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Neuronal Activity in Tumor Tissue

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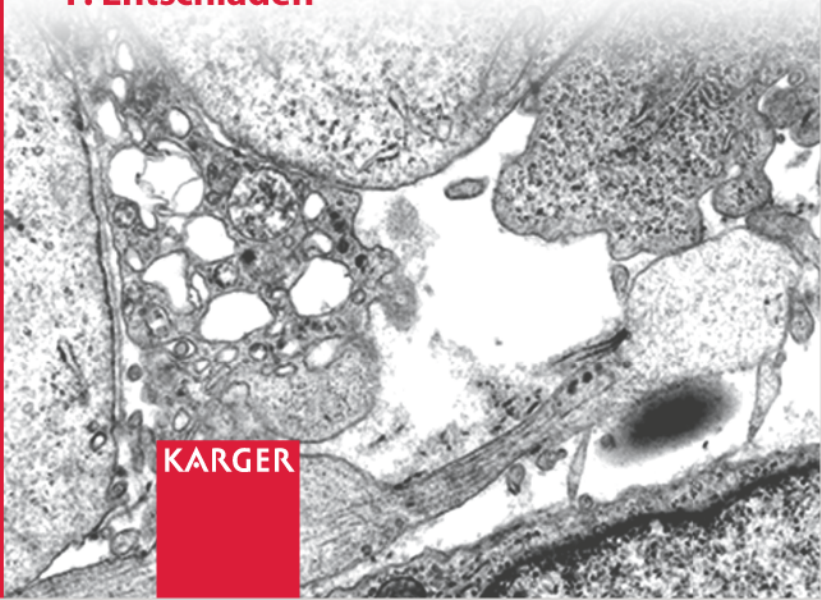

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Neuronal Activity in Tumor Tissue

Progress in Experimental Tumor Research

Vol. 39

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Neuronal Activity in Tumor Tissue

Volume Editors

Kurt S. Zänker *Witten*
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Contents

In Remembrance of Fritz Bender Esq. (1907–1986)

At this 100th anniversary of Fritz Benders birthday, it is my honorable duty, and soulful wish to reminisce and contemplate the heritage he generously bestowed upon us.

Fritz Bender was born in Heidelberg, the romantic and historical town on the banks of the Neckar. It is here, as well as in many cities strewn across the country, that one can find signs of his work as a unique master of the building trade. City council buildings in Mannheim, and Munich still bear evidence of his skill as plasterer and builder – a sorry consequence of the wide-spread damage caused by the bombings in the Second World War.

My earliest memories of Mr. Bender are those of his remarkably professional interpretation of a great variety of songs by Robert Schumann, Franz Schubert and Richard Strauß. His voice rang out in a timbre that filled the room, and was but an indication of his greatly facetted talent and character; for he bore and displayed a deep conviction of both symbolism and social aesthetics. He was a

masterful proponent of the Viennese Classical Period, something deeply rooted and richly lived in his life and work. He put great emphasis on both order and hierarchy, pronouncing a clear segregation of parts, colors and taking preference to simplicity rather than complexity. During the time of our friendship he would often quote Henrik Ibsen from the play 'Bygmester Soleness' (The Master Builder). It is with the words: 'a man could not build so high without paying the penalty of his hubris', that Mr Bender unveiled his wise, and fantastically clear understanding of personal and social responsibility.

On the eve of his death, his personal fight with cancer lead him to bequeath his estate for the endowment of an established trust, known as the Fritz Bender-Foundation. Within the genesis of this foundation lies the bold intention to unite the most renowned experts in the field of immunity and the control of cancer, as well as to provide the best resources needed for this battle. It should furthermore be fought by implementing the idea of holism in therapy, and thereby help relieve human suffering caused by maladies of both immunity and cancer. It is the duty of the foundation to ensure and secure the promotion, and attainment of these aims.

Those of us who knew Fritz Bender, and those who receive support from the Fritz Bender-Foundation are deeply beholden to carry forth his social, cultural and scientific legacy both now and beyond the anniversary of his 100th birthday.

Johanna Huber, Hans-Peter Huber, Franz Weigl, Kurt S. Zänker (Board of Directors Fritz Bender Foundation, Munich, Germany).

Foreword

In his book *The Structure of Scientific Revolution*, Kuhn [1] presented the idea that science does not evolve gradually towards truth, but instead undergoes periodic revolutions, which he called paradigm shifts. According to Kuhn, scientists do not use their puzzle-solving abilities to change old theories but take them for granted and use them as tools, often resulting in their enterprise itself becoming puzzle-solving. When the normal scientist is confronted with evidence that the current paradigm may not be correct, he/she tends to ignore it, blaming the mistake on experimental error instead of critically investigating the anomaly. The non-continuous model of scientific progress is exemplified by Judah Folkman's isolation of a tumor factor responsible for angiogenesis and, later on, for the development of a new class of drugs, the angiogenesis inhibitors. This was a fundamental correction of past errors that cytotoxic drugs were the only valid paradigm with no development of alternative theories. A similar change of paradigm is on the horizon in the 21st century, when scientists are trying to understand the interconnecting plasticity of tumor and neuronal cell signaling.

In adult organisms, the peripheral nervous system is highly dynamic and is able to regenerate. It can also respond and adapt to environmental influences not only by its main function – forwarding electrical membrane potentials as well as releasing of neurotransmitters and hormones – but by the nerve cells themselves which are able to adjust through morphological and metabolic changes. The wiring and organization of the peripheral nervous system and some of its key features and potential interactions with tumor cells forming a neuro-neoplastic synapsis are explained by Giehl (Aarhus) and von Düring and Fricke (Bochum) in the first two chapters of this book.

There are three modes in which neurotrophic factors and neurotransmitters play a role in tumor and neurotrophic cells:

(1) The tumor cells are able to secrete substances which act in an autocrine or paracrine loop when the acceptor cells express the appropriate receptors; here, we speak about an autocrine neuro-neoplastic synapsis. Functional responses provided by this type of autocrine activity are highlighted in the chapters by Chedotal (Paris) who deals with the neurotrophic factors and Lang and Bastian (Witten) who address the neurotransmitters.

(2) The tumor cells secrete neurotrophic factors and neurotransmitters, but are not necessarily sensitive to them. However, these secreted molecules can attract nerve cells to grow into the tumor tissue and, thus, adapt an innervation over time by the inducible expression of appropriate receptors. Palm and Entschladen (Witten) discuss this possibility in detail and Hagel and Stavrou (Hamburg) embarks on the presence of neuronal marker structures within a solid tumor tissue and highlights the corresponding prognostic value. Varner (San Diego, Calif., USA) shows parallels of the innervation process – also called neo-neurogenesis – with the process of neo-angiogenesis, which was first discovered 30 years ago.

(3) The tumor cells express receptors for neurotrophic factors and neurotransmitters and respond to the release of these substances by producing neurogenic phenotypes with cell functions such as increase/decrease of proliferation, apoptosis, and migration; the latter, when circumventing the immunosurveillance, leads to invasion and formation of metastases. Schuller (Knoxville, Tenn., USA) gives strong evidence in her contribution that carcinogenesis – mostly the initial step – is modulated by neurotransmitters such as acetylcholine and norepinephrine.

Last, but not least, and considering the hypotheses formulated and facts presented in this book, Muller (Poitiers) discusses some pharmacological approaches to inhibit the interaction between the nervous system and tumor; Zänker (Witten) puts forward the question whether there is substantiated evidence to coin the term ‘neuro-neoplastic synapsis’ and, by understanding the mode of action, whether it is a novel target structure for an antitumor therapy analogous to the inhibition of angiogenesis.

This book was only made possible because leading authorities from a number of relevant disciplines contributed to this most fascinating field at the frontier of cancer network research with special reference to the description of the ‘brain within the tumor’ [2]. The distinguished authors guarantee that this book will offer the reader sufficient insight into the cancer research problems of tomorrow; hopefully, the presentation of a whole lot of fascinating details may stimulate scientists in cancer research to keep a keen eye on this particular field.

We are grateful to the Karger Publishing House, Basel, Switzerland, and to J.R. Bertino, the Editor-in-Chief of this long-standing and well-recognized series *Progress in Experimental Tumor Research* for publishing this volume. We are thus able to render novel results in basic sciences of cancer research understandable to our clinicians, friends and politicians. If we cannot find the means of doing so, we researchers engaged in basic sciences may face the danger of losing support from the scientific community.

Frank Entschladen, PhD
Kurt S. Zänker, MD, DVM

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Neuronal Development

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Abstract

Biological tools that are unleashed in malignancies are employed in a controlled manner during neuronal development. By default, early embryonic cells would become neuronal stem cells, a path that is blocked by specific signaling pathways. The future nervous system only develops where this blockade is inhibited by inductive signals from the 'organizer'. Once the future brain and spinal cord regions are determined, the mitotic potential in this region must be maintained long enough to produce all cells required, but also be controlled to avoid excessive over-production of cells. Newly generated cells must then migrate to their future destination, they must know where to settle down, and they must differentiate. To shape the developing nervous system and to adapt its functionality to the postnatal environment, cell survival must be regulated, i.e. survival of some cells is supported while death of others is induced. Thus, inductive events, proliferation, cell migration, differentiation, cell survival and cell death are highly regulated during neuronal development, while these functions are de-regulated in malignancies. The molecular pathways for neuronal development mutually modulate each other and are still present in the adult nervous system. Because many of these pathways are implicated in tumors, neurons may affect these conditions.

On a logistic level, neuronal development takes place roughly in six steps. Firstly, the area in which the nervous system develops within the undifferentiated embryonic tissue has to be determined, a process called neuronal induction [1]. Secondly, the cellular components of the nervous system, i.e. neurons and glial cells, have to be generated from precursors and decisions have to be made as to which precursor gives rise to a neuron and which to a glial cell [1, 2]. Thirdly, already along with neuronal induction and the earliest stages of neurogenesis go early patterning decision [3–6], which, e.g. determine what becomes an output and what becomes an input area, what will be under control of our consciousness and what will be part of the autonomous nervous system, or simply what will be the front and what will be the back part of the nervous system.

Fourthly, newly generated cells have to migrate to their destination areas and to differentiate into a specific cell type [7–9]. Fifthly, the neurons have to establish the connectivity of the nervous system [10–12]. For example, a motoneuron has to send one process, the axon, to the muscle which it supplies with information, but it has also to grow other processes, the dendrites, that receive orders and feedback information, e.g. about the activity the axon is causing on the muscle. The primary generation of this connectivity as well as the generation of neurons occurs 'in excess'. This is not because the young embryo is so full of energy and just cannot hold back to embrace live, but to provide a framework of sufficient options to optimally adapt the nervous system to the postnatal requirements. Thus, the sixth goal to be achieved during development is to adapt this excess of neurons and connections to what is really needed [10, 12]. As implied, this may vary from individual to individual.

A network of signaling pathways that mutually modulate each other regulates these steps [13]. The spatial and temporal expressions of these signaling pathways has to occur in a precise orchestration because the functional effects of each pathway as well as its effects on the respective other pathways very much depend on the time point during development and on the area where the respective pathway is active. One pathway A, e.g. may well stimulate proliferation of precursors and suppress another pathway B promoting migration at an early time point of development, but at a later time point, the same pathway A may promote neuronal survival and activate pathway B that induces neuronal death by that time. As depicted initially, the cellular functions regulated during development – proliferation, cell fate determination, differentiation, migration, cell survival, and cell death – are out of control in malignancies, and the pathways regulating them in a proper manner during development are associated with a variety of cancers in adulthood [14–21]. Thus, understanding what controls these pathways and how they perform their functions in a physiological context will give us useful insights in the pathophysiology of malignancies and may even provide targets for future therapeutic intervention.

