular nucleus
MVeV	medial vestibular nucleus, ventral part
Op	optic nerve layer superior colliculus
OT	Nucleus optic tract
Pa5	Paratrigeminal nucleus
Pa6	Paraabducens nucleus
PCGS	Paracochlear glial substance
PCRt	Parvocellular reticular nucleus
PCRtA	Parvocellular reticular nucleus, pars alpha
PDTg	Posterodorsal tegmental nucleus
PL	Paralemniscal nucleus
PMR	Paramedian raphe
PN	Paranigral nucleus
Pn	Pontine nuclei
PnC	Pontine reticular nucleus, caudal
PnO	Pontine reticular nucleus, oral
PPT	Posterior pretectal nucleus
Pr5	Principal sensory trigeminal nucleus
Pr5DM	Principal sensory trigeminal nucleus, dorsalomedial part
Pr5VL	Principal sensory trigeminal nucleus, ventrolateral part
Prb	Probst bundle
PrH	Prepositus hypoglossal nucleus
py	Pyramidal tract
Pyx	Pyramidal tract decussation
R	red nucleus
Ramb	Retroambiguus nucleus
RAmb	Retroambiguus nucleus
Rli	rostral linear nucleus raphe
RMg	raphe magnus nucleus
Ro	Nucleus of Roller
ROb	raphe obscurus nucleus
Rpa	raphe pallidus nucleus
RPn	raphe pontis nucleus
RPO	rostral periolivary region
RR	Retrorubral nucleus
RRF	Retrorubral field
rs	Rubrospinal tract
RVL	Rostroventrolateral reticular nucleus
s5	Sensory root trigeminal nerve
scp	Superior cerebellar peduncle
SNC	Substantia nigra, pars compacta
SNL	Substantia nigra, pars lateralis
SNR	Substantia nigra, pars reticulata
SO	Superior olive
Sol	Nucleus solitary tract
SolC	Nucleus solitary tract, commissural part
SolL	Nucleus solitary tract, lateral part
SolM	nucleus solitary tract, medial part
sp5	spinal tract trigeminal nerve
Sp5C	spinal trigeminal nucleus, caudal division
Sp5I	spinal trigeminal nucleus, interpolar division
Sp5O	spinal trigeminal nucleus, oral division
Sph	sphenoid nucleus
Sph	sphenoid nucleus
SPTg	subpeduncular tegmental nucleus
SpVe	spinal vestibular nucleus
Su5	superior trigeminal nucleus
SubB	subbrachial nucleus
SubCA	subcoeruleus nucleus, alpha
SubCD	subcoeruleus nucleus, dorsal
SubCV	subcoeruleus nucleus, ventral
SuG	superficial gray layer superior colliculus
SuVe	superior vestibular nucleus
Tz	nucleus trapezoid body
Unc	uncinate fasciculus
VCA	ventral cochlear nucleus, anterior
VCP	ventral cochlear nucleus, posterior
VLL	ventral nucleus lateral lemniscus
VLPB	ventrolateral parabrachial nucleus
VLTg	ventrolateral tegmental nucleus
VTA	ventral tegmental area
VTg	ventral tegmental nucleus
X	X nucleus
xscp	Decussation superior cerebellar peduncle
Y	Y nucleus
Zo	Zonal layer superior colliculus

References

Adams, J.C. (1992) Biotin amplification of biotin and horseradish peroxidase signals in histochemical stains. *J. Histochem. Cytochem.* 40: 1457-1463.

Aldes, L.D., B. Shaw, R.B. Chronister, and J.W. Haycock (1990) Catecholamine-containing axon terminals in the hypoglossal nucleus of the rat: an immuno-electronmicroscopic study. *Exp Brain Res* 81: 167-178.

Aldes, L.D., R.B. Chronister, I. Shelton C., J.W. Haycock, L.A. Marco, and D.L. Wong (1988) Catecholamine innervation of the rat hypoglossal nucleus. *Brain Res Bull* 21: 305-312.

Alonso, G., O. Brun, J.L. Ridet, M. Geffard, and N. Chauvet (1995) Non-dopaminergic catecholaminergic neurons of mesencephalic and medullary nuclei contain different levels of dopamine. *J Chem Neuroanat* 9: 195-205.

Andén, N.-E., M.G.M. Jukes, A. Lundberg, and L. Vyklicky (1966) The effect of DOPA on the spinal cord. 1. Influence on transmission from primary afferents. *Acta Physiol. Scand.* 67: 373-386.

Andén, N.-E., M.G.M. Jukes, and A. Lundberg (1966) The effect of DOPA on the spinal cord. 2. A pharmacological analysis. *Acta Physiol. Scand.* 67: 387-397.

Ariano, M.A., and D.R. Sibley (1994) Dopamine receptor distribution in the rat CNS: elucidation using anti-peptide antisera directed against D_{1A} and D_3 subtypes. *Brain Res.* 649: 95-110.

Ariano, M.A., J. Wang, K.L. Noblett, E.R. Larson, and D.R. Sibley (1997) Cellular distribution of the rat D_4 dopamine receptor protein in the CNS using anti-receptor antisera. *Brain Res* 752: 26-34.

Ariano, M.A., R.S. Fisher, E. Smyk-Randall, D.R. Sibley, and M.S. Levine (1993) D_2 dopamine receptor distribution in the rodent CNS using anti-peptide antisera. *Brain Res.* 609: 71-80.

Armstrong, D.M., C.A. Ross, V.M. Pickel, T.H. Joh, and D.J. Reis (1982) Distribution of dopamine-, noradrenaline-, and adrenaline-containing cell bodies in the rat medulla oblongata: demonstrated by the immunocytochemical localization of catecholamine biosynthetic enzymes. *J Comp Neurol* 212: 173-187.

Asan, E. (1993) Comparative single and double immunolabeling with antisera against catecholamine biosynthetic enzymes: criteria for the identification of dopaminergic, noradrenergic and adrenergic structures in selected brain areas. *Histochemistry* 99: 427-442.

Aston-Jones, G., M.T. Shipley, and R. Grzanna (1995) The locus coeruleus, A5 and A7 noradrenergic cell groups. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, Inc., pp. 183-213.

Bandler, R., and M.T. Shipley (1994) Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? *TINS* 17: 379-389.

Bandler, R., P. Carrive, and S.P. Zhang (1991) Integration of somatic and autonomic reactions within the midbrain periaqueductal grey: Viscerotopic, somatotopic and functional organization. *Progress in Brain Res* 87: 269-305.

Barasi, S., and M.H.T. Roberts (1977) Responses of motoneurons to electrophoretically applied dopamine. *Br. J. Pharmacol.* 60: 29-34.

Barasi, S., M.M. Ben-Sreti, A.L. Clatworthy, K.N. Duggal, J.P. Gonzalez, J. Robertson, K.F. Rooney, and R.D.E. Sewell (1987) Dopamine receptor-mediated spinal antinociception in the normal and haloperidol pretreated rat: effects of sulpiride and SCH 23390. *Br. J. Pharmacol.* 90: 15-22.

Basbaum, A.I., F. Godefroy, and J. Weil-Fugazza (1987) A new microdissection technique for regional biochemical analysis of the rat spinal cord: serotonin, norepinephrine, dopamine and uric acid. *Brain Res.* 419: 229-238.

Batten, T.F.C., A. Maqbool, H.W.M. Steinbusch, and P.F.T. Vaughan (1990) Immunohistochemical and chromatographic detection of dopamine in the medulla oblongata of the cat brain. *J. Physiol.* 420?: 42P.

Baude, A., Z. Nusser, E. Molnar, R. McIlhinney, and P. Somogyi (1995) High-resolution immunogold localization of AMPA type glutamate receptor subunits at synaptic and non-synaptic sites in rat hippocampus. *Neuroscience* 69: 1031-1055.

Behbehani, M.M. (1995) Functional characteristics of the midbrain periaqueductal gray. *Progress in Neurobiology* 46: 575-605.

Beitz, A.J. (1982) The nuclei of origin of brainstem serotonergic projections to the rodent spinal trigeminal nucleus. *Neurosci. Lett.* 32: 223-228.

Beitz, A.J. (1985) The midbrain periaqueductal gray in the rat. I. Nuclear volume, cell number, density, orientation, and regional subdivisions. *J. Comp. Neurol.* 237: 445-459.

Berger, B., P. Gaspar, and C. Verney (1991) Dopaminergic innervation of the cerebral cortex: unexpected differences between rodents and primates. *TINS.* 1: 21-27.

Bishop, G.A., and R.H. Ho (1984) Substance P and serotonin immunoreactivity in the rat inferior olive. *Brain Res. Bull.* 12: 105-113.

Björklund, A., and A. Nobin (1973) Fluorescence histochemical and microspectrofluorometric mapping of dopamine and noradrenaline cell groups in the rat diencephalon. *Brain Res* 51: 193-205.

Björklund, A., and G. Skagerberg (1979) Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. *Brain Res.* 177: 170-175.

Björklund, A., and G. Skagerberg (1982) Descending monoaminergic projections to the spinal cord. In B. Sjolund and A. Bjorklund (eds): Brain Stem Control of Spinal Mechanisms, Vol. Elsevier Biomedical Press

Björklund, A., and O. Lindvall (1984) Dopamine-containing systems in the CNS. In A. Björklund and T. Hökfelt (eds):

Handbook of Chemical Neuroanatomy. Vol 2: Classical Transmitters in the CNS, Part 1, Vol. Elseviers Science, pp. 55-122.

Blaschko, H. (1939) The specific action of *L*-DOPA decarboxylase. *J. Physiol.* 96: 50-51.

Blessing, W., and J. Chalmers (1979) Direct projection of catecholamine (presumably dopamine)-containing neurons from hypothalamus to spinal cord. *Neurosci Lett* 11: 35-40.

Blessing, W.W., and J.P. Chalmers (1979) Direct projection of catecholamine (presumably dopamine)-containing neurons from hypothalamus to spinal cord. *Neurosci. Lett.* 11: 35-40.

Boundy, V.A., R.R. Luedtke, and P.B. Molinoff (1993a) Development of polyclonal anti-D_2 dopamine receptor antibodies to fusion proteins - inhibition of D_2 receptor-G protein interaction. *J. Neurochem.* 60: 2181-2191.

Boundy, V.A., R.R. Luedtke, R.P. Artymyshyn, T.M. Filtz, and P.B. Molinoff (1993b) Development of polyclonal anti-D_2 dopamine receptor antibodies using sequence-specific peptides. *Mol. Pharmacol.* 43: 666-676.

Bouthenet, M.-L., E. Souil, M.-P. Martres, P. Sokoloff, B. Giros, and J.-C. Schwartz (1991) Localization of dopamine D_3 receptor mRNA in the rat brain using *in situ* hybridization histochemistry: comparison with dopamine D_2 receptor mRNA. *Brain Res.* 564: 203-219.