The major pathways regulating neuronal development [13] – each of them being involved in malignancies – are the Wnt pathway, the receptor serine/threonine kinase pathway (transforming growth factor beta [TGFβ]-family pathway), the Hedgehog pathway, the Notch pathway, and the receptor tyrosine kinase (RTK) pathway. The first section of this chapter will describe these five pathways and their major constituents. These pathways are also in place during adulthood, partially in a functional context differing from development, and partially carrying out functions that resemble their developmental role. The neurotrophin/Trk receptor system, which belongs to the RTK pathway, is an example for a pathway exerting largely different functions in development and adult. While its prominent developmental function is the regulation of neuronal

survival and differentiation, this system mostly regulates neuronal transmission, plasticity, and learning during adulthood [9, 12]. The Wnt and Notch pathways [16, 18], on the other hand, are examples for pathways exerting many similar functions during development and adulthood. In both periods, the prominent features of these pathways are the maintenance of the proliferative capacity of stem cells and the suppression of differentiation. In this chapter, focus will be on the developmental functions of the respective pathways, which are also the functions most relevant in a cancer context. The remaining five sections of this chapter will describe the major steps during nervous system development, i.e. neuronal induction, neuro- and gliogenesis, early patterning, migration, the adaption of neurons to their innervation targets, and how the pathways described in the first section regulate these steps.

During the migration to their final destination and shortly afterwards, neurons grow out processes to establish the complex connectivity of the nervous system (see step five above) [10–12]. There are two types of processes, axons and dendrites. Axons represent the output structure for later synaptic transmission, while dendrites represent the receiving part of synaptic input. Depending on the type of neuron, i.e. interneuron or projecting neuron, the length of axons ranges from a few micrometers up to more than a meter respectively. The mechanisms governing the outgrowth and pathfinding of axons are covered by Chedotal in this issue and will, therefore, not be described here. In many central nervous system (CNS) areas, there is a so-called process of axon collateral elimination after the primary connectivity has been established [10]. Axon collateral elimination and, at a more local level, axonal pruning are adaptive events in order to optimize neuronal connectivity to the postnatal functional requirements. This aspect will be addressed in the section 'Adaption of Neurons to their Innervation Target' of this chapter together with another adaptive process serving the same functional purpose, programmed cell death [22]. The formation and maturation of the other type of neuronal process, the dendrites, takes place after axon formation. Some examples for the mechanisms governing dendrite formation and maturation are given in the section 'Neuronal Migration and Differentiation'.

Major Signaling Pathways in Neuronal Development

Wnt Pathway

The Wnt pathway has important functions for the development of all metazoan organisms, as reflected by its name, which is a contraction of Wingless, a Drosophila segment polarity gene, and its murine homolog Int-1, a proto-oncogene [18, 19]. The pathway is initiated by the binding of a Wnt ligand to its

receptor complex, which is composed of a Frizzled-family member and LRP-5/6, a member of the low-density lipoprotein (LDL) receptor family [23]. In mammals, there are about 20 different Wnt ligands and about 10 different Frizzels.

The canonical Wnt pathway, which is discussed here, acts by regulating the degradation of β-catenin [18, 19, 24]. β-Catenin also occurs in a different functional context regulating cell–cell adhesion. In this role, β-catenin is located at the plasma membrane complexing cadherins and actin microfilaments in desmosomes, adherens, and septate cell junctions. β-Catenin was also reported as a component of the γ-secretase complex whose cleavage activity releases the intracytoplasmatic tail of Notch and β-amyloid precursor protein for translocation and signaling to the nucleus [25]. The role of β-catenin in this context, however, is not understood. Without Wnt activity, intracytoplasmatic β-catenin quickly complexes with the scaffolding proteins tumor suppressors adenomatous coli (APC) and axin, which in turn recruit and activate the kinases CKI and GSK3-β that can now phosphorylate β-catenin [18, 19]. This complex is called the 'destruction complex'. Upon phosphorylation by the destruction complex, β-catenin undergoes rapid ubiquitin-mediated degradation in the proteasom. Activation of the Frizzeld/LRP-5/6-complex by Wnt disrupts the formation of a β-catenin/APC/axin complex, likely by recruiting axin to LRP-5/6 and thereby releasing the GSK3-β blocking activity of the axin-binding molecule Dishevelled (Dsh). The present model [18, 19, 23] is that without Wnt stimulation, Dsh is bound in an unphosphorylated form to axin, which prevents binding of axin to LRP-5/6. Wnt activation of the Frizzeld/LRP-5/6 complex leads to phosphorylation of Dsh, which induces dissociation of Dsh from axin and subsequent association of axin to LRP-5/6. The phosphorylation of β-catenin is, therefore, blocked (1) by dissociation of the destructor complex, but (2) also directly by phosphorylated Dsh, which inhibits GSK3-β. Being not phosphorylated and hence not degraded anymore, β-catenin can enter the nucleus and form a complex with transcription factors of the TCF/LEF family. TCF/LEF transcription factors suppress the expression of Wnt target genes in the absence of β-catenin, but promote their expression upon association with β-catenin. Wnt stimulates the expression of the constituents of its pathway, i.e. it acts in a positive feedback loop, which can however be suppressed by other pathways such as bone morphogenic protein (BMP), a member of the TGFβ-family pathway.

The Wnt pathway is involved in several aspects of development, largely depending on the time point of its action and the cell type it is acting on [18, 19]. A commonly regulated function of Wnt, however, is the promotion of cell proliferation and the suppression of differentiation [18, 19]. In Wnt1 deficient mice, e.g. the expansion of the CNS fails due to reduced mitogenic capacity of the neuronal progenitors. Wnt exerts this proliferative function via target genes

that control the cell cycle, e.g. cyclins [26]. Also during adulthood, Wnt signaling is required to maintain the proliferative capacity of the stem cell niche, ranging from bone marrow to gut and brain. This proliferation promoting effect of Wnt is reflected by the fact that mutations in constituents of the Wnt pathway that cause constitutive activation of the Wnt pathway result in cancer [18, 19]. The best-known example for this is colorectal cancer in the context of familial adenomatous polyposis. But mutations of the Wnt pathway are pathogenic also in the majority of sporadic colorectal cancer and several other malignancies such as medulloblastomas (a brain tumor). This underlines the importance of developmental signaling pathways for the development of malignancies. Specific Wnt effects and its major action during nervous system development are depicted in the later sections.

TGFβ-Family Pathway

This pathway is constituted by several ligand–receptor families, e.g. BMP, TGFβ, growth and differentiation factors, Veg-related proteins, nodal, and activin [13, 27]. The common characteristic of this pathway is that the dimeric receptors are serine/threonone kinases that recruit and activate Smad proteins upon ligand binding [13, 27]. The first step of the activation of this pathway is that the type II kinase of the receptor dimer phosphorylates the kinase domain of the type I kinase, which then phosphorylates Smad proteins. After phosphorylation, Smads form a complex that translocates to the nucleus to regulate the expression of target genes. The pathway can be regulated by negative and positive feedback loops. A typical feature of BMPs, the most prominent pathway of this family in the context of neural development, is that the transcription of BMP genes is stimulated and maintained in a positive feedback loop. Thus, blockade of BMP ligands will finally lead to silencing of BMP expression, a mechanism that is important for the regulation of BMP activities during development [1, 13].

Another typical feature of the pathway is that its ligands act via gradients over long distances [1, 13]. Correspondingly, many aspects of the signaling are related to the diffusibility and the interaction of the ligand with the extracellular matrix or extracellular binding partners. For example, TGFβs are synthesized as protein precursors that undergo differential processing in the Golgi apparatus [13, 27]. This results in a wide array of TGFβ isoforms that greatly differ in their diffusibility. Little is know how this processing is regulated. The ligands show also extensive interaction with the extracellular matrix, e.g. sulphated proteoglycans, which does not only affect the diffusion of the ligands but may also regulate their interaction with their receptors. In addition, it has been shown in Drosophila that there are secreted antagonists that bind to TGFβ pathway ligands and thereby prevent their interaction with the respective receptors. This

binding also mediates degradation through extracellular matrix metalloproteinases. Thus, the long-range effect of TGFβ pathway ligands can be influenced by processing of the ligand itself, by the extracellular matrix, and by the regulated interaction with secreted binding partners of the ligand.

The pathway is involved in many aspects of nervous system development [1, 13, 27]. Its major functions, however, are inductive events and its role for fate determination. To this end, induction or fate determination can be mediated by either a positive inductive effect, or by suppression of alternative fates, i.e. by an inhibitory effect. The most prominent effect of the TGFβ pathway is the suppression of the default neuroectoderm fate of early embryonic ectoderm by BMP. For the nervous system to develop, this suppression has to be overcome by antagonists of BMP, which are secreted by the organizer and inhibit BMP binding to its receptors by directly binding to BMP. This inhibition allows the ectoderm to unfold its default pathway, i.e. to develop into neuroectoderm. As a side note, it is interesting that this regulation does not primarily impinge on the intracellular signaling, but on the most prominent feature of the TGFβ pathway, its action via extracellular gradients.

Hedgehog Pathway

The pathway is named after the first ligand identified for the pathway, which is the Drosophila hedgehog [28, 29]. Absence of the ligand during fly development results in fly larvae with a surplus of spikes, therefore looking like a hedgehog. In vertebrates, there are many ligands of the hedgehog family such as Sonic hedgehog (SHH), Indian hedgehog, and Desert hedgehog. Hedgehog is secreted in a poorly diffusible 44 kDa pro-form. The C-terminal, larger part of the protein has a protease activity that cleaves off the smaller N-terminal part of the protein, which is the biologically active form of hedgehog and has better diffusion properties. The latter is important because hedgehog exerts its biologic activity, similar to the ligands of the TGFβ pathway, via long-range gradients.

Hedgehog acts via the heterodimeric receptor pair patched (Ptc) and smoothened (Smo) [28, 29]. While Ptc is the binding partner of hedgehog, Smo mediates the signal transduction of the ligand–receptor complex. Without bound hedgehog, Ptc inhibits Smo-mediated signal transduction. After hedgehog binding to Ptc, this inhibition is released and Smo activates a signaling cascade, which is only partially identified yet. Components of this cascade are the microtubule bound protein Fused kinase (Fu), inhibitor of Fu (Su), and the zinc-finger transcription factors Gli1–3 of the Ci/Gli family. Gli1–3 translocate to the nucleus in response to hedgehog binding to Ptc and initiate the transcription of their target genes. The pathway can be regulated at extra- and intracellular levels. First, hedgehog responding cells can express the transmembrane protein hedgehog-interacting protein, which binds hedgehog with similar

affinity as Ptc and thereby antagonizes the hedgehog pathway. There are also activators of the pathway. The extracellular matrix protein vibronectin, e.g. binds hedgehog and thereby increases its biological activity. At an intracellular level, protein kinase A is counteracting hedgehog activity by transforming Gli1–3 into a form that represses the expression of hedgehog target genes. Suppression of protein kinase A by activated Smo may contribute to the activation of the hedgehog pathway, i.e. the translocation of activating Gli1–3 to the hedgehog target genes.

The predominant functions of hedgehog during nervous system development are inductive events in a concentration-dependent manner, i.e. via gradients [28, 29]. In the neural tube, e.g. hedgehog is expressed basally in the floor plate and induces different motoneuron fates in the overlying neural tube areas. The respective fate hedgehog is inducing depends on the distance that the presumptive motoneuron has to the source of hedgehog expression. The effect is mediated by homeodomain genes, which specify regionalization during development. The concentration-dependence of the hedgehog effect is based on the differential effect of hedgehog on the expression of these homeodomain genes. Homedomain genes specify regional cell type identity by the relative expression of class I and class II homeodomain genes. While hedgehog suppresses the expression of class I genes, it stimulates the expression of class II genes. Thus, the class I/class II ratio of homeodomain expression in a specific cell depends on the hedgehog concentration to which this cell is exposed. Later during development, hedgehog also stimulates cellular proliferation, e.g. the proliferation of granule neuron progenitors during the development of the cerebellum. This proliferative effect may explain why some cancers, e.g. basal-cell carcinomas, are associated with a constitutively active hedgehog pathway. Interestingly, the proliferative effect of hedgehog appears to depend on the extracellular environment. During cerebellar development, laminin promotes the proliferative response of migrating granule cell precursors to hedgehog, while vibronectin binding to hedgehog switches the precursor response to exit the cell cycle and to enter differentiation.