Bouthenet, M.-L., M.-P. Martres, N. Sales, and J.-C. Schwartz (1987) A detailed mapping of dopamine D_2 receptors in rat central nervous system by autoradiography with [^{125}I]iodosulpiride. *Neuroscience* 20: 117-155.

Boyson, S.J., P. McGonigle, and P.B. Molinoff (1986) Quantitative autoradiographic localization of the D_1 and D_2 subtypes of dopamine receptors in rat brain. *J. Neurosci.* 6: 3177-3188.

Brichta, A.M., and G. Grant (1985) Cytoarchitectural organization of the spinal cord. In G. Paxinos (eds): The rat nervous system, Academic Press Australia, pp. 293-301.

Brock, J.W., S. Farooqui, K. Ross, and C. Prasad (1992) Localization of dopamine D_2 receptor protein in rat brain using polyclonal antibody. *Brain Res.* 578: 244-250.

Buijs, R.M., C.W. Pool, J.J. Van Heerikhuize, A.A. Sluiter, P.J. Van der Sluis, M. Ramkema, T.P. Van der Woude, and E. Van der Beek (1989) Antibodies to small transmitter molecules and peptides: production and application of antibodies to dopamine, serotonin, GABA, vasopressin, vasoactive intestinal peptide, neuropeptide Y, somatostatin and substance *P. Biomed. Res.* 10, suppl. 3: 213-221.

Buijs, R.M., M. Geffard, C.W. Pool, and E.M.D. Hoorneman (1984) The dopaminergic innervation of the supraoptic and paraventricular nucleus. A light and electron microscopic study. *Brain Res.* 32: 65-72.

Bunzow, J.R., H.H.M. Van Tol, D.K. Grandy, P. Albert, J. Salon, M. Christie, C.A. Machida, K.A. Neve, and O. Civelli (1988) Cloning and expression of a rat D_2 dopamine receptor cDNA. *Nature* 336: 783-787.

Burstein, R., R.J. Dado, and G.J. Giesler (1990) The cells of origin of the spinothalamic tract of the rat: a quantitative reexamination. *Brain Res.* 511: 329-337.

Carlsson, A. (1959) The occurrence, distribution and physiological role of catecholamines in the nervous system. *Pharm. Rev.* 11: 490-493.

Carp, J.S., and R.J. Anderson (1982) Dopamine receptor-mediated depression of spinal monosynaptic transmission. *Brain Res.* 242: 247-254.

Carp, J.S., Y. Ohno, and J.E. Warnick (1989) Prevention of phencyclidine-induced depression of the segmental reflex by *L*-3,4-dihydroxyphenylalanine in the rat spinal cord in vitro. *J. Pharmacol. Exp.* Ther. 248: 1048-1053.

Carrive, P. (1993) The periaqueductal gray and defensive behavior: functional representation and neuronal organization. Behav. *Brain Res.* 58: 27-47.

Chazot, P.L., A.J. Doherty, and P.G. Strange (1993) Antisera specific for D_2 dopamine receptors. *Biochem. J.* 289: 789-794.

Chitravanshi, V.C., and F.R. Calaresu (1992) Dopamine microinjected into the nucleus ambiguus elicits vagal bradycardia in spinal rats. *Brain Res.* 583: 308-311.

Ciliax, B.J., C. Heilman, L.L. Demchyshyn, Z.B. Pristupa, E. Ince, S.M. Hersch, H.B. Niznik, and A.I. Levey (1995) The dopamine transporter: immunochemical characterization and localization in brain. *J Neurosci 15:* 1714-23.

Ciliax, B.J., G.W. Drash, J.K. Staley, S. Haber, C.J. Mobley, G.W. Miller, E.J. Mufson, D.C. Mash, and A.I. Levey (1999) Immunocytochemical localization of the dopamine transporter in human brain. *J Comp Neurol* 409: 38-56.

Civelli, O., J.R. Bunzow, D.K. Grandy, Q.-Y. Zhou, and H.H.M. van Tol (1991) Molecular biology of the dopamine receptors. *Eur J Pharmacol - Mol Pharmacol Section* 207: 277-286.

Clatworthy, A., and S. Barasi (1987) Intrathecally administered apomorphine or LY171555 reduces nociceptive responses recorded from ventrobasal thalamic neurones in urethane anaesthetised rats. *Neurosci. Lett.* 75: 308-312.

Commissiong, J.W., and E.M. Sedgwick (1974) A pharmacological study of the adrenergic mechanisms involved in the stretch reflex of the decerebrate rat. *Br. J. Pharmacol.* 50: 365374.

Commissiong, J.W., and N.H. Neff (1979) Current status of dopamine in the mammalian spinal cord. *Biochem. Pharmacol.* 28: 1569-1573.

Commissiong, J.W., C.L. Calli, and N.H. Neff (1978) Differentiation of dopaminergic and noradrenergic neurons in rat spinal cord. *J Neurochem* 30: 1095-1099.

Compoint, C., and C. Buisseret-Delmas (1988) Origin, distribution and organization of the serotoninergic innervation in the inferior olivary complex of the rat. *Arch. Ital. Biol.* 126: 99-110.

Coote, J.H., V.H. Macleod, S. Fleetwood-Walker, and M.P. Gilbey (1981) The response of individual sympathetic preganglionic neurones to microelectrophoretically applied endogenous monoamines. *Brain Res.* 215: 135-145.

Copray, J.C., G.J. Ter Horst, R.S. Liem, and J.D. van Willigen (1990b) Neurotransmitters and neuropeptides within the mesencephalic trigeminal nucleus of the rat: an immunohistochemical analysis. *Neuroscience* 37: 399-411.

Copray, J.C., R.S. Liem, G.J. Ter Horst, and J.D. van Willigen (1990a) Dopaminergic afferents to the mesencephalic trigeminal nucleus of the rat: a light and electron microscope immunocytochemistry study. *Brain Res.* 514: 343-348.

Dahl, S., O. Edvarsen, and I. Sylte (1991) Molecular dynamics of dopamine at the D_2 receptor. *Proc Natl Acad Sci USA* 88: 8111-8115.

Dahlström, A., and K. Fuxe (1964) Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol. Scand.* 62, Suppl. 232: 5-55.

Day, H.E., S. Campeau, S.J.J. Watson, and H. Akil (1997) Distribution of alpha 1a-, alpha 1b- and alpha 1d-adrenergic receptor mRNA in the rat brain and spinal cord. *J Chem Neuroanat* 13: 115-139.

de Groat WC, Yoshiyama M, Ramage AG, Yamamoto T, Somogyi GT(1999) Modulation of voiding and storage reflexes by activation of alpha1-adrenoceptors. *Eur Urol*;36 Suppl 1:68-73

De Jong, W. (1974) Noradrenaline: central inhibitory control of blood pressure and heart rate. *Eur J. Pharmacol.* 29: 179-181.

Dearry, A., J. Gingrich, P. Falardeau, R. Fremau Jr, M. Bates, and M. Caron (1990) Molecular cloning and expression of the gene for a human D_1 dopamine receptor. *Nature* 347: 72-75.

Defagot, M.C., E.L. Malchiodi, M.J. Villar, and M.C. Antonelli (1997) Distribution of D_4 dopamine receptor in rat brain with sequence- specific antibodies. *Brain Res Mol Brain Res* 45: 1-12.

Demirjian, C., R. Grossman, R. Meyer, and R. Katzman (1976) The catecholamine pontine cellular groups locus coeruleus, A4, subcoeruleus in the primate Cebus apella. *Brain Res.* 115: 395-411.

Dietl, M., M. Arluison, P. Mouchet, C. Feuerstein, M. Manier, and J. Thibault (1985) Immunohistochemical demonstration of catecholaminergic cell bodies in the spinal cord of the rat. *Histochemistry* 82: 385-389.

Doyle, C.A., and D.J. Maxwell (1991) Ultrastructural analysis of noradrenergic nerve terminals in the cat lumbosacral spinal dorsal horn: a dopamine-β-hydroxylase immunocytochemical study. *Brain Res.* 563: 329-333.

Dubois, A., M. Savasta, O. Curet, and B. Scatton (1986) Autoradiographic distribution of the D_1 agonist [3H]SKF 38393, in the rat brain and spinal cord. Comparison with the distribution of D_2 dopamine receptors. *Neuroscience* 19: 125-137.

Dupelj, M., and J. Geber (1981) Dopamine as a possible neurotransmitter in the spinal cord. *Neuropharmacology* 20: 145-148.

Espey MJ, Du HJ, Downie JW (1998) Serotonergic modulation of spinal ascending activity and sacral reflex activity evoked by pelvic nerve stimulation in cats. Brain Res. 798:101-8.

Faye-Lund, H. and K.K. Osen (1985) Anatomy of the inferior colliculus in rat. *Anat. Embryol.* 171: 1-20.

Feldman, P.D., and F.J. Galiano (1995) Evidence against a hemodynamic role for serotonin in the dorsal motor neucleus of the vagus. *Brain Res. Bull.* 37: 457-462.

Ferré, S., and K. Fuxe (1992) Dopamine denervation leads to an increase in the intramembrane interaction between adenosine A2 and dopamine D_2 receptors in the neostriatum. Brain Res 594: 124-130.

Ferré, S., R. Cortes, and F. Artigas (1994) Dopaminergic regulation of the serotonergic raphe-striatal pathway: microdialysis studies in freely moving rats. *J. Neurosci.* 14: 4839-4846.

Fisher, R.S., M.S. Levine, D.R. Sibley, and M.A. Ariano (1994) D_2 dopamine receptor protein location: golgi impregnation-gold toned and ultrastructural analysis of the rat neostriatum. *J. Neurosci. Res.* 38: 551-564.

Fleetwood-Walker, S.M., and J.H. Coote (1981) Contribution of noradrenaline-, dopamine- and adrenaline-containing axons to the innervation of different regions of the spinal cord of the cat. *Brain Res.* 206: 95-106.

Fleetwood-Walker, S.M., P.J. Hope, and R. Mitchell (1988) Antinociceptive actions of descending dopaminergic tracts on cat and rat dorsal horn somatosensory neurones. *J. Physiol. (Lond.)* 399: 335-348.

Frazer, A., and J.G. Hensler (1994) Serotonin. In G.J. Siegel *et al.* (eds): Basic Neurochemistry: Molecular, Cellular, and Medical Aspects, Vol. New York: Raven Press, Ltd, pp. 283-308.

Fremeau, R.T., Jr, G.E. Duncan, M.-G. Fornaretto, A. Dearry, J.A. Gingrich, G.R. breese, and M.G. Caron (1991) Localization of D_1 dopamine receptor mRNA in brain supports a role in cognitive, affective, and neuroendocrine aspects of dopaminergic neurotransmission. *Proc. Natl. Acad. Sci. USA* 88: 3772-3776.

Fritschy, J.-M., and R. Grzanna (1989) Immunohistochemical analysis of the neurotoxic effects of DSP-4 identifies two populations of noradrenergic axon terminals. *Neuroscience* 30: 181-197.