Notch Pathway

While the ligands activating the pathways described in the previous sections were secreted and diffusible proteins, the ligand in the Notch pathway is a transmembrane protein only acting on neighboring cells [16, 20, 30]. Notch is the receptor mediating signal transduction in the pathway, the ligands are Delta (Dl) and Serrate (Ser) in Drosophila. Vertebrates have four Notch receptors (Notch1–4), three Dl homologs called Delta-like (DLL-1, 3, and 4), and two Ser homologs called Jagged (JAG1 and 2). Notch signaling can be modified at the ligand receptor level by fringe proteins [16, 31]. Fringes are glycosyl transferases

that add fucose residues to the extracellular domain of the Notch receptor. After this modification, Notch can only be stimulated by JAGs but not by DLLs. Notch is synthesized as a single protein but intracellularly cleaved by a furin-like convertase so that it appears as a heterodimeric receptor complex at the cell surface [16]. Interaction with one of its ligands causes a specific cleavage of Notch that initiates signal transduction. The ligand-induced cleavage of Notch occurs in two steps [16, 32]. First, the metalloprotease tumor necrosis factor α-converting enzyme cleaves off the extracellular part of Notch close to the plasma membrane. Then, the intracellular domain of Notch (Notch-ICD) is cleaved off by the γ-secretase complex, which also cleaves off the intracellular domains for signaling to the nucleus of other transmembrane receptors such as the β-amyloid precursor protein and the common neurotrophin receptor p75 [25, 33]. After the second cleavage step, the Notch-ICD translocates to the nucleus where it binds to the transcription factor CSL [16]. In the absence of the Notch-ICD, CSL is part of a repressor complex of Notch target genes. If Notch-ICD translocates to the nucleus, it displaces components of this repressor-complex by associating with CSL and thereby converts CSL to an activator of Notch targets. The primary Notch targets belong to the family of basic helix-loop-helix (bHLH) transcription factors [16, 34]. The bHLH transcription factors in turn activate downstream targets of Notch.

The first identified function of Notch, which is still one of its predominant functions, is the mediation of divergent cell fate decision between two neighboring cells [20, 30]. The mechanism is mediated by bHLH genes and was described in Drosophila [34]. Before differentiation of the neuroectoderm, ectodermal cells of the Drosophila embryo have identical Notch and Delta levels, i.e. the Notch–Delta circuit between neighboring cells is balanced [20, 30]. At the time of neuroectodermal induction, the levels of the Notch ligand Delta increase in some ectodermal cells. The primary cause of this increase is unknown, but it is known that the expression of Delta is stimulated by bHLH transcriptional activators of the achaete–scute complex. Upon stimulation by Delta, Notch activates – in addition to other bHLH targets that maintain an undifferentiated state and suppress the progression to a neuroblast fate – the expression of the bHLH transcriptional repressor enhancer of split proteins. Enhancer of split proteins now blocks the transcription of the achaete–scute complex. Thus, the cell that initially receives a higher Notch stimulation will down-regulate its Delta expression. Vice versa, the cell having higher Delta levels will receive less Delta signal from the neighboring cell and, therefore, less repression of its achaete–scute complex. The consequence of this circuit is that the cell with higher Delta levels and lower Notch stimulation will progress to a neuroblast fate, while the cell receiving higher Notch stimulation and lowering its Delta expression will continue to suppress a neuroblast fate. This first

example of Notch biology shows two important aspects of Notch function. Firstly, it mediates inductive cell fate decisions. Secondly, it promotes an undifferentiated progenitor state. The inductive properties of Notch are not always exerted by the above-described imbalance-induction between two cells expressing both Notch and Delta. It is also possible that a Delta (but not Notch) expressing cell induces a certain fate in a cell that expresses Notch (but not Delta). For example, mouse thymocytes induce a T-cell fate in early lymphocyte precursors expressing Notch1. Finally to add more complexity, even though stem cell maintenance is Notch's prominent feature, Notch can also function as an inducer of terminal differentiation. For example, mouse neural crest stem cells differentiate into glial cells upon Notch stimulation.

Consistent with the above functions, mutations of the Notch pathway are associated with cancer, e.g. T-cell lymphomas, breast cancer, and colon adenocarcinomas [16, 20]. The effect of a constitutive Notch pathway activation in these cancers seems to be mostly based on Notch's role for the maintenance of a stem cell fate. Thus, cells with high Notch activity may not properly respond to differentiation signals from the environment. Pathological Notch activity, however, is not sufficient in most cases to induce cancer. Other mutations that, e.g. induce the high mitotic rates typical for tumor cells must occur. Some of the Notch targets are anti-apoptotic genes, e.g. the survival promoting phosphatidylinositol 3-kinase (PI3) or members of MAPK kinase pathway. Thus, the cancer promoting effect of constitutive Notch activity may also be elicited by Notch-mediated protection of malignant cells from apoptosis. Notch can also act as tumor suppressor. This might be partially explained by its capability not only to promote stem cell fate, but also to induce terminal differentiation. In the context of tumor suppressive properties of Notch, also indirect effects via the Wnt and hedgehog pathways are conceivable because both pathways can be suppressed by Notch. However, deregulated Notch expression may also result in constitutive activation of the hedgehog and Wnt pathways and thereby promote malignancies.

RTK Pathway

The RTK pathway encompasses several receptor families and their ligands [13]. The most important RTK pathways for nervous system development are the receptor families of epidermal growth factors, fibroblast growth factors (FGFs), platelet-derived growth factors, and the neurotrophin tropomyosin kinase receptor family (Trks) [35–39]. The common feature of the RTK pathway is that their ligands induce the formation of a receptor dimer (mostly homodimer) that causes the intracytoplasmatic tyrosine kinase domains of the receptors to phosphorylate each other at specific tyrosine residues [13]. Upon phosphorylation, the initial components of several signal transduction pathways

are bound to the phosphorylated residues, which activates the respective pathway. The best characterized pathways activated by RTKs are the ras/MAPK pathway, the PI3K/Akt pathway, and the phospholipase C-γ pathway. These pathways mediate a variety of functions. For example, the ras/MAPK pathway regulates neuronal differentiation and process outgrowth, the phospholipase C-γ pathway regulates activity dependent plasticity of neurons, and the PI3K/Akt pathway promotes neuronal survival [36].

Whether all, some, or only one of the above pathways is stimulated depends on several factors. Extensively studied examples to this end are the Trks and their ligands, the neurotrophins [36, 40]. So far, three Trk receptors have been characterized in vertebrates, TrkA, TrkB, and TrkC. They are activated by neurotrophins with nerve growth factor activating TrkA, brain-derived neurotrophic factor (BDNF) and neurotrophin 4 activating TrkB, and neurotrophin 3 (NT-3) activating TrkC. NT-3 also interacts with TrkA and TrkB, albeit with lower affinity than with TrkC. Depending on the tissue context and the developmental time point of activation, the different Trk ligands can induce different responses through Trks. On the same cell type, e.g. TrkB stimulation via BDNF may stimulate only survival promotion, while its stimulation through neurotrophin 4 promotes neurite outgrowth. Also differential interaction of Trk with the common neurotrophin receptor p75 may alter the effect that a neurotrophin mediates via its Trk. A third possibility is based on the fact that Trks occur in several isoforms due to differential splicing events. Signaling of TrkC, e.g. can be affected by differential insertions of short amino acid sequences into the tyrosine kinase domain of the receptor. Also, truncated forms of Trks that do not contain the tyrosine kinase domain are widely expressed in the nervous system. The role of these truncated receptor forms is not completely understood, but they may act as dominant negative regulators of Trk tyrosine kinase-mediated signaling because this requires association of the two RTK domains upon ligand binding. Finally, the extracellular domain of Trks is subjected to extensive splicing events, which may alter the ligand/receptor specificity as well as the functional output of Trk activation.

The biological purpose of RTKs may be completed and directed by co-receptors, as exemplified by the neurotrophin receptor families [36, 40–43]. As depicted, the common neurotrophin receptor p75 functions as a co-receptor for all Trks to modulate their signaling output but also to alter their interaction with their ligands. In particular, p75 increases the affinity of Trks to their respective ligands, and it can convert the low-affinity interactions of NT-3 with TrkA and TrkB into a high-affinity interaction. RTK co-receptors may, however, also have an independent role that functionally completes RTK activity. Best characterized in this respect are the neurotrophin co-receptors p75 and sortilin. Neurotrophins are produced as precursors that are processed intracellularly by

furin into their mature form. These precursors can, however, also be secreted in an unprocessed form into the extracellular space, where they can either be processed to their mature form by extracellular matrix metalloproteinases or plasminogen, or where they can act as specific neurotrophin ligands for p75 to induce cell death. As depicted, it is important to regulate cell survival during development, i.e. to remove some cells but to maintain others. Regulation of cell survival is one of the major functions of the neurotrophins and their receptors. Thus, in order to achieve the full competence of survival regulation, active death induction of a pro-neurotrophin via p75 functionally completes the survival-promoting function of the Trks. To exert its death-inducing activity, p75 needs the VPS10q-domain family receptor sortilin as a co-receptor. Sortilin was originally described as a sorting receptor and is now well-recognized as an additional co-receptor of the neurotrophin receptor families. Sortilin renders the low-affinity interaction of p75 with a pro-neurotrophin into a high-affinity interaction by forming a trimeric ligand–receptor complex consisting of pro-neurotrophin, p75, and sortilin. The formation of this complex is required for the active death-induction by neurotrophins. Upon formation of this complex, p75 activates the JNK pathway for death induction. Whether sortilin is only required for the formation of death complex, or whether it has also independent signaling function in this context is unknown.

Induction of the Nervous System

The nervous system begins to develop in the gastrula stage when cells from the embryonic surface, the ectoderm, ingress into the interior of the embryo to form the mesoderm and the endoderm [1]. The latter tissues give rise to intestinal organs, muscles, and the skeleton, while the ectoderm mainly forms the skin and the nervous system. The ingression of tissue giving rise to meso- and endoderm occurs in all vertebrate species, however, the morphology of this process may vary. The origin of the ingression is the blastopore in amphibians, the embryonic shield in teleosts, and the primitive streak in reptiles, birds, and mammals. Importantly, a very specific region of these structures – the dorsal blastopore lip in amphibians and the Hensen's node in vertebrates – is crucial for the development of the nervous system. This was first discovered in amphibians by Hilde Mangold and Hans Spemann in the early 1920s, when they demonstrated that the dorsal blastopore lip of one embryo implanted into the prospective belly region of another embryo at the gastrula stage causes a complete second nervous system to develop – at the ventral side of the host embryo and in addition to its 'proper' nervous system at the dorsal side. Besides the fact that this is one of the most beautiful experiments

ever (imagine the moment of realizing – for the first time – that your experimental manipulation causes a complete nervous system to develop), this experiment established the concept of neuronal induction (the concept of induction was introduced earlier by Hans Spemann when he discovered that certain regions of the developing brain are able to induce organs in other germ layers, e.g. the pituitary gland or the eye). The region causing neuronal induction was termed the organizer.

On a morphological level, neural induction occurs in two directions, vertically (vertical induction) and horizontally (planar induction) with the first steps initiated by the organizer and progressing horizontally into the mesoderm [44, 45]. The inductive signals from the organizer initiate the formation of a cord-like structure in the mesoderm, the so-called notochord. The notochord defines the dorsal longitudinal axis and directly underlies the ectoderm. From the notochord, inductive and fate determining signals are sent vertically to the overlying ectoderm, which transforms into the neuroectoderm. Vertical and planar inductions are not exclusive, but parallel processes. The concurrence of horizontal and vertical signals in the presumptive neuroectoderm as well as the proper neuroectoderm are important for inductive events, early fate determination, and early patterning of the developing nervous system, which will be discussed exemplarily in the remaining part of this section and the subsequent two sections.