Fritschy, J.-M., and R. Grzanna (1990) Demonstration of two separate descending noradrenergic pathways to the rat spinal cord: evidence for an intragriseal trajectory of locus coeruleus axons in the superficial layers of the dorsal horn. *J. Comp. Neurol.* 291: 553-582.

Fritschy, J.-M., and R. Grzanna (1991) Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? *Progr. Brain Res.* 88: 257-268.

Fritschy, J.-M., M. Geffard, and R. Grzanna (1990) The response of noradrenergic axons to systemically administered DSP-4 in the rat: an immunohistochemical study using antibodies to noradrenaline and dopamine-B-hydroxylase. *J. Chem. Neuroanat.* 3: 1-13.

Fritschy, J.-M., W.E. Lyons, C.A. Mullen, B.E. Kosofsky, M.E. Molliver, and R. Grzanna (1987) Distribution of locus coeruleus axons in the rat spinal cord: a combined anterograde transport and immunohistochemical study. *Brain Res.*

437: 176-180.
Fung, S.J., K. Reddy, R.H. Liu, Z. Wang, and C.D. Barnes (1994) Existence of glutamate in noradrenergic locus coeruleus neurons of rodents. *Brain Res. Bull.* 35: 505-512.
Fuxe, K. (1965) Evidence for the existence of monoamine neurons in the central nervous system. III. The monoamine nerve terminal. *Zeitschrift fur Zellforschung* 65: 573-596.
Fuxe, K., and T. Hokfelt (1966) Further evidence for the existence of tubero-infundibular dopamine neurons. *Acta Physiol Scand* 66: 245-246.
Fuxe, K., B. Tinner, B. Bjelke, L.F. Agnati, A. Verhofstad, H.G.W. Steinbusch, M. Goldstein, and M. Kalia (1990) Monoaminergic and peptidergic innervation of the intermediolateral horn of the spinal cord. *Eur. J. Neurosci.* 2: 430-450.
Fuxe, K., T. Hokfelt, O. Johansson, G. Jonsson, P. Lidbrik, and A. Ljungdahl (1974) The origin of the dopamine nerve terminals in limbic and frontal cortex. Evidence for mesocortico dopamine neurons. *Brain Res* 82: 349-355.
Gabella, G. (1995) Autonomic nervous system. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, Ca, USA: Academic Press, Inc., pp. 39-45.
Gajendiran, M., P. Seth and D.K. Ganguly (1996) Involvement of the presynaptic dopamine D_2 receptor in the depression of spinal reflex by apomorphine. *Neuroreport.* 7: 513-516
Gandelman, K.-Y., S. Harmon, R.D. Todd, and K.L. O'Malley (1991) Analysis of the structure and expression of the human dopamine D_2 receptor gene. *J. Neurochem.* 56: 1024-1029.
Gasbarri, A., C. Verney, R. Innocenzi, E. Campana, and C. Pacitti (1994) Mesolimbic dopaminergic neurons innervating the hippocampal formation in the rat: a combined retrograde tracing and immunohistochemical study. *Brain Res.* 668: 71-79.
Geffard, M., R.M. Buijs, P. Seguela, C.W. Pool, and M. Le Moel (1984) First demonstration of highly specific and sensitive antibodies against dopamine. *Brain Res.* 294: 161-165.
Geffard, M., S. Patel, J. Dulluc, and A.-M. Rock (1986) Specific detection of noradrenaline in the rat brain by using antibodies. *Brain Res.* 363: 395-400.
Gerfen, C.R., T.M. Engber, L.C. Mahan, Z. Susel, T.N. Chase, F.J. Monsma Jr., and D.R. Sibley (1990) D_1 and D_2 dopamine receptor-regulated gen expression of striatonigral and striatopallidal neurons. *Science* 250: 1429-1431.
Gingrich, J.A., and M.G. Caron (1993) Recent advances in the molecular biology of dopamine receptors. *Annu. Rev. Neurosci.* 16: 299-321.
Giros, B., P. Sokoloff, M.-P. Martres, J.-F. Riou, L.J. Emorine, and J.-C. Schwartz (1989) Alternative splicing directs the expression of two D_2 dopamine receptor isoforms. *Nature* 342: 923-926.
Glazer, E.J., and L.L. Ross (1980) Localization of noradrenergic terminals in sympathetic preganglionic nuclei of the rat: demonstration by immunocytochemical localization of dopamine-β-hydroxylase. *Brain Res.* 185: 39-49.
Gobert, A., J.-M. Rivet, A. Audinot, A. Newman-Tancredi, L. Cistarelli, and M.J. Millan (1998) Simultaneous quantification of serotonin, dopamine and noradrenaline levels in single frontal cortex dialysates of freely-moving rats reveals a complex pattern of reciprocal auto- and heteroreceptor-mediated control of release. *Neuroscience* 84: 413-429.
Granata, A.R., and G.N. Woodruff (1982) Dopaminergic mechanisms in the nucleus tractus solitarius and effects on blood pressure. *Brain Res. Bull.* 8: 483-488.
Grandy, D., and O. Civelli (1992) G-protein coupled receptors: the new dopamine receptor subtypes. Current Opinion in Neurobiology 2: 275-281.
Grant, G. (1995) Primary afferent projections to the spinal cord. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, Ca, USA: Academic Press, Inc., pp. 61-66.
Grant, G., B. Wiksten, K.J. Berkley, and H. Aldskogius (1982) The location of cerebellar-projecting neurons within the lumbosacral spinal cord in the cat. An anatomical study with HRP and retrograde chromatolysis. *J. Comp. Neurol* 204: 336-348.
Green, J.P. (1994) Histamine. In G.J. Siegel *et al.* (eds): Basic Neurochemistry: Molecular, Cellular, and Medical Aspects, Vol. New York: Raven Press, Ltd, pp. 309-319.
Grillner, S. (1975) Locomotion in vertebrates: central mechanisms and reflex interaction. *Physiol. Rev.* 55: 247-304.
Grzanna, R., and J.-M. Fritschy (1991) Efferent projections of different subpopulations of central noradrenaline neurons. *Progr in Brain Res* 88: 89-101.
Grzanna, R., W.K. Chee, and E.W. Akeyuson (1987) Noradrenergic projections to brainstem nuclei: evidence for differential projections from noradrenergic subgroups. *J. Comp. Neurol.* 263: 76-91.
Hagihira, S., E. Senba, M. Yoshida, M. Tohyama, and I. Yoshiya (1990) Fine structure of noradrenergic terminals and their synapse in the rat spinal dorsal horn: an immunohistochemical study. *Brain Res.* 526: 73-80.
Haines, D.E. (1997) Fundamental Neuroscience. New York: Churchill Livingstone
Halliday, G., A. Harding, and G. Paxinos (1995) Serotonin and tachykinin systems. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, Inc., pp. 929-974.
Hedeman, L.S., M.K. Shellenberger, and J.H. Gordon (1974) Studies in experimental spinal cord trauma. Part I: Alterations in catecholamine levels. *J. Neurosurg.* 40: 37-43.
Hedreen, J. (1980) Terminal degeneration demonstrated by the Fink-Heimer method following lateral ventricular injection of 6-hydroxydopamine. *Brain Res. Bull.* 5: 425-436.

Herbert, H., and C. Saper (1992) Organization of medullary adrenergic and noradrenergic projections to the periaqueductal gray matter in the rat. *J. Comp. Neurol.* 315: 34-52.

Herbert, H., M.M. Moga, and C.B. Saper (1990) Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. *J. Comp. Neurol.* 293: 540-580.

Herkenham, M. (1987) Mismatches between neurotransmitter and receptor localizations in brain: observations and implications. *Neurosci* 23: 1-38.

Hersch, S.M., B.J. Ciliax, C.A. Gutekunst, H.D. Rees, C.J. Heilman, K.K. Yung, J.P. Bolam, E. Ince, H. Yi, and A.I. Levey (1995) Electron microscopic analysis of D_1 and D_2 dopamine receptor proteins in the dorsal striatum and their synaptic relationships with motor corticostriatal afferents. *J. Neurosci.* 15: 5222-1537.

Hoffman, B.J., S.R. Hansson, E. Mezey, and M. Palkovits (1998) Localization and dynamic regulation of biogenic amine transporters in the mammalian central nervous system. *Front Neuroendocrinol* 19: 187-231.

Hökfelt, T., O. Johansson, and M. Goldstein (1984) Central catecholamine neurons as revealed by immunohistochemistry with special reference to adrenaline neurons. In A. Björklund and T. Hökfelt (eds): Handbook of chemical neuroanatomy., Vol. 2, part1. Amsterdam: Elsevier, pp. 157-276.

Hökfelt, T., O. Phillipson, and M. Goldstein (1979) Evidence for a dopaminergic pathway in the rat descending from the A11 cell group to the spinal cord. *Acta Physiol. Scand.* 107: 393-395.

Holets, V., and R. Elde (1982) The differential distribution and relationship of serotoninergic and peptidergic fibers to sympathoadrenal neurons in the intermediolateral cell column of the rat: a combined retrograde axonal transport and immunofluorescence study. *Neuroscience* 7: 1155-1174.

Holstege, G. (1991) Descending motor pathways and the spinal motor system: limbic and non- limbic components. *Progr. Brain Res.* 87: 307-421.

Holstege, J.C. (1996) The ventro-medial medullary projections to spinal motoneurons: ultrastructure, transmitters and functional aspects. *Progr. Brain Res. 107:* 159-181.

Holstege, J.C. (1996) The ventromedial medullary projections to spinal motoneurons: ultrastructure, transmitters and functional aspects. In G. Holstege, R. Bandler and C.B. Saper (eds): The Emotional Motor System. Progress in Brain Research, Vol. 107. Amsterdam: Elsevier, pp. 159-181.

Holstege, J.C., and H.G.J.M. Kuypers (1987) Brainstem projections to spinal motoneurons: an update. *Neuroscience* 23: 809-821.

Holstege, J.C., H. van Dijken, R.M. Buijs, H. Goedknegt, T. Gosens, and C.M.H. Bongers (1996) Distribution of dopamine immunoreactivity in the rat, cat, and monkey spinal cord. *J. Comp. Neurol.* 376: 631-652.

Hosoya, Y., N. Okado, Y. Sugiura, and K. Kohno (1991) Coincidence of "ladder-like patterns" in distributions of monoaminergic terminals and sympathetic preganglionic neurons in the rat spinal cord. *Exp. Brain Res.* 86: 224-228.

Hozawa, K., and T. Takasaka (1993) Catecholaminergic innervation in the vestibular labyrinth and vestibular nucleus of guinea pigs. *Acta Otolaryng. Suppl.* 503: 111-113.

Hyde, T.M., M.B. Knable, and A.M. Murray (1996) Distribution of dopamine D_1-D_4 receptor subtypes in human dorsal vagal complex. *Synapse* 24: 224-32.