The quest for the molecular mechanisms underlying neuronal induction was a tale of frustration for many decades. This changed in the 1990s when it was discovered that Noggin, Follistatin, Chordin, Cerebrus, Drm, Dan, and Ogon/Sizzled are secreted from the organizer to bind to and to antagonize the activity of BMP4, the ligand that suppresses neural fate in ectodermal cells [1]. The idea was born that neuronal induction is caused by the neutralization of a signal inhibiting a neural default fate of ectodermal cells. Once suppression of BMP signaling is initiated, the subsequent promotion of a neuronal fate is amplified by the property of the BMP pathway to be maintained by a positive feedback loop. If the inhibition of the BMP pathway is experimentally prevented, the development of the nervous system is almost completely prevented. The fact, however, that uncontrolled BMP signaling does not completely prevent neural development suggested that the default model of neuronal induction does not encompass the complete mechanism underlying neuronal induction. Indeed, it was found that there are also inducing factors (FGF family members) that are important to sensitize ectodermal cells for a neuronal fate before BMP regulation takes place.

There is still controversy how BMPs and FGFs interact in the context of neuronal induction, but there are several examples as to how this interaction may work [1]. First, one target of the signaling cascade initiated by FGF are Smad proteins, effectors of BMP signaling. Stimulation of the FGF pathway

induces a phosphorylation of Smad1, which inhibits Smad1 activation by BMP. It was also reported that FGF has a direct suppressing effect on BMP transcription at the gastrula stage, but a positive effect on the transcription of the inhibitory BMP interaction partners Chordin and Noggin. Finally, FGFs can even directly stimulate a neuronal fate. The precise mechanism of BMP and FGF mediated neuronal induction, however, is not established yet.

The regulatory machinery upstream of BMP and FGF is poorly characterized. The Wnt pathway seems to be involved [1]. First, it was shown that Wnt favors neuroectodermal fate by suppressing the transcription of BMP at the blastula stage. Thus, the cascade initiating neuronal induction may even be activated earlier than originally assumed. Apparently contradictory, the above-mentioned FGF mediated suppression of the BMP pathway requires parallel suppression of Wnt signaling. This discrepancy may, however, be explained by stage dependent differences of the effects mediated by the respective pathways.

Generation of Neurons and Glia

The inductive events described in the preceding section result in the formation of the neural plate, an epithelial cell assembly consisting of neural stem cells [2, 15]. Neural stem cells are mitotically active cells that possess the potential of self-renewal and generate neural and glial precursors, which in turn give rise to neurons and glial cells respectively. By definition, a neuronal stem cell is able to give rise to all types of neurons and glial cells needed in the nervous system. This characteristic may be fulfilled by the very early neural stem cells immediately after induction of the neuroectoderm. It is, however, very likely that there are, in parallel to the above-described inductive events, signals that already convey very early fate restricting information to the neural stem cells. In other words, the stem cells receive patterning information that restricts their developmental potential and proliferation to the requirements of the topographical region they are located in. Examples for these patterning signals will be given in the next section. This section focuses on aspects specifically important for the genesis of precursor cells.

As indicated, most, if not all, initial neuroectodermal cells are neural stem cells. They form a single layered epithelium that can be morphologically distinguished from the neighboring ectoderm by its greater thickness [46]. The cells in this epithelium are rapidly proliferating, initially mostly symmetrically, i.e. the mitosis of one neural stem cell gives rise to two identical daughter cells – two new neural stem cells. While the apical and basal processes of these stem cells remain contact to the inner (apical) and outer (basal) limit of the neural plate epithelium respectively, their cell bodies and nuclei are cycling between

the inner and outer surface with the actual mitoses occurring apically, i.e. in the immediate neighborhood of the surface of the embryo (and of the ventricular surface after neurulation, as described below). As these mitoses progress, the neural plate rapidly increases its area and folds inward in the midline axis of the embryo, and slightly outward at the lateral borders of the neural plate to the ectoderm. This lip-like, extruding lateral part of the neural plate is called the neural crest. This folding process, called neurulation, proceeds and the neural plate becomes successively a neural groove and finally a neural tube. The neural tube closes at the former lateral borders of the neural plate and descends inwardly towards the center of the embryo remaining close to its ectodermal surface, which also closes over the descending neural tube.

After neurulation is completed, all neuroectodermal tissue is located inside the embryo, the former outer neuroectodermal surface is now located centrally and forms the ventricular walls, and the entire embryo is surrounded by ectoderm [46]. Alike the neural tube, the above-mentioned neural crest also descends inwardly being initially located dorso-laterally between neural tube and ectoderm. The neural crest give rise to all the neurons whose cell body is located in the peripheral nervous system, to all glial cells of the peripheral nervous system, to the adrenal medulla, to melanocytes, and to some connective tissue of the head. Thus, these cells display the highest migratory activity in the developing body. The neural tube changes its appearance during further development. First, it develops three vesicle at its anterior end, the prosencephalic (future forebrain), mesencephalic (future midbrain), and rhombencephalic (future pons, cerebellum, and medulla oblongata) vesicles. Two additional vesicles (the lateral vesicles) are budding off from the prosencephalic vesicle during development. The original medial prosencephalic vesicle develops into the diencephalon, the two lateral vesicles develop into the telencephalon. The cells of the neural tube (and the brain vesicles) give rise to all spinal cord and brain cells and to peripherally projecting neurons whose cell body is located in the CNS. By definition, the border between central and peripheral nervous system is at the point where the cranial nerves (exception: optic nerve, which is not really a nerve but a protrusion of a brain fiber tract system. Accordingly, the myelinating cells of the optic nerve are oligodendrocytes) and the spinal cord ventral and dorsal roots leave the brain and spinal cord respectively. This definition is not ‘artificial’ because also the cell types that sheet and myelinate axons changes at this location. While CNS axons are myelinated by oligodendrocytes (one oligodendrocyte myelinates several axon-segments derived from different neurons), peripheral nervous system axons are myelinated by Schwann cells (one Schwann cell myelinates one axon segment of one neuron). To differentiate between these two types of myelinating glial cells is important from a functional point of view because oligodendrocytes inhibit the regeneration of

damaged axons while Schwann cells promote their regeneration [47–49]. The clinical pendant to this difference is obvious: there is no significant functional and structural regeneration after spinal cord injury, while peripheral nerve lesions can completely regenerate.

During neurulation, the future nervous system is a single-layered epithelium consisting mostly of mitotically active neural stem cells [46]. Parallel to the closure of the neural tube and at subsequent stages of neural development, the mitoses in the neuroepithelium become increasingly asymmetrical, i.e. a neural stem cell mitosis gives rise to a stem cell and a progenitor, either neural or glial. The first products of these asymmetrical divisions are specialized glial cells, the radial glial cells, that have one process maintaining contact to the ventricular surface, and one process maintaining contact to the basal membrane of the neuroepithelium, the later external limitans membrane of the brain and spinal cord surface. Subsequent cohorts of progenitor cells migrate along these radial glial cells and settle down outside the mitotically active ventricular zone (there are exceptions). The latter zone is also called the matrix zone. Thus, the matrix zone contains the stem cells and produces the progenitor cells. The next population to be produced after the population of radial glial cells is the population of neural progenitors, which is followed by glial progenitors [34, 46]. The progenitor cells leave the matrix zone and form an additional layer surrounding the matrix zone, the so-called marginal zone [46]. Thus, the marginal zone inserts itself between the ventricular zone and the outer limitans membrane. As development proceeds, the marginal zone becomes thicker and acquires the very complex structure of the postnatal nervous system consisting of fiber tract systems (white matter) and neuronal cell bodies that are grouped and organized either in nuclei (corresponds to ganglia in the periphery) or in layers, which are the so-called cortical structures of the brain (e.g. cerebellum, neocortex). Both nuclei and cortices constitute the gray matter of the nervous system. It should be noted that the matrix zone is the primary area of neurogenesis, but that there are additional ‘matrix zones’ during development, i.e. stem cell containing and progenitor producing zones, which are secondarily established from cells that originate in the periventricular matrix zone. The best known example is the dorsal anlage of the cerebellum in which cerebellar granule cells are generated. The regulation of cell migration, which builds up the marginal zone and its adult successors, will be discussed in the next but not one section. The mechanisms directing the insertion of white matter between and into the different gray matter structures, i.e. axonal growth and pathfinding, will be discussed in the chapter of Chedotal.

The two major zones of the developing nervous system, the matrix and the marginal zones, dramatically change their size proportion to each other during development [46]. While the matrix zone is the clearly dominating ‘neural

structure' immediately after neurulation, the marginal zone becomes increasingly dominant during subsequent development until it finally forms the mature brain and spinal cord after development. In the mature brain, only the flat epithelium of the ventricular walls, the ependyma, and a few cell layers immediately neighboring the ependyma are the remnants of the embryonic matrix zone. As we know today, this zone still contain neural stem cells in the adult [2].

What are the molecular mechanisms that underlie such a powerful neurogenic potential of the matrix zone? Before depicting our present knowledge towards this end, it is helpful to define the functional framework for neuro- and gliogenesis [2, 15]. Three important requirements have to be considered in this context. Firstly, the proliferation of stem cells has to be regulated, i.e. there should be neither excessive over-production nor too little production of new cells. Secondly, at least a portion of stem cells has to be prevented from differentiating into precursors, neurons, and glial cells because premature differentiation would lead to a depletion of the stem cell pool before development is completed (and also extinguish those stem cells that are maintained through adulthood as a regenerative reserve pool). Thirdly, there have to be mechanisms that adapt the potential developmental fates to the regional and stage-dependent requirements, i.e. achieve an adequate patterning of the nervous system and produce the right type of cell at the right time and the right place. Even though the signaling pathways regulating these requirements may mutually influence each other and contribute to each of these requirements, each requirement is functionally dominated by a specific subset of signaling pathways. The Notch and Wnt pathway are most crucial to maintain stem cells, i.e. to prevent their differentiation [18, 30]. Members of the RTK pathways affect primarily proliferation (mostly promoting it), but they may also initiate differentiation, stage dependent fate decisions, and promote stem cell survival [9, 13, 36, 50]. For example, bFGF stimulates the proliferation of cortical progenitors, yet a member of another RTK family, the neurotrophin NT-3 induces these progenitors to exit the cell cycle and to differentiate into a neural precursor [51]. Important mediators of these regulatory pathways (especially of the Notch pathway) are transcription factors of the bHLH family of transcription factors [34, 52].

The name bHLH transcription factor is based on the structural motif shared by these molecules, which mediates their dimerization and DNA binding [34, 52]. The human genome contains approximately 125 bHLH factors. The effects of these factors on stem cell maintenance, proliferation, and fate decision will be exemplarily described for cortical development. Both stem cell maintenance and progression into differentiation depend on bHLH factors. To maintain neural stem cell fate and to promote their proliferation, two classes of inhibitory bHLH factors are important. Firstly, the Hes factors, which are Notch

targets, and secondly the Id factors, which are Wnt targets. Hes and Id factors counteract proneuronal bHLH factors and promote stem cell maintenance and proliferation.

Hes factors use two means to repress differentiation [34, 52]. First, they form hetero- and homodimers that directly bind to DNA at so-called N-boxes of proneuronal genes and thereby repress the expression of these genes. In addition to Hes, the repressor complex contains transcriptional co-repressors that belong to the Groucho-transducin-like enhancer of split family (Gro/Tle). The second means of repression is exerted by the ability of Hes factors to bind to transcriptional activators of proneuronal genes and thereby repress their activity. For this mode of suppression, Hes does not need to interact directly with DNA, the recruitment of Gro/Tle transcriptional co-repressors, however, is required. Hes factor mediated mechanisms are essential for the ability of Notch to block differentiation and to maintain neural stem cell fate. Remembering the characteristic signaling properties of the Notch pathway, coupling of Notch signaling to Hes factors allows that neighboring cells undergo asymmetrical fate decisions. The stem cell expressing more of the Notch ligand Delta will stimulate Notch signaling and thereby promote stem cell fate in its neighbor cell, the neighbor cell in contrast will suppress Delta expression in response to its Notch stimulation and therefore convey less Notch stimulation to the first cell. Thus, the cell expressing more Delta will have suppressed Notch signaling and is thereby enabled to undergo differentiation into a neural precursor. This mechanism, which is called lateral inhibition, will allow one portion of the stem cell population to stay in an undifferentiated stem cell stage, while the other portion of stem cells can take off to a neuronal fate.

Id factors have a similar role as Hes factors, they use different mechanisms, however, and are under control of the Wnt pathway [34, 52]. Id factors have the bHLH motif for hetero- and homodimerization with other bHLH factors, but they lack the motif for DNA binding. Accordingly, they do not interact directly with repressor or promoter regions of genes, but rather affect the activity of transcriptional activator/repressor complexes by selectively interacting with components of these complexes. One mechanism of Ids to suppress neuronal and glial differentiation is to sequester E proteins from transcriptional activator complexes of E box containing genes, many of which are required for neuronal and glial differentiation (e.g. Mash, Neurogenin, NeuroD, Olig family members). Ids also affect another aspect of stem cell propagation, the progression through the cell cycle. To this end, they interact with retinoblastoma (Rb) family members and thereby prevent the binding of Rb to E2F transcriptional activator complexes, which would suppress the progression through the cell cycle. It is likely that this mechanism is, at least in part, the basis of Wnt-meditated promotion of cell cycle progression.

As indicated above, the Hes and Id factors counteract proneuronal and neuronal differentiation bHLH factors [34, 52]. Neurogenesis is initiated if this balance is moved towards the proneuronal bHLH factors, which are expressed in the matrix zone. The neuronal differentiation bHLH factors that specify, which subtype of neuron is generated, are predominantly expressed in the marginal zone. Proneuronal (e.g. Neurogenins, Mash) as well as neuronal differentiation bHLH factors (e.g. NeuroD) form heterodimeric complexes with E proteins and bind to so-called E-boxes in the promoter regions of target genes. Activation of an active transcriptional promoter complex requires the association of additional co-activators. The mechanism regulating the precise timing and location of proneuronal and neuronal differentiation bHLH expression and activity are largely unknown. Because these bHLH factors have potential phosphorylation sites for GSK3, which is regulated by the Wnt and FGF pathways, it is proposed that Wnts and FGFs are involved in the activation of proneuronal and neuronal differentiation bHLH factors. In any case, the increase in proneuronal bHLH factor activity initiates neurogenesis. At later stages of development, astrocytes are generated. The neuronal and neuronal differentiation bHLH factors are not involved in astrocyte differentiation, but it seems that the Hes factors are important to induce the generation of astrocytes. This is surprising because Hes factors also cause maintenance of a neuronal stem cell fate. The precise mechanisms underlying astrocyte generation by Hes factors are unknown, but it is assumed that the function of Hes factors switches from promoting stem cell fate to promote astrocyte fate after initiation of a neurogenic wave by proneuronal bHLH factors. This is in line with the observation that also the Hes regulator Notch promotes astrocyte fate at these stages.

The development of cortical oligodendrocytes and cortical GABAergic interneurons provides a good example that the build-up of the marginal zone does not always follow the initially depicted rules of radial migration along radial glial fibers [8, 34]. GABAergic cortical interneurons and cortical oligodendrocytes arise from the matrix zones of the ventral telencephalon, i.e. a region that is mainly concerned with the build-up of the basal ganglia. These precursors, therefore, undergo extensive tangential migration and settle down in their dorsal cortical target areas. The mechanism of oligodendrogenesis has recently been discovered [34]. Generation of these cells is induced by the bHLH factors Olig1 and Olig2 after cortical astrogenesis is completed. Similar to proneuronal bHLH factors, Olig1 and Olig2 are E-protein interacting molecules.

Early Patterning of the Nervous System

This section describes the influence of the hedgehog and BMP pathways on the early dorso-ventral patterning of the nervous system. Understanding the

geometry of neurulation is prerequisite for understanding the mechanisms of dorso-ventral patterning of the nervous system. In the previous section, it was described that the neural plate invaginates along its anterio-posterior axis and successively forms the neural groove and neural tube [46]. During the entire process of neurulation, the notochord underlies the anterio-posterior axis of the neural plate/tube in the midline, directly bordering the floor plate (=central portion of the neural plate/tube). In anterio-posterior direction, the border between ectoderm and neural plate defines those aspects of the neural plate that become dorsal after the completion of neurulation, i.e. the closure of the neural tube. The most dorsal neural tube area is called roof plate.

The inductive signals for dorso-ventral patterning of the developing nervous system originate in structures along and parallel to the anterio-posterior axis of the neural plate/tube, the ecto-neuroectoderm border/roof plate and the notochord/floor plate [45, 46]. The ecto-neuroectoderm border and roof plate define the 'dorsal' aspect of the neural plate/tube (=dorsal patterning center), while the notochord/floor plate define the ventral aspect of the neural plate/tube (ventral patterning center). Diffusible substances A and B secreted from the dorsal (A) and ventral (B) patterning centers can, therefore, build-up concentration gradients in the neural plate/tube with opposing directions for A and B. Thus, each dorso-ventral level will have a specific concentration for A and B, which is used to specify the neuronal subtypes needed in the respective dorso-ventral levels. The known dorso-ventral patterning molecules are BMPs, which are produced in the dorsal patterning center, and SHH, which is produced in the ventral patterning center [28, 46]. While BMPs predominantly specify neuronal subtypes in the dorsal half of the neural tube, i.e. sensory neuron subtypes, SHH specifies the neuronal subtypes in the ventral half of the neural tube, i.e. motor neuron subtypes. It is interesting to note that suppression of BMP expression in the neural tube after neuroectoderm induction is the basis for a dorso-ventral BMP gradient within the neural tube. From the principle depicted above, it is conceivable that the specific ratio of SHH and BMPs at the respective dorso-ventral levels contributes to the dorso-ventral patterning. Whether this is the case, however, is not known. The following will exemplarily describe how SHH regulates motoneuron subtype specification in the ventral tube.

SHH is expressed in the notochord and floor plate exactly at the time when these two structures have the capacity to induce ventralization of the neural plate and neural tube [28]. As depicted above, SHH has distinct effects on the expression of class I and class II homeodomain genes with suppressing the expression of class I and promoting the expression of class II genes. The graded signaling of SHH thus causes the differential expression of the class I genes Pax6, Irx3, and Dbx1/2 and the class II genes Nkx2.2 and Nkx6.1 in the ventricular zone of the ventral neural tube. This establishes the region-specific

expression from ventral to dorsal of Nkx2.2/Nkx6.1, Nkx6.1, Irx3/Nkx6.1, Dbx2/jIrx3, and Dbx1/2/Irx3, which specifies the development of Sim1, Isl1, Chx10, En1, and Evx1/2 neuronal marker expressing subpopulation of motoneurons respectively. Whether SHH has an instructive effect on the specification of motoneurons in the more anterior parts of the neural tube, i.e. the future forebrain areas, is not known. After the generation of motoneurons is completed, SHH has an inductive function for the generation of oligodendrocytes, which are all produced in ventral areas of the developing nervous system in areas where SHH is expressed, or in areas immediately neighboring SHH expression. The requirement of SHH for oligodendrocyte induction may also explains why these cells have to undergo the above-described extensive tangential migration from the ventral to the dorsal forebrain areas; they have to be induced ventrally because SHH is expressed there (there is also SHH expression dorsally in the cortex, which is however counteracted by dorsally expressed BMP) [53]. Consistently, SHH also promotes the generation of cortical GABAergic interneurons, which have to perform a similar tangential migration from the ventral matrix center to their dorsal cortical target areas [54, 55]. Thus, the necessity of tangential migration for these subtypes of cells may depend on the inductive role of ventrally expressed SHH, but also on the suppressive role of dorsally expressed BMPs.

As indicated in the previous paragraph, there are additional centers in the dorsal nervous system expressing SHH at later developmental stages [28]. The function of SHH expressed at these later developmental stages may, however, not be restricted to the induction of specific cell fates. In the context of cerebellar development, e.g. SHH can promote granule cell precursor proliferation and differentiation, depending on the nature of the extracellular matrix; in a laminin-rich environment, SHH promotes proliferation, while it induces exit from the cell cycle and differentiation in a vibronectin-rich environment. The proliferative effects of SHH are likely mediated via Ptc interaction with cyclin B1, while the switch of the SHH response to differentiation requires the activation of the cAMP response element binding protein. Similar roles of SHH on proliferation have been proposed in the context of eye development.

Neuronal Migration and Differentiation

Once the different regions and subdivision of the developing nervous system are specified, the newly generated precursors from the matrix zone are set and ready for migration [8, 56]. There are two types of migration, the radial and the tangential migration. In the context of radial migration, cells migrate from the matrix zone along the radial glia scaffold towards the surface of the developing

nervous system. In contrast, tangential migration is characterized by a direction of cell migration which is orthogonal to the orientation of the glia scaffold. The following will describe these migratory modes in the context of cortical development, which is one of the best-studied examples of mechanisms that direct migration. It should be noted that the cortex is a layered structure. Thus, the principle mechanism governing neuronal migration may differ in areas that are organized in a nuclear arrangement.

Radial migration is the primary mode how newly generated cells leave the matrix zone to build-up the nervous system [8]. This is reflected by the structure of the adult brain, which is organized in a radial manner. The key component of radial migration is the population of radial glial cells. Radial glia cells derive from the first wave of progenitors generated by the matrix zone and maintain processes to the ventricular and pial surface of the developing nervous system. These cells do not only serve as a scaffold for migrating neurons, they also have stem cell-like characteristics. Their cell body is located in the matrix zone and can undergo mitosis to generate new neurons. Thus, radial glia cells can be regarded as an intermediate stage between stem cell and progenitor stage. After neurogenesis is completed, radial glial cells differentiate into astrocytes. The primary mode of radial migration is the glial-guided mode of locomotion (subsequently described mechanisms refer to this mode). This mode is characterized by the physical attachment of the migrating cell to the radial glia cell. The migrating cell uses the radial glia then as a 'highway' to move in salutatory locomotion patterns (rapid forward movements followed by a resting phase) towards the pial surface. Another mode of radial migration, which is used by cells generated very early during development, is the somal translocation. In this mode, the migrating cell first extends a process along the radial glia scaffold towards the pial surface. Once this process attaches to the pial surface, the cell loses its contact to the ventricular surface and 'pulls' its cell body continuously towards the pial surface. The mode of somal translocation is also used in the last phase of glial guided locomotion, when the leading process of the migrating cell reaches the pial surface.

In cortical development, the very first wave of neurons forms the so-called preplate [8]. The second wave of neurons settles down in the middle of the preplate and thereby splits the preplate into two layers, the marginal zone (in a narrower sense of cortical development) at the pial surface and the subplate, which is close to the ventricular zone. The marginal zone contains a population of cells that is very important for the subsequent layering of the cortex, the population of Cajal-Retzius cells. Cajal-Retzius cells secrete an extracellular matrix associated protein, reelin, which is required for the subsequent waves of newly generated neurons to build-up a properly layered cortex (the mechanism is described below and applies to all layered structures of the nervous system).

The very typical feature of the development of the neocortex is the so-called 'inside first, outside last' mode of cortical development [46]. This means that a newly generated cohort of neurons has to migrate through the population of neurons that was created before. Thus, each new layer settles down between the antecedent layer and the marginal zone. The consequence of this pattern is that the oldest layer of the cortex lies deepest, whereas the youngest layer lies most superficial. If Cajal-Retzius cells do not secrete a functional reelin molecule, this layering cannot be built up [8]. The preplate is not split into marginal zone and subplate, and newly generated neurons cannot properly cross the cell layers that have been generated before. The consequence is a mislayered and disorganized cortex.