Ikai, Y., M. Takada, Y. Shinonaga, and N. Mizuno (1992) Dopaminergic and non-dopaminergic neurons in the ventral tegmental area of the rat project, respectively, to the cerebellar cortex and deep cerebellar nuclei. *Neuroscience* 51: 719-728.

Jacobs, B.L., and C.A. Fornal (1999) Activity of serotonergic neurons in behaving animals. *Neuropsychopharmacology* 21: 9S-15S.

Jacobs, B.L., and E.C. Azmitia (1992) Structure and function of the brain serotonin system. *Phyiological Reviews* 72: 165-227.

Jaeger, C.B., D.A. Ruggiero, V.R. Albert, D.H. Park, T.H. Joh, and D.J. Reis (1984) Aromatic L-amino acid decarboxylase in the rat brain: immunocytochemical localization in neurons of the brain stem. *Neuroscience* 11: 691-713.

Jankowska, E. (1992) Interneuronal relay in spinal pathways from proprioceptors. *Progr. Neurobiol.* 38: 335-378.

Jensen, T.S. (1986) Endogenous antinociceptive systems: studies on spinal and supraspinal modulating mechanisms with particular reference to monoaminergic and opioid systems. *Acta Neurol. Scand.* 74 Suppl: 108: 6-34.

Johnson RA, Johnson SM, Mitchell GS (1998) Catecholaminergic modulation of respiratory rhythm in an in vitro turtle brain stem preparation. *J Appl Physiol* 85:105-14

Jonsson, G. (1983) Chemical lesioning techniques: monoamine neurotoxins. In A. Björklund and T. Hökfelt (eds): Methods in chemical neuroanatomy, Handbook of chemical neuroanatomy, Vol. I. Amsterdam: Elsevier, pp. 463-496.

Jurna, I., and A. Lundberg (1968) The influence of an inhibitor of dopamine-beta-hydroxylase on the effect of dopa on transmission in the spinal cord. In C. von Euler, S. Skoglund and U. Söderberg (eds): Structure and function of inhibitory neuronal mechanisms, Wenner-Gren Center International Symposion Series, Vol. 10. Oxford: Pergamon Press, pp. 469-472.

Kalia, M., K. Fuxe, and M. Goldstein (1985) Rat medulla oblongata. II. Dopaminergic, noradrenergic (A1 and A2) and adrenergic neurons, nerve fibers, and presumptive terminal processes. *J. Comp. Neurol.* 233: 308-332.

Kalia, M., K. Fuxe, M. Goldstein, A. Harfstrand, L.F. Agnati, and J.T. Coyle (1984) Evidence for the existence of putative dopamine-, adrenaline- and noradrenaline-containing vagal motor neurons in the brainstem of the rat.

Neurosci Lett 50: 57-62.
Kamei, I., S. Shiosaka, E. Senba, H. Takagi, M. Sakanaka, S. Inagaki, K. Takasuki, K. Nakai, H. Imai, T. Itakura, N. Komai, and M. Tohyama (1981) Comparative anatomy of the distribution of catecholamines within the olivary complex from teleosts to primates. *J. Comp. Neurol.* 202: 125-133.
Kamijo, N., T. Nagao, and H. Ono (1993) Depression of the monosynaptic reflex by apomorphine or bromocriptine is not mediated by D_1/D_2 receptors. *Neuropharmacology* 32: 777-783.
Kanaseki, T. and J.M. Sprague (1974) Anatomical organization of pretectal nuclei and tectal laminae in the cat. *J. Comp. Neurol.* 158: 319-337.
Kaneko, T., H. Akiyama, I. Nagatsu, and N. Mizuno (1990) Immunohistochemical demonstration of glutaminase in catecholaminergic and serotoninergic neurons of rat brain. *Brain Res.* 507: 151-154.
Karoum, F., J.C. Commissiong, N.H. Neff, and R.J. Wyatt (1981) Regional differences in catecholamines formation and metabolism in the rat spinal cord. *Brain Res.* 212: 361-366.
Kebabian, J.W. and D.B. Calne (1979) Multiple receptors for dopamine. *Nature* 227: 93-96.
Kennis JH and Holstege JC (1997) A differential and time-dependent decrease in AMPA-type glutamate receptor subunits in spinal motoneurons after sciatic nerve injury. *Exp Neurol* 147:18-27
Kessler, J.P., and A. Jean (1986) Effect of catecholamines on the swallowing reflex after pressure microinjections into the lateral solitary complex of the medulla oblongata. *Brain Res.* 386: 69-77.
Khan, Z.U., A. Gutierrez, R. Martin, A. Penafiel, A. Rivera, and A. De La Calle (1998) Differential regional and cellular distribution of dopamine D_2-like receptors: an immunocytochemical study of subtype-specific antibodies in rat and human brain. *J Comp Neurol* 402: 353-71.
Kiritsy-Roy, J.A., B.C. Shyu, P.J. Danneman, T.J. Morrow, C. Belczynsky, and K.L. Casey (1994) Spinal antinociception mediated by a cocaine-sensitive dopaminergic supraspinal mechanism. *Brain Res.* 644: 109-116.
Klepper, A., and H. Herbert (1991) Distribution and origin of noradrenergic and serotonergic fibers in the cochlear nucleus and inferior colliculus of the rat. *Brain Res.* 557: 190-201.
Kojima, M., and Y. Sano (1983) The organization of serotonin fibers in the anterior column of the mammalian spinal cord. An immunohistochemical study. *Anat. Embryol.* 167: 1-11.
Kojima, M., and Y. Sano (1984) Sexual differences in the topographical distribution of serotonergic fibers in the anterior column of the rat lumbar spinal cord. *Anat. Embryol.* 170: 117-121.
Kojima, M., T. Matsuura, A. Tanaka, T. Amagai, J. Imanishi, and Y. Sano (1985) Characteristic distribution of noradrenergic terminals on the anterior horn motoneurons innervating the perineal striated muscles in the rat. *Anat. Embryol.* 171: 267-273.
Kojima, M., Y. Takeuchi, M. Goto, and Y. Sano (1983a) Immunohistochemical study on the localization of serotonin fibers and terminals in the spinal cord of the monkey (*Macaca fuscata*). *Cell Tissue Res.* 229: 23-36.
Kojima, M., Y. Takeuchi, M. Kawata, and Y. Sano (1983b) Motoneurons innervating the cremaster muscle of the rat are characteristically densely innervated by serotonergic fibers as revealed by combined immunohistochemistry and retrograde fluorescent DAPI-labeling. *Anat. Embryol.* 168: 41-49.
Kolta, A., R. Dubuc, and J.P. Lund (1993) An immunocytochemical and autoradiographic investigation of the serotonergic innervation of trigeminal mesencephalic and motor nuclei in the rabbit. *Neuroscience* 53: 1113-1126.
Kondo, M., H. Fujiwara, and C. Tanaka (1985) Autoradiographic evidence for dopaminergic innervation in guinea pig spinal cord. *Jap. J. Pharmacol.* 38: 442-444.
Kontani, H., T. Inoue, and T. Sakai (1990) Effects of apomorphine on urinary bladder motility in anesthetized rats. *Jpn. J. Pharmacol.* 52: 59-67.
Kromer, L.F., and R.Y. Moore (1980) Norepinephrine innervation of the cochlear nuclei by locus coeruleus neurons in the rat. *Anat. Embryol.* 158: 227-244.
Krukoff, T.L. (1987) Peptidergic inputs to sympathetic preganglionic neurons. *Can. J. Physiol. Pharmacol.* 65: 1619-1623.
Kuypers, H.G.J.M. (1981) Anatomy of the descending pathways. In J.M. Brookhart, V.B. Mountcastle, V.B. Brooks and S.R. Geiger (eds): Handbook of Physiology, the Nervous System Vol. II, Motor Control part I, Vol. Bethesda, Maryland: American Physiological Society, pp. 597-665.
Kuzuhara, S., and S.M. Chou (1980) Localization of the phrenic nucleus in the rat: a HRP study. *Neurosci. Lett.* 16: 119-124.
Lahlou, S., and P. Demenge (1993) Cardiovascular responses to intrathecal dopamine receptor agonists after spinal transection in conscious rats. Cardiovasc. Res. 27: 222-230.
Lahlou, S., P. Petitjean, G. Pellissier, P. Mouchet, C. Feuerstein, and P. Demenge (1990) Rostrocaudal localization of cardiovascular responses induced by intrathecal administration of apomorphine in conscious, freely moving rats. *J. Cardiovasc. Pharmacol.* 16: 331-337.
LaMotte, C.C., and N.C. Lanerolle (1983) Ultrastructure of chemically defined neuron systems in the dorsal horn of the monkey. III. Serotonin immunoreactivity. *Brain Res.* 274: 65-77.
Lanau, F., M.T. Zenner, O. Civelli, and D.S. Hartman (1997) Epinephrine and norepinephrine act as potent agonists at the recombinant human dopamine D_4 receptor. *J. Neurochem.* 68: 804-812.
Lawrence, A.J., E. Krsrtew, and B. Jarrott (1995) Functional dopamine D_2 receptors on rat vagal afferent neurones. *Br. J. Pharmacol.* 114: 1329-1334.

Lazarov, N.E., and C.N. Chouchkov (1995) Serotonin-containing projections to the mesencephalic trigeminal nucleus of the cat. *Anat. Rec.* 241: 136-142.

Levey, A.I., S.M. Hersch, D.B. Rye, R.K. Sunahara, H.B. Niznik, C.A. Kitt, D.L. Price, R. Maggio, M.R. Brann, and B.J. Ciliax (1993) Localization of D_1 and D_2 dopamine receptors in brain with subtype-specific antibodies. *Proc. Natl. Acad. Sci., U.S.A.* 90: 8861-8865.

Lewis, D.I., and J.H. Coote (1990) Excitation and inhibition of rat sympathetic preganglionic neurones by catecholamines. *Brain Res.* 530: 229-234.

Liem, R.S., J.C. Copray, and J.D. van Willigen (1993) Serotonin-immunoreactive terminals in the mesencephalic trigeminal nucleus of the rat: an electron microscopic immunocytochemical study. *Acta Anat.* 148: 34-41.

Lindvall, O., A. Björklund, and G. Skagerberg (1983) Dopaminecontaining neurons in the spinal cord: anatomy and some functional aspects. *Ann. Neurol.* 14: 255-260.

Liu, Q.-S., J.-T. Qiao, and N. Dafny (1992) D_2 dopamine receptor involvement in spinal dopamine-produced antinociception. *Life Sci.* 51: 1485-1492.

Liu, R.-H., S.J. Fung, V.K. Reddy, and C.D. Barnes (1995) Localization of glutamatergic neurons in the dorsolateral pontine tegmentum projection to the spinal cord of the cat with a proposed role of glutamate on lumbar motoneuron activity. *Neuroscience* 64: 193-208.