There are several functionally distinct processes in the context of radial migration [8]. First, the cells have to initiate movement, then they have to attach to the radial glial cell, they have subsequently to move along the radial glial cell, and finally they have to detach once they reached their final destination. Thus, the major duties to be performed are cell movement and attachment. Movement of migrating cells can be regulated by two classes of extracellular signals, neurotransmitters (GABA, Glutamate) and members of the RTK pathway (neurotrophins, epidermal growth factor). GABA, e.g. appears to contribute to the initiation of movement out of the ventricular zone via $GABA_{A/C}$ receptors, and to regulate cell movement from the ventricular zone to the cortical plate via $GABA_B$ receptors. Also neurotrophins modulate the movement of migrating cells. The modulation of cell movement involves intracellular Ca^{2+} signaling. The adhesion/detachment is influenced by a great variety of ligand/receptor systems that partially belong to the classical families of cell adhesion molecules (e.g. integrins), but also to the LDL receptor family (Apolipoprotein E receptor 2 [Apoer2], very-low-density lipoprotein receptor [VLDLR]). As it is now well-established, the migratory roles of reelin are mediated by a receptor complex containing Apoer2 and VLDLR [57]. Upon reelin binding, Apoer2 and VLDLR recruit the cytoplasmatic adaptor protein disabled 1 (Dab1) to a specific motif of their intracellular domain, the NPxY motif. The NPxY motif is characteristic for all LDL family receptors and is used in the context of lipid metabolism for the internalization of lipoprotein/lipoprotein receptor complexes. In the functional context of migration, the NPxY motif does not mediate internalization, but activation of an intracellular signaling pathway that regulates several important aspects of proper migration. Initiation of this pathway requires the docking of Dab1 to Apoer2/VLDLR and subsequent phosphorylation of Dab1. The phosphorylation step requires a kinase activity, which is most likely constituted by Fyn and Src-family kinases through association of Apoer2/VLDLR with a co-receptor (potentially integrins). It is not resolved yet how exactly reelin influences migration and proper laminar

positioning through Apoer2 and VLDLR. Three main possibilities are discussed [8, 57]. Firstly, reelin could act as a stop signal for neurons that arrive at their destinations close to the marginal zone. In this case, however, one would not expect accumulation of newly generated neurons at more distant locations to the marginal zone, as it occurs in reeler mutant mice, which express a non-functional reelin. Secondly, reelin might affect the adhesive properties of glial cells. Finally, and conceptually similar to the second possibility, reelin may regulate the detachment of migrating neurons from the radial glial cells. Then, previously generated neurons that would not detach would create a physical barrier for newly incoming neurons. This scenario is in agreement with the phenotype observed in reeler mice. Reelin's influence on the adhesive properties could be mediated by its interaction with other proteins such as integrin receptors, which would be consistent with the above-described necessity for Dab1 phosphorylation to recruit a kinase activity to Apoer2/VLDLR. The reality, however, is probably more complicated because knockouts of relevant candidate integrin receptors do not display the lamination effects that are observed in reeler mice, Apoer2/VLDLR double knockouts, or Dab1 deficient mice.

Tangential migration employs different mechanisms than radial migration [8, 56]. In some cases, it is directed by specific interactions of migrating cells with extracellular matrix proteins, in other cases, the tangential migration uses pre-existing axonal projections as guidance cues. As in the context of radial migration, however, the tangential migration of cells is influenced by extracellular signals and mediated by cell adhesion and pathfinding molecules (e.g. integrins, Eph family tyrosine kinases and their membrane associated ephrin ligands, semaphorins and their neurophilin receptors), many of which are also employed in the context of axonal pathfinding and described in the section of Chedotal.

Once migration is completed and the neurons reach their final destination, they differentiate into the mature type of neurons they are designated for. This is regulated by the above-described neuronal differentiation bHLH factors, which are under control of Notch and Wnt pathways [34]. Important mediators of terminal differentiation are several members of the RTK pathway, which are in turn – at least partially – also under control of bHLH factors [9, 34, 36]. The expression of Trk receptors, e.g. is regulated by NeuroD. RTK pathways affect many aspects of terminal differentiation such as the neurotransmitter types used by the respective neurons or their dendritic morphology. In the visual cortex, e.g. the neurotrophins regulate the dendritic morphology of pyramidal neurons and the formation of occular dominance columns [58, 59]. Another aspect of terminal differentiation is the adaption of the neuronal projections to their innervation targets, which is described in the next section. Neurotrophins may not only modulate the transmitter phenotype of a neuron, they may also be important for the

implementation of a mature response to neurotransmitter stimulation. For example, GABAergic transmission during neuronal development is not hyperpolarizing as it is in the mature brain, but depolarizing [60]. The nature of a response to GABA stimulation, i.e. whether it is hyper- or depolarizing, largely depends on the intracellular chloride concentration of the cell receiving a GABA signal. If the intracellular chloride concentration is high, the response upon GABA will be depolarizing, if the concentration is low, the response will be hyperpolarizing. The transition of an immature depolarizing type of GABA transmission to a mature hyperpolarizing type is mediated by the onset of expression of the neuronal cation-chloride co-transporter KCC2 in maturing neurons [61]. KCC2 extrudes chloride from the cells and is, therefore, responsible for setting the intracellular chloride concentration to the low level typically observed in mature neurons. As it was shown recently, the neurotrophin BDNF is, at least in part, required for the late-developmental up-regulation of KCC2 [62].

Adaption of Neurons to Their Innervation Target

As depicted initially, the constituents of neuronal connectivity – neurons and their axonal projections – are created in excess during neuronal development. It is thought that this over-production serves to create a reserve potential for the postnatal fine tuning of connectivity to the actual requirements. There are principally two means to achieve this fine tuning, postnatal programmed cell death [22] and axon collateral elimination [10]. Postnatal programmed cell death was the first described of these mechanisms, and is still much better understood than the process of collateral elimination.

In the late 1950s, Rita Levi-Montalcini, Stanley Cohen, and Victor Hamburger discovered and described nerve growth factor as a molecule that regulates the survival of certain neuronal subpopulations during early postnatal development [63]. Based on their finding, the classical neurotrophin hypothesis was formulated. This hypothesis states that the survival of a neuron depends on survival factors that are produced in limited amounts in the innervation target of the respective neuron. Only neurons that have sufficient access to this factor, i.e. display optimal target innervation, survive while the other neurons die. Thus, survival regulation was regarded as a function of availability of a survival factor for a neuron. This mechanism was thought to adapt a neuronal population to its innervation target. Indeed, it was shown that removal of innervation targets increases the amount of postnatal death in those neuronal populations that would have innervated the removed target. Vice versa, addition of surplus innervation targets reduced the amount of cell death. It has later been recognized by Yves-Alain Barde and co-workers [64, 65] that neurotrophins also possess

active death inducing capacities. It is now an accepted concept that neurotrophins regulate neuronal survival antagonistically [41, 43, 66–69]. The present model to this end is that the mature form of a neurotrophin promotes neuronal survival via Trk/p75, while the pro-form of a neurotrophin induces death via p75/sortilin. This mechanism surely expands the biological applicability of neurotrophin mediated survival regulation to other developmental contexts. In the developing optic system, e.g. neurotrophin mediated death induction is used in a morphogenic sense as a certain subpopulation of RGC is removed in order to create space for the outgrowing optic nerve axons. At present, there is no convincing evidence that neurotrophin-mediated survival regulation also applies to normal adult CNS neurons. There are some examples where neurotrophins regulate the survival of adult neurons after injury [70]. However, injury induces many embryonic regulatory programs for repair processes [49, 71] and the neurotrophin-mediated survival regulation more likely represents the re-activation of a developmental program for survival regulation than a mechanism representative for the survival regulation of undamaged, normally aging neurons.

The validity of neurotrophin-mediated survival regulation as a mechanism for developmental adaption of a neuronal projection system to its target has been shown mostly in the peripheral nervous system. CNS neurons seem to employ different mechanisms. The best studied examples are the long-projecting cortical layer V pyramidal neurons [10]. These cells initially grow an elaborate axon collateral system to many subcortical targets. In the process of maturation, many of these collaterals are eliminated and only those collaterals are maintained that are important for the proper function of the respective system. For example, layer V pyramidal neurons of the visual cortex and the primary motor cortex initially project, among others, to the spinal cord and to the optic tectum. During maturation, the visual layer V neurons lose their connection to the spinal cord but maintain their connection to the optic tectum. Conversely, the motor layer V neurons lose their connection to the optic tectum, but maintain their connection to spinal cord motor centers. Similar processes are also observed at a finer level [46]. For example, the lateral geniculate nucleus is innervated by the optic nerve fibers of both eyes. This innervation is initially overlapping, i.e. both eyes largely occupy the same innervation fields. Later in development, this process is refined by axon pruning (equivalent to axon collateral elimination) so that each eye innervates specific sub-areas in the nucleus. It is known for a while that neuronal activity governs the processes of axon pruning/collateral elimination, but the underlying molecular mechanism was obscure for a long time. It was recently shown that major histocompatibility complex (MHC) class I receptors are required for the activity dependent pruning of geniculate innervation by the optic nerve [72].

MHC class I molecules are important in the context of the adaptive immune response [73]. They intracellularly bind fragments of digested proteins and present these at the cell surface to monitoring cytotoxic T lymphocytes. In the immune system, this mechanism serves for self and non-self recognition and is central to our immune response to viral and bacterial infections, but also in cancer development. It has long been assumed that MHC class I molecules are not expressed in the nervous system. Recent more sensitive methods have, however, well established that there is a broad and very complex expression of these molecules in the developing as well as in the mature nervous system. The precise mechanism of MHC class I mediated regulation of activity dependent plasticity is not deciphered yet. The experimental design of the study revealing this novel MHC I function suggests that the machinery required for MHC I function in the immune system is also involved in a neuronal context. Instead of knocking out specific MHC I genes, β2m (obligatory light chain of most MHC I molecules) and TAP1 (transporter required for peptide loading of MHC I molecules) were knocked out. This prevented localization of MHC I molecules to the cell surface and thereby their ability to involve in activity dependent axon pruning. Whether this mechanism applies to axon pruning and/or axon collateral elimination in other systems than the optic system has not been shown yet. Also, it is not clear what type of molecules are loaded onto the MHC I molecule for surface delivery, and whether MHC I receptors in this functional context act in concert with co-receptors, as they do in the immune system.

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Organization of Peripheral Nerves in Skin, Musculoskeletal System and Viscera

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Abstract

Skin, musculoskeletal system and all organs of the body are supplied by nerve fibers of the somatic and autonomic nervous system, each of the systems with its specific nerve fiber types, fiber composition, fiber density and targets. Experimental data support the hypothesis that tumor tissue might interact with nerve fibers. The peripheral nervous system possesses an extraordinary cellular equipment to protect the axons against pathological stimuli. Only restricted areas lacking a cellular barrier are weak points within the nervous network. Therefore, this article focuses on the functional morphology of the peripheral nervous system and its regional differences.

Skin, musculoskeletal system and all organs of the body are supplied by nerve fibers of the somatic and autonomic nervous system, each of the systems with its specific nerve fiber types, fiber composition, fiber density and targets. The peripheral nerves therefore consist of a varying number of myelinated and unmyelinated neurons whose perikarya are located in brain, spinal cord or peripheral ganglia. The organ specific peripheral innervation pattern is ontogenetically established in a time- and space-specific manner following the individual genetic program in combination and interaction with various epigenetic influences. The organ-specific innervation pattern seems to be one of the prerequisites for the central nervous system networks to optimize and adapt to life challenges.

In this chapter, we will focus on the histological organization of peripheral nerves and the enteric nervous system. The three connective tissue compartments covering the peripheral nerve and the establishment of the 'blood-nerve barrier' in the conductive part of the nerve fibers guarantee an undisturbed signal conduction. The striking difference in the organization of the enteric nervous system is the absence of cellular barriers to the surrounding microenvironment.

To fulfill their proper functions these ganglionated and interconnecting plexus need the free communication with this local microenvironment.

Compartments of the Peripheral Nerve

Peripheral nerves are enclosed by three connective tissue compartments, the epineurium, perineurium and endoneurium, which protect the nerve fibers along their pathway to their targets against external stimuli and which guarantee a controlled microenvironment for the axons and Schwann cells. Peripheral nerves have their own vascular supply – the vasa nervorum – composed of the epidural vascular plexus providing endoneural microvessels. Due to the characteristic composition of the three compartments differing in amount and composition of collagen fibers, microfibrils, elastic fibers and extracellular matrix components the nerves exhibit great tensile strength but lack resistance to compression (fig. 1).