Lorang, D., S.G. Amara, and R.B. Simerly (1994) Cell-type-specific expression of catecholamine transporters in the rat brain. *J Neurosci* 14: 4903-14.

Lu, Y., Y.-J. Du, B.-Z. Qin, and J.-S. Li (1993) The subdivisions of the intermediolateral nucleus in the sacral spinal cord of the cat. *Brain Res.* 632: 351-355.

Lyons, W.E., and R. Grzanna (1988) Noradrenergic neurons with divergent projections to the motor trigeminal nucleus and the spinal cord: a double retrograde neuronal labeling study. *Neuroscience* 26: 681-693.

Lyons, W.E., J.-M. Fritschy, and R. Grzanna (1989) The noradrenergic neurotoxin DSP-4 eliminates the coeruleospinal projection but spares projections of the A5 and A7 groups to the ventral horn of the rat spinal cord. *J. Neurosci.* 9: 1481-1489.

MacDonald, E., and M. Scheinin (1995) Distribution and pharmacology of alpha 2-adrenoceptors in the central nervous system. *J Physiol Pharmacol* 46: 241-258.

Maeda, T., K. Kitahama, and M. Geffard (1994) Dopaminergic innervation of rat locus coeruleus: a light and electron microscopic immunohistochemical study. *Microscopy research and technique* 29: 211-218.

Magnusson, T. (1973) Effect of chronic transection on dopamine, noradrenaline and 5-hydroxytryptamine in the rat spinal cord. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 278: 13-22.

Maitra, K.K., P. Seth, M. Thewissen, and H.-G. Ross (1993) Dopaminergic influence on the excitability of antidromically activated Renshaw cells in the lumbar spinal cord of the rat. *Acta Physiol. Scand.* 148: 101-107.

Mansour, A., F. Meng, J. Meador-Woodruff, L. Taylor, O. Civelli, and H. Akil (1992) Site-directed mutagenesis of the human dopamine D_2 receptor. *Eur J Pharmacol* 227: 205-214.

Mansour, A., J.H. Meador-Woodruff, J.R. Bunzow, O. Civelli, H. Akil, and S.J. Watson (1990) Localization of dopamine D_2 receptor mRNA and D_1 and D_2 receptor binding in the rat brain and pituitary: An *in situ* hybridization-receptor autoradiographic analysis. *J. Neurosci.* 10: 2587-2600.

Mansour, A., J.H. Meador-Woodruff, Q. Zhou, O. Civelli, H. Akil, and S.J. Watson (1992) A comparison of D_1 receptor binding and mRNA in rat brain using receptor autoradiographic and *in situ* hybridization techniques. *Neuroscience* 46: 959-971.

Maqbool, A., T.F.C. Batten, P.A. Berry, and P.N. McWilliam (1993) Distribution of dopamine-containing neurons and fibres in the feline medulla oblongata: a comparative study using catecholamine-synthesizing enzyme and dopamine immunohistochemistry. *Neuroscience* 53: 717-733.

Marlier, L., F. Sandillon, P. Poulat, N. Rajaofetra, M. Geffard, and A. Privat (1991) Serotonergic innervation of the dorsal horn of rat spinal cord: light and electron microscopic immunocytochemical study. *J. Neurocytol.* 20: 310-322.

Matsushita, M., and T. Ueyama (1973) Ventral motor nucleus of the cervical enlargement in some mammals; its specific afferents from the lower cord levels and cytoarchitecture. *J. Comp. Neurol.* 150: 33-52.

Matsushita, M., and Y. Hosoya (1979) Cells of origin of the spinocerebellar tract in the rat, studied with the method of retrograde transport of horseradish peroxidase. *Brain Res.* 173: 185-200.

McCune, S.K., M.M. Voigt, and J.M. Hill (1993) Expression of multiple alpha adrenergic receptor subtype messenger RNAs in the adult rat brain. *Neuroscience* 57: 143-151.

McKenna, K.E., and I. Nadelhaft (1986) The organization of the pudendal nerve in the male and female rat. *J. Comp. Neurol.* 248: 532-549.

McRae-Deguerce, A., L. Rosengren, K. Haglid, I. Augustsson, and A. Dahlström (1988) Dopaminergic terminals in the rat locus coeruleus: an immunocytochemical investigation. *Acta Physiol. Scand.* 133: 583-585.

McVittie, L.D., M.A. Ariano, and D.R. Sibley (1991) Characterization of anti-peptide antibodies for the localization of D_2 dopamine receptors in rat striatum. *Proc. Natl. Acad. Sci., U.S.A.* 88: 1441-1445.

Meador-Woodruff, J., A. Mansour, D. Healy, R. Kuehn, Q.-Y. Zhou, J. Bunzow, H. Akil, O. Civelli, and S. Whatson (1991) Comparison of the distributions of D_1 and D_2 dopamine receptor mRNAs in rat brain. *NeuroPsychopharmacology* 5: 231-242.

Meador-Woodruff, J.H., A. Mansour, J.R. Bunzow, H.H.M. van Tol, S.J. Watson Jr., and O. Civelli (1989) Distribution

of D_2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci.* 86: 7625-7628.
Meister, B., T. Hökfelt, H.W.M. Steinbusch, G. Skagerberg, O. Lindvall, M. Geffard, T.H. Joh, A.C. Cuello, and M. Goldstein (1988) Do tyrosine hydroxylase-immunoreactive neurons in the ventrolateral arcuate nucleus produce dopamine or only *L*-DOPA. *J. Chem. Neuroanat.* 1: 59-64.
Mendlin, A., F. Martin, and B. Jacobs (1998) Involvement of dopamine D_2 receptors in apomorphine-induced facilitation of forebrain serotonin output. *Eur J Pharmacol* 351: 291-298.
Molander, C., and G. Grant (1995) Spinal cord cytoarchitecture. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, Ca, USA: Academic Press, Inc., pp. 39-45.
Molander, C., C. Rivero-Melian, and G. Grant (1989) Cytoarchitectonic organization of the spinal cord in the rat: II. The cervical and upper thoracic cord. *J Comp Neurol* 239: 375-385.
Molander, C., Q. Xu, and G. Grant (1984) The cytoarchitectonic organization of the spinal cord in the rat I. The lower thoracic and lumbosacral cord. *J Comp Neurol* 230: 133-141.
Monsma, J., F.J., L.D. McVittie, C.R. Gerfen, L.C. Mahan, and D.R. Sibley (1989) Multiple D_2 dopamine receptors produced by alternative RNA splicing. *Nature* 342: 926-929.
Montagu, K.A. (1957) Catechol compounds in rat tissues and in brains of different animals. *Nature* 180: 244-245.
Mooney, R.D., C. Bennett-Clarke, N.L. Chiaia, N. Sahibzada and R.W. Rhoades (1990) Organization and actions of the noradrenergic input to the hamster's superior colliculus. *J. Comp. Neurol.* 292: 214-230.
Moons, L., J. van Gils, E. Ghijsels, and F. Vandesande (1994) Immunocytochemical localization of *L*-DOPA and dopamine in the brain of the chichen (Gallus Domesticus). *J. Comp. Neurol.* 346: 97-118.
Moore, R.Y., and F.E. Bloom (1979) Central catecholamine neuron systems: Anatomy and physiology of the norepinephrine and epinephrine systems. Ann. Rev. Neurosci. 2: 113-168.
Mouchet, P., B. Guerin, and C. Feuerstein (1982) Dissociate destruction of noradrenaline and dopamine descending projections in the thoracic spinal cord of the rat. *Life Sci.* 30: 373-381.
Mouchet, P., M. Manier, and C. Feuerstein (1992) Immunohistochemical study of the catecholaminergic innervation of the spinal cord of the rat using specific antibodies against dopamine and noradrenaline. *J. Chem. Neuroanat.* 5: 427-440.
Mouchet, P., M. Manier, M. Dietl, C. Feuerstein, A. Berod, M. Arluison, L. Denoroy, and J. Thibault (1986) Immunohistochemical study of catecholaminergic cell bodies in the rat spinal cord. *Brain Res. Bull.* 16: 341-353.
Murakoshi T, Suzue T, Tamai S (1985) A pharmacological study on respiratory rhythm in the isolated brainstem-spinal cord preparation of the newborn rat. *Br J Pharmacol* 86:95-104
Nagy, J.I., and E. Senba (1985) Neural relations of cremaster motoneurons, spinal cord systems and the genitofemoral nerve in the rat. *Brain Res. Bull.* 15: 609-627.
Nakagawa, S. (1980) Onuf's nucleus of the sacral cord in a south american monkey (Saimiri): its location and bilateral cortical input from area 4. *Brain Res.* 191: 337-344.
Newman-Tancredi, A., V. Audinot-Bouchez, A. Gobert, and M.J. Millan (1997) Noradrenaline and adrenaline are high affinity agonists at dopamine D_4 receptors. *Eur. J. Pharmacol.* 319: 379-383.
Newton, B.W. (1990) Peptidergic innervation of the cremaster nucleus I. A sexually dimorphic population of substance Pcontaining intraspinal neurons exists in the substance P pathway to the rat cremaster nucleus. *Brain Res.* 537: 187--196.
Newton, B.W., and W. Hamill (1988) Neuropeptide Y immunoreactivity is preferentially located in rat lumbar sexually dimorphic nuclei. *Neurosci. Lett* 94: 10-16.
Nicholas, A.P., V. Pieribone, and T. Hokfelt (1993) Distributions of mRNAs for alpha-2 adrenergic receptor subtypes in rat brain: an *in situ* hybridization study. *J Comp Neurol* 328: 575-594.
Nicholas, A.P., V.A. Pieribone, U. Arvidsson, and T. Hokfelt (1992) Serotonin-, substance P- and glutamate/aspartate-like immunoreactivities in medullo-spinal pathways of rat and primate. *Neurosci* 48: 545-559.
Nieuwenhuys, R. (1985). In R. Nieuwenhuys (eds): Chemoarchitecture of the brain. Berlin, Heidelberg, New York, Tokio. Springer-Verlag, pp. 11-44
Norgren, R. (1995) Gustatory system. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, pp. 751-771.
Nosjean, A., B. Franc, and R. Laguzzi (1995) Increased sympathetic nerve discharge without alteration in the sympathetic baroreflex response by serotonin3 receptor stimulation in the nucleus tractus solitarius of the rat. *Neurosci. Lett.* 186: 41-44.
O'Dowd, B. (1992) Repeat sequences in the gene encoding the human D_4 dopamine receptor. *Gene* 118: 301-302.
O'Dowd, B.F. (1993) Structures of dopamine receptors. *J. Neurochem.* 60: 804-816.
Olazabal, U.E. and J.K. Moore (1989) Nigrotectal projection to the inferior colliculus: horseradish peroxidase transport and tyrosine hydroxylase immunohistochemical studies in rats, cats, and bats. *J. Comp. Neurol.* 282: 98-118.
O'Malley, K.L., K.J. Mack, K.-Y. Gandelman, and R.D. Todd (1990) Organization and expression of the rat D_2A receptor gene: Identification of alternative transcripts and a variant donor splice site. *Biochemistry* 29: 1367-1371.
Ono, H., and H. Fukuda (1984) Effect of methamphetamine on rat spinal cord. *Neuropharmacology* 23: 637-642.
Panagopoulos, N.T., G.C. Papadopoulos, and N.A. Matsokis (1991) Dopaminergic innervation and binding in the rat cerebellum. *Neurosci. Lett.* 130: 208-212.
Paré, M., L. Descarries, and L. Wiklund (1987) Innervation of rat inferior olive by neurons containing serotonin and

substance P: an immunohistochemical study after 5,6-dihydroxytryptamine lesioning. *J. Neurocytol.* 16: 155-167.
Pearson, J.C., and L. Jennes (1988) Localization of serotonin- and substance P-like immunofluorescence in the caudal spinal trigeminal nucleus of the rat. *Neurosci. Lett.* 88: 151-156.
Pehek, E.A., J.T. Thompson, and E.M. Hull (1989) The effects of intrathecal administration of the dopamine agonist apomorphine on penile reflexes and copulation in the male rat. *Psychopharmacol.* 99: 304-308.
Pellissier, G., and P. Demenge (1991) Hypotensive and bradycardic effects elicited by spinal dopamine receptor stimulation: effects of D_1 and D_2 receptor agonists and antagonists. *J. Cardiovasc. Pharmacol.* 18: 548-555.
Petitjean, P., P. Mouchet, G. Pellissier, M. Manier, C. Feuerstein, and P. Demenge (1984) Cardiovascular effects in the rat of intrathecal injections of apomorphine at the thoracic spinal cord level. *Eur. J. Pharmacol.* 105: 355-359.
Plug, M.J., J. Dijk, J.A. Maassen, and W. Möller (1992) An anti-peptide antibody that recognizes the dopamine D_2 receptor from bovine striatum. *Eur. J. Biochem.* 206: 123-130.
Polistina, D.C., M. Murray, and M.E. Goldberger (1990) Plasticity of dorsal root and descending serotoninergic projections after partial deafferentation of the adult rat spinal cord. *J. Comp. Neurol.* 299: 349-363.
Poulat, P., F. Sandillon, L. Marlier, N. Rajaofetra, C. Oliver, and A. Privat (1992) Distribution of thyrotropin-releasing hormone in the rat spinal cord with special reference to sympathetic nuclei: a light- and electron-microscopic immunocytochemical study. *J. Neurocytol.* 21: 157-170.
Qian, M., A.E. Johnson, L. Kallstrom, H. Carrer, and P. Sodersten (1997) Cholecystokinin, dopamine D_2 and N-methyl-D-aspartate binding sites in the nucleus of the solitary tract of the rat: possible relationship to ingestive behavior. *Neuroscience* 77: 1077-1089.
Rainbow, T.C., B. Parsons, and B.B. Wolfe (1984) Quantitative autoradiography of beta 1- and beta 2-adrenergic receptors in rat brain. *Proc Natl Acad Sci USA* 81: 1585-1589.
Rajaofetra, N., J.-G. Passagia, L. Marlier, P. Poulat, F. Pellas, F. Sandillon, B. Verschuere, D. Gouy, M. Geffard, and A. Privat (1992a) Serotonergic, noradrenergic and peptidergic innervation of Onuf's nucleus of normal and transected spinal cords of baboons (Papio papio). *J. Comp. Neurol.* 318: 1-17.
Rajaofetra, N., J.-L. Ridet, P. Poulat, L. Marlier, F. Sandillon, M. Geffard, and A. Privat (1992b) Immunocytochemical mapping of noradrenergic projections to the rat spinal cord with an antiserum against noradrenaline. *J. Neurocytol.* 21: 481-494.
Ramón y Cajal, S. (1909) Histologie du système nerveux de l'homme et des vertebrés, Vol. 1 Paris: A. Maloine.
Rapport, M.M., A.A. Green, and I.H. Page (1948) Serum vasoconstrictor (serotonin). IV. Isolation and characterization. *J. Biol. Chem.* 176: 1243-1251.
Rexed B (1952) The cytoarchitectonic organization of the spinal cord in the cat. *J. Comp. Neurol* 96: 415-495
Rexed B (1954) The cytoarchitectonic organization of the spinal cord in the cat. *J. Comp. Neurol* 100: 297-379
Ribeiro-da-Silva, A. (1995) Substantia gelatinosa of spinal cord. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, Ca, USA: Academic Press, Inc., pp. 47-59.
Ridet, J.-L., F. Sandillon, N. Rajaofetra, M. Geffard, and A. Privat (1992) Spinal dopaminergic system of the rat: light and electron microscopic study using an antiserum against dopamine, with particular emphasis on synaptic incidence. *Brain Res.* 598: 233-241.
Romagnano, M.A., and R.W. Hamill (1984) Spinal sympathetic pathway : a enkephalin ladder. *Science* 225: 737-739.
Ross, C.A., D.A. Ruggiero, M.P. Meeley, D.H. Park, T.H. Joh, and D.J. Reis (1984) A new group of neurons in hypothalamus containing phenylethanolamine N-methyltransferase (PNMT) but not tyrosine hydroxylase. *Brain Res.* 306: 349-353.
Ross, C.A., D.M. Armstron, D.A. Ruggiero, V.M. Pickel, T.H. Joh, and D.J. Reis (1981) Adrenaline neurons in the rostral ventrolateral medulla innervate thoracic spinal cord: A combined immunocytochemical and retrograde transport demonstration. *Neurosci Lett* 25: 257-262.
Ruda, M.A., J. Coffield, and H.W.M. Steinbusch (1982) Immunocytochemical analysis of serotonergic axons in laminae I and II of the lumbar spinal cord of the cat. *J. Neurosci.* 2: 1660-1671.
Rueter, L.E., C.A. Fornal, and B.L. Jacobs (1997) A critical review of 5-HT brain microdialysis and behavior. *Rev Neurosci* 8: 117-37.
Ruggiero, D.A., C.A. Ross, M. Anwar, D.H. Park, T.H. Joh, and D.J. Reis (1985) Distribution of neurons containing phenylethanolamine N-methyltransferase in medulla and hypothalamus of rat. *J. Comp. Neurol.* 239: 127-154.
Ruggiero, D.A., V.M. Pickel, T.A. Milner, M. Anwar, K. Otake, and E.P. Mtui (1994) Viscerosensory processing in nucleus tractus solitarii: structural and neurochemical substrates. In I.R.A. Barraco (eds): Nucleus of the solitary tract, Vol. Boca Raton: CDC Press Inc, pp. 3-34.
Ruigrok, T.H., and F. Cella (1995) Precerebellar nuclei and red nucleus. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, Inc., pp. 277-308.
Sachar, E. J. (1985) Disorders of thought: the schizophrenic syndromes. In E.R.Kandel and J.H. Schwartz (eds): Principles of neural science, New York: Elsevier, pp. 704-716
Sachs, B. (1995) Placing erection in context: the reflexogenic-psychogenic dichotomy considered. *Neurosci Biobehav Rev* 19: 211-24.
Saper, C.B. (1995) Central autonomic system. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, pp. 107-135.
Sato, H. and Y. Kayama (1983) Effects of noradrenaline applied iontophoretically on rat superior collicular neurons.

Brain Res. Bull. 10: 453-457.

Sato, M., N. Mizuno, and A. Konishi (1978) Localization of motoneurons innervating perineal muscles: a HRP study in cat. *Brain Res.* 140: 149-154.

Savasta, M., E. Ruberte, J. Palacios, and G. Mengod (1989) The colocalization of cholecystokinin and tyrosine hydroxylase mRNAs in mesencephalic dopaminergic neurons in the rat brain examined by *in situ* hybridization. *Neuroscience* 29: 363-369.

Sawchenko, P.E., and L.W. Swanson (1982) Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J. Comp. Neurol.* 205: 260-272.

Scatton, B., T. Dennis, R. L'Heureux, J.-C. Monfort, C. Duyckaerts, and F. Javoy-Agid (1986) Degeneration of noradrenergic and serotonergic but not dopaminergic neurones in the lumbar spinal cord of parkinsonian patients. *Brain Res.* 380: 181-185.

Scheinin, M., J.W. Lomasney, D.M. Hayden-Hixson, U.B. Schambra, M.G. Caron, R.J. Lefkowitz, and R.T.J. Fremeau (1994) Distribution of alpha 2-adrenergic receptor subtype gene expression in rat brain. *Brain Res Mol Brain Res* 21: 133-149.

Schrøder, H.D. (1980) Organization of the motoneurons innervating the pelvic muscles of the male rat. *J. Comp. Neurol.* 192: 567-587.

Schuerger, R.J., and C.D. Balaban (1993) Immunohistochemical demonstration of regionally selective projections from locus coeruleus to the vestibular nuclei in rats. *Exp. Brain Res.* 92: 351-359.

Seeman, P., H.-c. Guan, O. Civelli, H. van Tol, R. Sunahara, and H. Niznik (1992) The cloned dopamine D_2 receptor reveals different densities for dopamine receptor antagonist ligands. Implications for human brain positron emission tomography. *Eur J Pharmacol - Mol Pharmacol Sect* 227: 139-146.

Sesack, S.R., C. Aoki, and V.M. Pickel (1994) Ultrastructural localization of D_2 receptor-like immunoreactivity in midbrain dopamine neurons and their striatal targets. *J. Neurosci.* 14: 88-106.

Shirouzu, M., T. Anraku, Y. Iwashita, and M. Yoshida (1990) A new dopaminergic terminal plexus in the ventral horn of the rat spinal cord. Immunohistochemical studies at the light and electron microscopic levels. *Experientia* 46: 201-204.

Sibley, D., and F. Monsma Jr (1992) Molecular biology of dopamine receptors. *Trends Pharmacol. Sci* 13: 61-69.

Sibley, D. (1999) New insights into dopaminergic receptor function using antisense and genetically altered animals. *Annu. Rev. Pharmacol. Toxicol.* 39: 313-341.

Siegel, G.J. (1994) Basic Neurochemistry. New York: Raven Press

Singewald, N., and A. Philippu (1998) Release of neurotransmitters in the locus coeruleus. *Prog Neurobiol* 56: 237-67.

Skagerberg, G., A. Bjorklund, O. Lindvall, and R.H. Schmidt (1982) Origin and termination of the diencephalo-spinal dopamine system in the rat. *Brain Res. Bull.* 9: 237-244.

Skagerberg, G., and O. Lindvall (1985) Organization of diencephalic dopamine neurons projecting to the spinal cord in the rat. *Brain Res.* 342: 340-351.

Skagerberg, G., B. Meister, T. Hökfelt, O. Lindvall, M. Goldstein, T. Joh, and A.C. Cuello (1988) Studies on dopamine-, tyrosine hydroxylase- and aromatic *L*-amino acid decarboxylase-containing cells in the rat diencephalon: comparison between formaldehyde-induced histofluorescence and immunofluorescence. *Neuroscience* 24: 605-620.