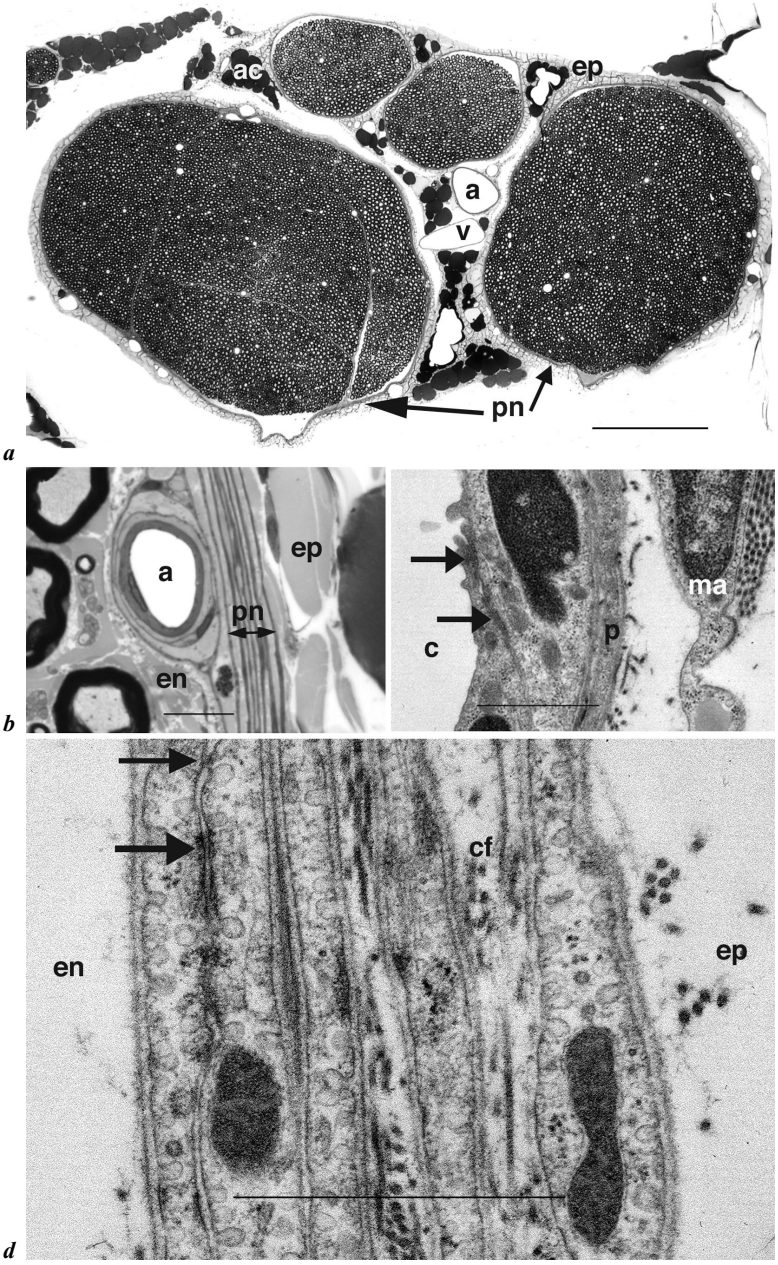
Epineurium

The epineurium is the outermost compartment of peripheral nerves which surrounds each nerve fiber bundle and which binds together the nerve fascicles to a nerve. It is mainly composed of dense connective tissue with only some very fine elastic fibers. The amount of adipose tissue within the epineurium varies depending on the developmental stage and topography. The epineurium houses the epineural vascular plexus, lymphatic vessels and perivascular mast cells. In the proximal part of the nerve the epineurium is relatively thick, but up to the smaller branches its thickness is gradually reduced.

Perineurium

The perineurium is composed of one to several layers of perineural cells, their basement laminae, the extracellular matrix and the collagen fiber network. Junctional complexes are established between the perineural cells. The perineurium builds up a highly organized and selective barrier between the inner compartment (endoneurium) and outer compartment (epineurium) of the nerve fiber bundles [1–3]. It follows the cranial and spinal nerves up to their final terminations close to the target. Only at these termination sites molecules and cells from outside have direct access to the axonal and Schwann cell membranes.

The perineurium is one part of the blood-nerve barrier and guarantees myelinated and non-myelinated axons a stable microenvironment within the endoneurium independent from a changing microenvironment of the surrounding tissue compartments. Because of the established tight junctions between the perineurial cells a paracellular transport of molecules is impossible. During the



a

b

c

d

last 20 years, the molecular components of the junctional complexes as tight-, gap- and adherens junctions between the perineural lamellae came into the focus of interest (fig. 1b, d). Molecular biological techniques made it possible to analyze and describe the complex structures in their molecular dimension and interaction and to define their special role in the formation and establishment of the barrier properties.

Pummi et al. [4] investigated the expression pattern of the different tight junction proteins claudin-1, -3, -5, zonula occludens-1 (ZO-1) and occludin of the human fetal perineural sheath. They found that in the perineurium the mature junctional complexes with their distribution and expression pattern are not established before 35 weeks of gestation. Occludin and claudin seem to contribute to the tightness of the barrier, whereas the tight junction plaque protein ZO-1 seems to organize the coupling of cytoskeletal proteins as actin filaments to the plasma membrane. Nevertheless, up to 24 weeks postnatal the perineurium is still permeable [5].

The perineurium however, allows a selective transport of molecules and substances from the external environment to the endoneurium. One of the best-analyzed pathways is the glucose transport across the perineurial cells using the glucose transporters 1 (GLUT1) [6–8]. Morphologically the high amount of caveolae as well as coated pits may be correlated with transcellular transport pathways of the perineural cells [2, 9, 10] (fig. 1d). The occurrence of connexin43 in all layers of the perineurium reflects a distinct metabolic coupling of the individual perineural laminae [11].

Schwann cells play an important role in the ontogenetic formation of the perineurium. Its configuration especially needs desert hedgehog protein (dhh) secreted by Schwann cells. Moreover, this protein is of fundamental importance for an intact barrier [12–14]. In the knock out mouse (dhh–/–) the epineurium is reduced in thickness, the perineurium is morphologically disturbed, lacks connexin43 and displays barrier dysfunction allowing cells and molecules

Fig. 1. Compartments of the peripheral nerve. ***a*** Cross section of the sciatic nerve of the rat with four fascicles each surrounded by the perineurium (pn), epineurium (ep) with adipose tissue (ac), epidural vessels with arteriole (a) and venule (v): bar 500 μm. ***b*** Higher magnification of the perineurium (pn) with its slender cytoplasmic lamellae separating endoneurium (en) and epineurium (ep). Endoneurial small arteriole (a). Semithin section, bar 10 μm. ***c*** Endothelial cells with junctional complexes (arrows), pericyte (p) and macrophage (ma) of the endoneurium. Capillary (c). Electron micrograph, bar 1 μm. ***d*** Junctional complexes (arrows) between single perineurial cells. Note the membrane vesiculation. Fine collagen fibrils (cf) occur regularly between the perineural lamellae, endoneurium (en), epineurium (ep). Electron micrograph, bar 1 μm.

access to the endoneurium thus compromising the homeostasis of the endoneurial compartment [15].

Endoneurium

The endoneurial connective tissue in which myelinated and non-myelinated axons are integrated is organized by reticular and collagen fibers. Reticular fibers form a delicate three-dimensional meshwork in close association to the Schwann cell basement laminae and additionally with the arterioles, capillaries and venules. Collagen fibrils smaller than in the epineurium form a further supplementary fibrillar network which is mainly oriented in the longitudinal axis of the nerve fiber bundles. Beside the fibrillar nets the fibroblastic cells are in contact with each other and build up a wide meshed cellular network with their long and slender cytoplasmic processes. A distinct layer of these fibroblastic cells demarcates the perineurium. Resident macrophages and immunocompetent cells are morphologically inconspicuous and are regularly found close to the vessel wall. Mast cells may occur occasionally. Arterioles, capillaries and venules are present in the endoneurium whereas lymphatic capillaries are lacking. The density of the endoneurial capillaries which are not innervated differs among the various nerves [16–18].

Capillary endothelial cells are connected by tight and gap junctions and exhibit GLUT1 transporters in their plasma membrane [7, 8] (fig. 1a, c). Therefore, the capillary endothelium is an effective barrier for hydrophilic substances but permeable for glucose. Endothelial pinocytosis is present at the luminal and abluminal side of the cell. A nearly continuous layer of pericytes covers the external surface of the capillary endothelium. Intravasal HRP (horseradish peroxidase) and evans blue injections revealed, that in mouse and rat a functional mature blood-nerve barrier of the endoneurial vessel wall is established in the postnatal period days 16–24 [5, 19–21]. Hirakawa et al. [22] showed that the functional tightness of the microvessels is not uniform. They demonstrated that in dorsal root ganglia the blood-nerve barrier is tight in the nerve fiber rich area, whereas the capillaries in the cell body rich area are leaky. Their results are in concordance with the different expression of the tight-junction proteins, claudin-1 and occludin in the fiber rich part, only claudin-5 expression in the leaky part.

Blood-Nerve Barrier

The blood-nerve barrier is established in two locations: First, the perineurium, which protects the nerve fibers against diffusion of substances from the external environment into the nerve fascicles; second, the endoneurial capillaries

with their tight junctions avoiding the direct transport of substances from the blood circulation into the endoneurium. As we know from experimental work, the expression of special tight junction proteins in the perineurial cells and capillary endothelial cells are responsible for the tightness of the blood-nerve barrier.

Glial Cells of the Peripheral Nervous System

Peripheral glia consists of Schwann cells and satellite cells. Satellite cells are supporting cells surrounding the perikarya of neurons in sensory, enteric and autonomic ganglia, whereas Schwann cells ensheath peripheral axons. Different types of Schwann cells occur: First, the myelinating Schwann cell which forms compact myelin by multiple wraps of the plasmalemma; second, the non-myelinating Schwann cell (Remak Schwann cell) which engulfs multiple unmyelinated axons; third, the terminal Schwann cells at the termination sites of the myelinated and unmyelinated axons.

Myelinating Schwann Cell

Phylogenetically, myelinating Schwann cells appear first in gnastostomes. Agnatha do not have myelinating glial cells at all [23, 24]. Myelination of the axons is a prerequisite for rapid saltatory signal propagation in the neuronal network. During the ontogenetic development myelination of axons occurs in complex interactive networks where neurons and glial cells are dependent on reciprocal signals in a time and a space specific manner [25]. The molecular analysis of the myelination process is a hot topic and under intense investigation [26, 27]. The myelinating Schwann cell is a remarkable polarized cell (fig. 2a). During the developmental process it forms the compact myelin and wraps up the axon with loops of its cytoplasmic processes which after extrusion of the cytoplasm and the reduction of extracellular space form the tightly apposed membranes [28]. The axon defines the thickness of the myelin sheath and the internodal length. The longer and thicker the nerve fiber is, the longer is the internodal segment. The node of Ranvier is defined as a small gap where the myelin sheath is interrupted between successive Schwann cells along the axon (fig. 2c). In this location, the axonal membrane is only partially covered by few and small interdigitating cell processes of the adjoining Schwann cells. At these sites the compact myelin of the Schwann cell breaks up into paranodal loops and several glial endfeet are sealed to the paranodal axonal membrane via the septate-like junctions. The neuronal glycoprotein Contactin associated protein