Skoog, B., and B.R. Noga (1995) Dopaminergic control of transmission from group II muscle afferents to spinal neurones in the cat and guinea pig. *Exp. Brain Res.* 105: 39-47.

Sladek, J.R.J., and J.P. Bowman (1975) The distribution of catecholamines within the inferior olivary complex of the cat and rhesus monkey. *J. Comp. Neurol.* 163: 203-213.

Smiley, J., S. Williams, K. Szigeti, and P. Goldman-Rakic (1992) Light and electron microscopic characterization of dopamine-immunoreactive axons in human cerebral cortex. *J. Comp. Neurol.* 321: 325-335.

Smiley, J.F., A.I. Levey, B.J. Ciliax, and P.S. Goldman-Rakic (1994) D_1 dopamine receptor immunoreactivity in human and monkey cerebral cortex: Predominant and extrasynaptic localization in dendritic spines. *Proc. Natl. Acad. Sci.* 91: 5720-5724.

Smith, D.O., D. Lowe, R. Temkin, P. Jensen, and H. Hatt (1995) Dopamine enhances glutamate-activated currents in spinal motoneurons. *J. Neurosci.* 15: 3905-3912.

Sofic E, Riederer P, Gsell W, Gavranovic M, Schmidtke A, Jellinger K (1991) Biogenic amines and metabolites in spinal cord of patients with Parkinson's disease and amyotrophic lateral sclerosis. *J Neural Transm Park Dis Dement Sect*; 3:133-42

Sokoloff, P., B. Giros, M.-P. Martres, M.-L. Bouthenet, and J.-C. Schwartz (1990) Molecular cloning and characterization of a novel dopamine receptor D_3 as a target for neuroleptics. *Nature* 347: 146-151.

Srinivasan, M., Y. Yamamoto, E. Brodin and H. Persson (1991) Chronic treatment with SCH-23390, a selective dopamine D_1 receptor blocker decreases preprotachykinin-A mRNA levels in nucleus tractus solitarii of the rabbit: role in respiratory control. *J. Appl. Physiol.* 85: 946-54.

Stefanick, M.L., E.R. Smith, J.T. Clark, and J.M. Davidson (1982) Effects of a potent dopamine receptor agonist, RDS-127, on penile reflexes and seminal emission in intact and spinally transected rats. *Physiol. Behav.* 29: 973-978.

Steinbusch, H.W.M. (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat - cell bodies and terminals. *Neuroscience* 6: 557-618.

Steinbusch, H.W.M. (1991) Distribution of histaminergic neurons and fibers in rat brain. *Acta Otolaryngol (Stockh)* Suppl. 479: 12-23.

Steinbusch, H.W.M., and F.J.H. Tilders (1987) Immunohistochemical techniques for light-microscopical localization of dopamine, noradrenaline, adrenaline, serotonin and histamine in the central nervous system. In H.W.M. Steinbusch (eds): Monoaminergic neurons: light microscopy and ultrastructure, Vol. Chichester: John Wiley, pp. 125-165.

Steinbusch, H.W.M., S.P. Van Vliet, J.G.J.M. Bol, and J. De Vente (1991) Development and application of antibodies to primary (DA, *L*-DOPA) and secondary (cGMP) messengers: a technical report. In A. Calas and D. Eugene (eds): Neurocytochemical Methods, noradrenalineTO ASI Series, Vol. H 58. Berlin: Springer-Verlag, pp. 1-27.

Storm-Mathisen, J., A.K. Leknes, A.T. Bore, J.L. Vaaland, P. Edminson, F.H.S. Haug, and O.P. Otterson (1983) First visualization of glutamate and GABA in neurons by immunohistochemistry. *Nature* 301: 517-520.

Strack, A.M., W.B. Sawyer, L.M. Marubio, and A.D. Loewy (1988) Spinal origin of sympathetic preganglionic neurons in the rat. *Brain Res.* 455: 187-191.

Strange, P. (1991) Interesting times for dopamine receptors. *TINS* 14: 43-45.

Sulzer, D., M.P. Joyce, L. Lin, D. Geldwert, S.N.Haber, T. Hattori and S. Rayport (1998) Dopamine neurons make glutamatergic synapses in vitro. *J. Neurosci.* 18: 4588-602

Sunahara, R., H. Niznik, D. Weiner, T. Stormann, M. Brann, J. Kennedy, J. Gelernter, R. Rozmahel, Y. Yang, Y. Israel, P. Seeman, and B. O'Dowd (1990) Human dopamine D_1 receptor encoded by an intronless gene on chromosome 5. *Nature* (London) 347: 80-83.

Sunahara, R.K., H.-C. Guan, B.F. O'Dowd, P. Seeman, L.G. Laurier, G. Ng, S.R. George, J. Torchia, H.H.M. van Tol, and H.B. Niznik (1991) Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1. *Nature (London)* 350:

Swanson, L.W., and B.K. Hartman (1975) The central adrenergic system: an immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-β-hydroxylase as a marker. *J. Comp. Neurol.* 163: 467-506.

Swanson, L.W., and S. McKellar (1979) The distribution of oxytocin and neurophysin stained fibers in the spinal cord of the rat and monkey. *J. Comp. Neurol.* 188: 87-106.

Swanson, L.W., P.E. Sawchenko, A. Berod, B.K. Hartman, K.B. Helle, and D.E. Vanorden (1981) An immunohistochemical study of the organization of catecholaminergic cells and terminal fields in the paraventricular and supraoptic nuclei of the hypothalamus. *J. Comp. Neurol.* 196: 271-285.

Takada, M. (1993) Widespread dopaminergic projections of the subparafascicular thalamic nucleus in the rat. *Brain Res. Bull.* 32: 301-309.

Takada, M., Z. Li, and T. Hattori (1988) Single thalamic dopaminergic neurons project both the neocortex and spinal cord. *Brain Res* 455: 346-352.

Tallaksen-Greene, S.J., R. Elde, and M.W. Wessendorf (1993) Regional distribution of serotonin and substance P co-existing in nerve fibers and terminals in the brainstem of the rat. *Neuroscience* 53: 1127-1142.

Thor, K.B., A. Blitz-Siebert, and C.J. Helke (1992) Autoradiographic localization of $5HT_1$ binding sites in autonomic areas of the rat dorsomedial medulla oblongata. *Synapse* 10: 217-227.

Tiberi, M., K.R. Jarvie, C. Silvia, P. Falardeau, J.A. Gingrich, N. Godinot, L. Bertrand, T.L. Yang-Feng, R.T. Fremeau Jr., and M.G. Caron (1991) Cloning, molecular characterization, and chromosomal assignment of a gene encoding a second D_1 dopamine receptor subtype: Differential expression pattern in rat brain compared with D_{1A} receptor. *Proc. Natl. Acad. Sci. USA* 88: 7491-7495.

Todd, A.J., and R.C. Spike (1993) The localization of classical transmitters and neuropeptides within neurons in laminae I-III of the mammalian spinal dorsal horn. *Progr. Neurobiol.* 41: 609-645.

Todd, R., K. Mack, K.-Y. Gandelman, J. Hickok, and K. O'Malley (1992) Dopamine D_2 receptor subtypes couple to different second messenger systems. *Neurochem Int* 20: 23S-26S.

Todd, R.D., and K.L. O'Malley (1993) Family ties: The dopamine D_2-like receptor genes. *Neurotransmissions* IX: 1-4.

Tracey, D.J. (1995) Ascending and descending pathways in the spinal cord. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, Ca, USA: Academic Press, Inc., pp. 67-80.

Uda, K., H. Okamura, F. Kawakami, and Y. Ibata (1986) Sexual differences in the distribution of substance P immunoreactive fibers in the ventral horn of the rat lumbar spinal cord. *Neurosci. Lett.* 64: 157-162.

Ueda, S., N. Ihara and Y. Sano (1985) The organization of serotonin fibers in the mammalian superior colliculus. An immunohistochemical study. *Anat. Embryol.* 173: 13-21.

Ueyama, T., N. Mizuno, S. Nomura, A. Konishi, K. Itoh, and H. Arakawa (1984) Central distribution of afferent and efferent components of the pudendal nerve in cat. *J. Comp. Neurol.* 222: 38-46.

van Dijken, H., J. Dijk, P. Voorn, and J.C. Holstege (1996) Localization of dopamine D_2 receptor in rat spinal cord identified with immunocytochemistry and *in situ* hybridization. *Eur. J. Neurosci.* 8: 621-628.

van Giersbergen, P.L.M., M. Palkovits, and W. de Jong (1992) Involvement of neurotransmitters in the nucleus tractus solitarii in cardiovascular regulation. *Physiol. Rev.* 72: 789-824.

van Tol, H.H.M., J.R. Bunzow, H.C. Guan, R.K. Sunahara, and P. Seeman *et al.* (1991) Cloning of the gene for a human dopamine D_4 receptor with high affinity for the antipsychotic clozapine. *Nature* 350: 610-614.

Vankov, A., A. Herve-Minvielle, and S.J. Sara (1995) Response to novelty and its rapid habituation in locus coeruleus neurons of the freely exploring rat. *Eur J Neurosci* 7: 1180-7.

Verburgh, C.A. (1990) Ascending and descending axon-collaterals from cervical spinal neurons. A retrograde fluorescent double-labeling study in the rat. Erasmus University Medical School

Verhage, M., W.E.J.M. Ghijsen, F. Boomsma, and F.H. Lopes da Silva (1992) Endogenous noradrenaline and dopamine in nerve terminals of the hippocampus: differences in levels and release kinetics. *J. Neurochem.* 59: 881-887.

Verney, C., M. Baulac, B. Berger, C. Alvarez, A. Vigny, and K.B. Helle (1985) Morphological evidence for a dopaminergic terminal field in the hippocampal formation of young and adult rat. *Neuroscience* 14: 1039-1052.

Versteeg, D.H., J. Van Der Gugten, W. De Jong, and M. Palkovits (1976) Regional concentrations of noradrenaline and dopamine in rat brain. *Brain Res* 113: 563-74.

Vibert, N., M. Serafin, O. Crambes, P.P. Vidal, and M. Mutlethaler (1995) Dopaminergic agonists have both presynaptic and postsynaptic effects on the guinea-pig's medial vestibular nucleus neurons. *Eur. J. Neurosci.* 7: 555-562.

Voogd, J. (1995) Cerebellum. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, Ca, USA: Academic Press, Inc., pp. 309-350.

Voorn, P., and R.M. Buijs (1987) Ultrastructural demonstration of dopamine in the central nervous system. In H.W.M. Steinbusch (eds): Monoaminergic neurons: light microscopy and ultrastructure, Vol. Chichester: John Wiley, pp. 241-264.

Waite, P.M.E., and D.J. Tracey (1995) Trigeminal sensory system. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, Inc., pp. 705-724.

Wang, B.-R., E. Senba, and M. Tohyama (1989) Ultrastructural investigation of substance P-, leucine-enkephalin- and 5-hydroxytryptamine-like immunoreactive terminals in the area of the cremaster motoneurons of the male rat. *Neuroscience* 28: 711-723.

Weil-Fugazza, J., and F. Godefroy (1991) Further evidence for the involvement of the diencephalo-dopaminergic system in pain modulation: a neurochemical study on the effect of morphine in the arthritic rat. *Int. J. Tiss. Reac.* 13: 305--310.

Weiner, D.M., A.I. Levey, R.K. Sunahara, H.B. Niznik, B.F. O'Dowd, P. Seeman, and M.R. Brann (1991) D_1 and D_2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci.* 88: 1859-1863.

Weinshank, R., N. Adham, M. Macchi, M. Olsen, T. Branchek, and P. Hartig (1991) Molecular cloning and characterization of a high affinity dopamine receptor (d1b) and it pseudogene. *J Biological Chem* 266: 22472-22435.

Westerink, B.H.C., and J.B. De Vries (1985) On the origin of dopamine and its metabolite in predominantly noradrenergic innervated brain areas. *Brain Res.* 330: 164-166.

Westerink, B.H.C., and T.B.A. Mulder (1981) Determination of picomole amounts of dopamine, noradrenaline, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid in nervous tissue after one step purification on sephadex G-10, using high performance liquid chromatography with a novel type of electrochemical detection. *J. Neurochem.* 36: 1449-1462.

Westlund, K.N., R.M. Bowker, M.G. Ziegler, and J.D. Coulter (1984) Origins and terminations of descending noradrenergic projections to the spinal cord of monkey. *Brain Res.* 292: 1-16.

Westlund, K.N., R.M. Bowker, M.G. Ziegler, and J.D. Coulter (1983) Noradrenergic projections to the spinal cord of the rat. *Brain Res.* 263: 15-31.

White, S.R. (1985) A comparison of the effects of serotonin, substance P and thyrotropin-releasing hormone on excitability of rat spinal motoneurons in vivo. *Brain Res.* 335: 63-70.

White, S.R., and R.S. Neuman (1980) Facilitation of spinal motoneuron excitability by 5-hydroxytryptamine and noradrenaline. *Brain Res.* 188: 119-127.

White, S.R., S.J. Fung, D.A. Jackson, and K.M. Imel (1996) Serotonin, norepinephrine and associated neuropeptides: effects on somatic motoneuron excitability. In G. Holstege, R. Bandler and C.B. Saper (eds): The emotional motor system, Progress in Brain Research, Vol. 107. Amsterdam: Elsevier, pp. 182-???(in press).

Wichmann, T., and K. Starke (1988) Uptake, release, and modulation of release of noradrenaline in rabbit superior colliculus. *Neuroscience* 26: 621-634.

Willcockson, W.S., J.M. Chung, Y. Hori, K.H. Lee, and W.D. Willis (1984) Effects of iontophoretically released amino acids and amines on primate spinothalamic tract cells. *J. Neurosci.* 4: 732-740.

Willis, W.D., K.N. Westlund, and S.M. Carlton (1995) Pain. In G. Paxinos (eds): The rat nervous system, Vol. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press, pp. 725-740.

Yang, R.H., Y. Igarashi, J.M. Wyss, and Y.F. Chen (1990) Dopamine D_2 receptors in the posterior region of the nucleus tractus solitarius mediate the central pressor action of quinpirole (LY171555). *Brain Res. Bull.* 24: 97-103.

Yokoyama, C., H. Okamura, T. Nakajima, J.-I. Taguchi, and Y. Ibata (1994) Autoradiographic distribution of [^{3}H]YM-09151-2, a high-affinity and selective antagonist ligand for the dopamine D_2 receptor group, in the rat brain and spinal cord. *J. Comp. Neurol.* 344: 121-136.

Yoshida, M., and M. Tanaka (1988) Existence of new dopaminergic terminal plexus in the rat spinal cord: assessment by immunohistochemistry using anti-dopamine serum. *Neurosci. Lett.* 94: 5-9.

Zandberg, P., W. De Jong, and D. De Wied (1979) Effect of catecholamine-receptor stimulating agents on blood pressure after local application in the nucleus tractus solitarii of the medulla oblongata. *Eur. J. Pharmacol.* 55: 43-56.

Zardetto-Smith, A.M., and A.K. Johnson (1995) Chemical topography of efferent projections from the median preoptic nucleus to pontine monoaminergic cell groups in the rat. *Neurosci. Lett.* 199: 215-219.

Zhou, Q.-Y., D. Grandy, L. Thambi, J. Kushner, H. van Tol, R. Cone, D. Pribnow, J. Salon, J. Bunzow, and O. Civelli

(1990) Cloning and expression of human and rat D_1 dopamine receptors. *Nature* 347: 76-79.

Zoli, M., A. Jansson, E. Sykova, and K. Fuxe (1999) Volume transmission in the CNS and its relevance for neuropsychopharmacology. *TIPS* 20: 142-150.

List of publications

Brouwer, N., van Dijken, H., Ruiters, M.H.J., van Willigen, J.-D. and ter Horst, G.J. (1992) Localization of dopamine D_2 receptor mRNA with non-radioactive *in situ* hybridization histochemistry. *Neurosci. Lett.* 142: 223-227.

de Jongste, M.J.L. and van Dijken, H. (1992) Epidurale ruggemergstimulatie als aanvullende therapie voor anderszins onbehandelbare ernstige angina pectoris. *Hart Bulletin* 23: 61-66.

Holstege, J.C., H. van Dijken, R.M. Buijs, H. Goedknegt, T. Gosens, and C.M.H. Bongers (1996) Distribution of dopamine immunoreactivity in the rat, cat, and monkey spinal cord. *J. Comp. Neurol.* 376: 631-652.

Luiten, P.G.M., de Jong, G.I., Van der Zee, E.A. and van Dijken, H. (1996) Ultrastructural localization of cholinergic muscarinic receptors in rat brain cortical capillaries. *Brain Res* 720:225-9

Toonen M, van Dijken H, Holstege JC, Ruigrok TJ, Koekkoek SK, Hawkins RK, Teune TM, v d Burg J, De Zeeuw CI (1998) Light microscopic and ultrastructural investigation of the dopaminergic innervation of the ventrolateral outgrowth of the rat inferior olive. *Brain Res* 802:267-73.

van Dijken, H. and de Jongste, M.J.L. (1992) De rol van afferente sensorische innervatie van het hart bij coronair ischemie. *Hart Bulletin* 23: 61-66.

van Dijken, H., J. Dijk, P. Voorn, and J.C. Holstege (1996) Localization of dopamine D_2 receptor in rat spinal cord identified with immunocytochemistry and *in situ* hybridization. *Eur. J. Neurosci.* 8: 621-628.

van Dijken, H. and J.C. Holstege (2000). Distribution Of Dopamine D_2 Receptor In The Rat Brain Stem Using Anti-Peptide Antibodies. *Thesis.*

van Dijken, H. and J.C. Holstege (2000). The Distribution Of Dopamine Immunoreactive Fibers And Presumptive Terminals In The Rat Brain Stem. A Comparison With The Distribution Of Dopamine-ß-Hydroxylase. *Thesis.*

Nawoord

Na 5 jaar voltijd onderzoek in Rotterdam en 3½ jaar lang s'avonds en in het weekend doorploeteren aan de afronding van mijn proefschrift is het eindelijk zover: het is af! Graag wil ik iedereen bedanken die heeft bijgedragen aan het tot stand komen van dit proefschrift. Enkele personen wil ik in het bijzonder noemen. Joan Holstege, copromotor en dagelijks begeleider. Jan Voogd, mijn promotor. Mijn dank voor het gestelde vertrouwen in het slagen van dit project. Paul Luiten, ik ben verheugd dat je wilde deelnemen in de kleine promotiecommissie. Chris de Zeeuw, mijn enige "echte" collega. Erika, Richard en Corlinda. Jullie hebben mij terzijde gestaan op het lab. Erika, ook naast het werk ben je een gezellige babbeltante. Collega-AIO's Thea en Pierre. Jullie promotie heeft mij erg gestimuleerd bij de afronding van mijn proefschrift. Pierre, bedankt voor het wijzen op de *in situ* hybridisatie techniek. Pieter Voorn, bedankt voor de samenwerking bij de *in situ* experimenten. Doortje Voorhuis, bedankt voor het wijzen op het dopamine D_2 antilichaam. Jan Dijk, dank voor het D_2 antilichaam en het zuiveren daarvan. Eddie en Hans, ik ben jullie beiden zeer erkentelijk voor de assistentie bij de operaties en de voorbereidingen daarvan. Eddie, ook bedankt voor je assistentie bij al het afdrukwerk, al dan niet in digitale vorm. Edith, bedankt voor secretariele ondersteuning en de gezelligheid.

Tot slot: mijn meeste dank gaat uit naar Mechteld, omdat je me tot het eind toe bent blijven (onder)steunen.

Curriculum Vitae

Henk van Dijken werd op 13 april 1966 te Emmen geboren. Hij bezocht de Openbare Scholengemeenschap in Stadskanaal en legde in 1983 het eindexamen HAVO met goed gevolg af. In datzelfde jaar begon hij met de studie HLO-medisch aan de IHBO te Emmen. De medische laboratoriumopleiding werd afgerond in 1987 met als hoofdrichting klinische chemie. Na zijn militaire dienst als medisch laborant in de Militair Geneeskundige Dienst te Wezep begon hij in 1988 aan de studie Biologie aan de RijksUniversiteit Groningen. Tijdens de doctoraalfase verrichtte hij een doctoraalonderwerp neuroanatomie bij de vakgroep Dierfysiologie, subfaculteit Biologie, RUG onder supervisie van Dr. P.G.M. Luiten. Een tweede doctoraalonderwerp werd gedaan bij de vakgroep Kindergeneeskunde onder leiding van Prof. Dr. R.J. Vonk. Het doctoraalexamen Medische Biologie werd afgelegd in augustus 1991. Vanaf 1 september 1991 was hij werkzaam als assistent in opleiding op de afdeling Anatomie (Hoofd: Prof. Dr. J. Voogd) aan de Erasmus Universiteit van Rotterdam, alwaar hij de dopaminerge innervatie van de hersenstam en ruggemerg onderzocht onder begeleiding van Dr. J.C. Holstege. De resultaten hiervan zijn beschreven in dit proefschrift. In oktober 1994 ondernam hij een 5 weekse werkbezoek aan het laboratorium van Dr. A.I. Levey, Department of Neurology, Emory University, Atlanta middels een SIR-beurs van NWO. In november 1996 is hij begonnen als software ontwikkelaar bij XCESS expertise center, alwaar hij momenteel de functie Manager Research & Development heeft.