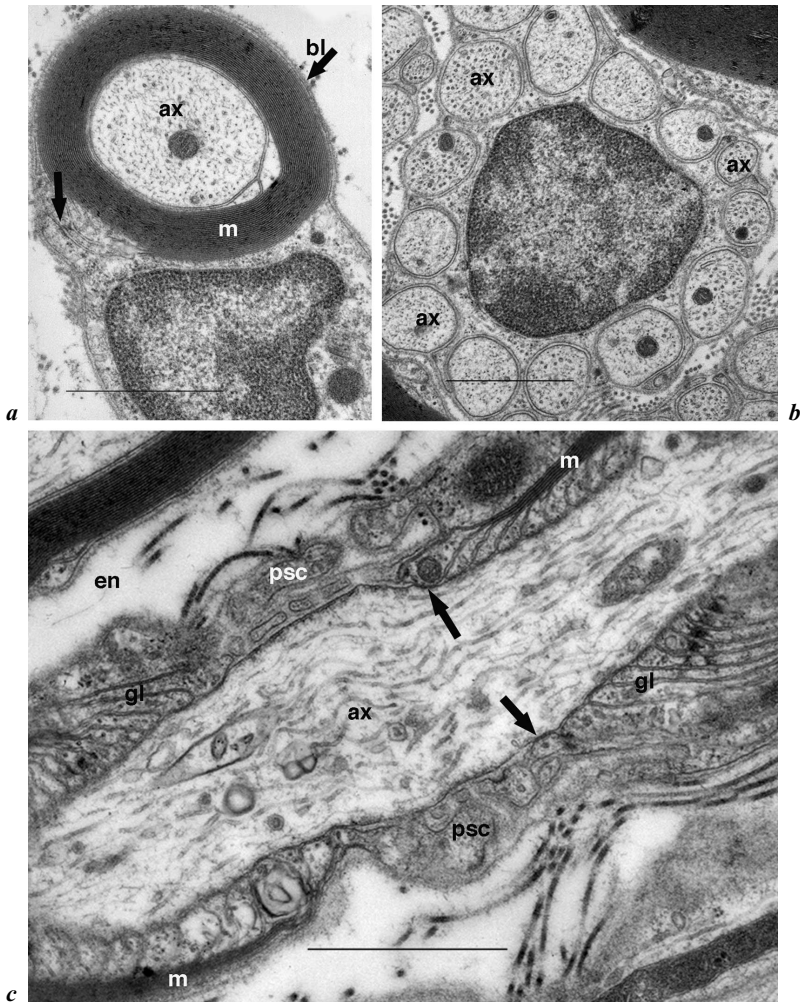


Fig. 2. Myelinating Schwann cell and non-myelinating Schwann cell. ***a*** Myelinating Schwann cell with the inner and outer mesaxon (arrrow). Note the basement lamina (bl) in close apposition with fine collagen fibrils of the endoneurium. Compact myelin (m), axon (ax). Electron micrograph, bar 1 μm. ***b*** Non-myelinating Schwann cell with numerous axons (ax) invaginated in its cytoplasm. Electron micrograph, bar 1 μm. ***c*** Node of Ranvier and paranodal segment with paranodal strands and inserting glial lamellae (gl). Arrows indicate paranodal strands. Small cytoplasmic processes of the adjoining Schwann cells (psc) cover the node of Ranvier. Axon (ax), myelin (m), endoneurium (en) with collagen fibrils. Electron micrograph, bar 1 μm.

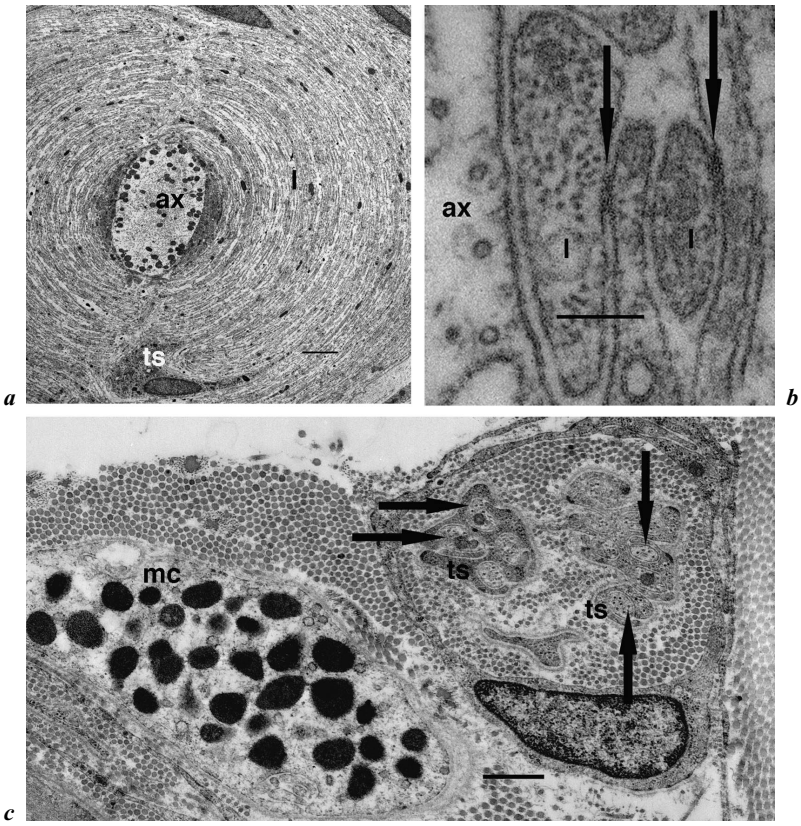
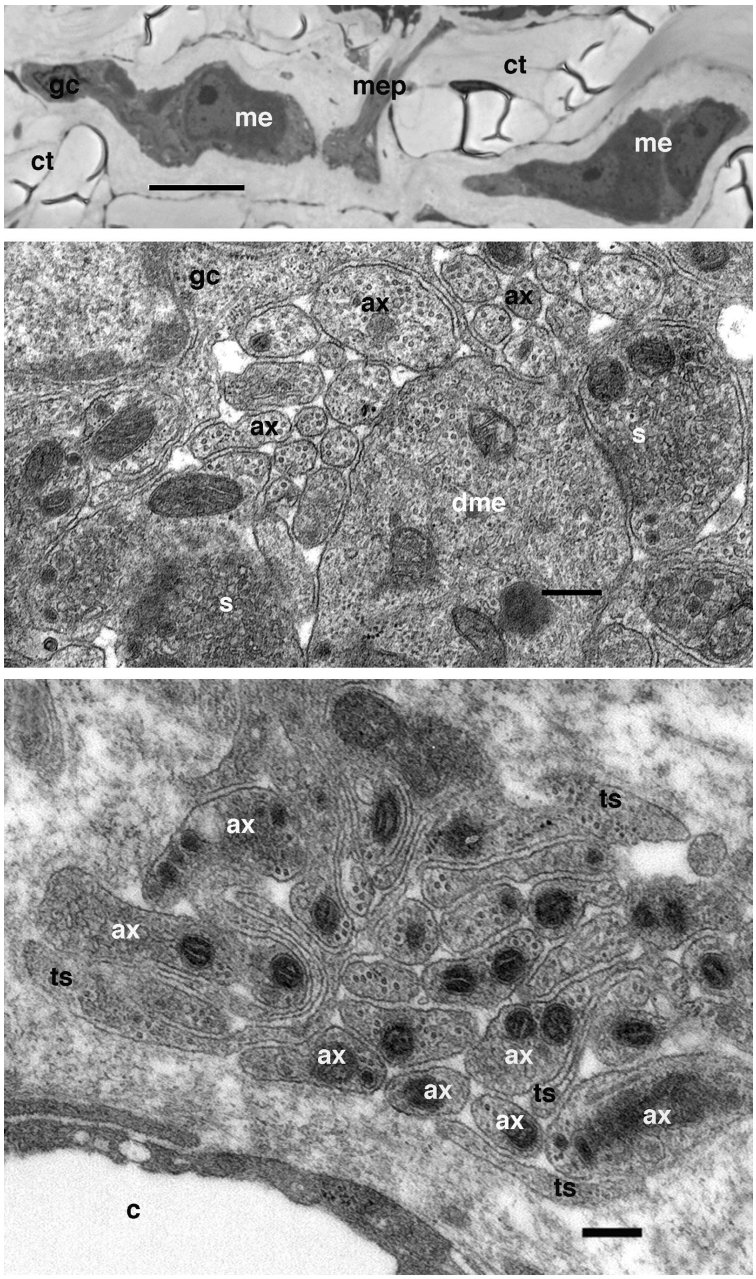


Fig. 3. Two types of terminal Schwann cells of a mechanoreceptor and a nociceptor. ***a*** Terminal Schwann cells (ts) of the Vater Pacini corpuscle form the inner core (l) around the sensory axon terminal (ax). ***b*** Gap junctions (arrows) are numerous between the inner core lamellae (l). Electron micrograph, bar 100 nm. ***c*** Numerous nociceptive axons (arrows) close to a mast cell (mc) in the Achilles tendon. Terminal Schwann cell (ts). Electron micrograph, bar 1 μm.

(Caspr)/paranodin, Caspr2 and protein 4.1B are essential for the structure and function of these axo-glial junctions [29] in interaction with neurofascin of the Schwann cell [30]. These septate-like junctions are necessary to concentrate the voltage-gated Na^+ channels of the node of Ranvier and to avoid their lateral diffusion. Multiple adherens junctions are obvious between the glial endfeet containing E-cadherin, catenin and F-actin [31]. Schmidt-Lanterman incisures are small cytoplasmic funnels of non-compact myelin. In this area, the individual lamellae are connected by gap junctions (connexin32) allowing several metabolic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short-circuits between the outer and inner cytoplasmic compartments of the Schwann cell [32].

Non-Myelinating Schwann Cell or Remak Cell

Non-myelinating Schwann cells are at least the most frequent glial cells in the adult peripheral nervous system. In contrast to the myelinating Schwann cell one non-myelinating Schwann cell may ensheath more than 20 unmyelinated axons of different size and function (fig. 2b). This means that autonomous nerve fibers and sensory nerve fibers run in the same Remak cell [33]. Nearly every unmyelinated axon is surrounded by Schwann cell cytoplasm. The non-myelinating Schwann cell is completely covered by a continuous basement lamina. Morphologically non-myelinating Schwann cells are coupled by small gap junctions.

Terminal Schwann Cells

The terminal Schwann cell is a specialized non-myelinating glial cell at the termination sites of myelinated and unmyelinated sensory and motor nerve fibers. Depending on the type of afferent or efferent axons the morphology of the terminal Schwann cell differs [33–42] (fig. 3). Neurons and their terminal Schwann cells are dependent on each other showing complex reciprocal interactions especially during development as it is shown with modern molecular biological and genetic techniques. This functional relationship is time controlled and coordinated and involves the secretion of trophic molecules from both the axon and terminal glial cell [43, 44]. As shown for neuromuscular junctions the terminal Schwann cell plays an important role in nerve terminal growth and maintenance, synaptic modulation, axonal sprouting and regeneration [45–47]. The axon terminal, as well as the nodes of Ranvier, are regions where axonal sprouting may occur. Furthermore, sensory axon and terminal

Fig. 4. Enteric ganglia. ***a*** Submucosal enteric plexus of Meissner with ganglionic cells (me), glia cell (gc) and fiber plexus (mep) embedded in the collagen tissue (ct) of the rat jejunum. Semithin section, bar 10 μm. ***b*** Organization of the enteric neuropil resembles the neuropil of the CNS. Preterminal axons with synaptic vesicles (s), ganglionic cell dendrite (dme). Glia cell (gc), axon (ax). Note the lack of connective tissue inside of the ganglionic cell complex. Electron micrograph, bar 0.2 μm. ***c*** Termination of enteric axons close to a capillary (c) in the lamina propria mucosae of the rat jejunum. Note the free axon terminals (ax) only partly covered by their terminal glia cell (ts). Electron micrograph, bar 0.2 μm.

Schwann cell interactions play a crucial role for the maturation of the mechanoreceptors [48, 49]. Preventing apoptosis of the terminal Schwann cells in transected nerves by in vivo injection of neuregulin – a glial growth factor – will allow axon regeneration and receptor formation [50]. Terminal Schwann cells express the neuregulin receptors ErB2 and ErB3 [51].

Nociceptors belong to the main group of sensory unmyelinated axons (C-fibers) terminating in all tissue compartments of the body except the brain. Terminal Schwann cells of nociceptors cover the receptive parts of the axons incomplete exposing the axon membrane directly to the tissue microenvironment [33, 35, 52]. This configuration allows controlled interactions between axon terminal and tissue environment. Besides their afferent function nociceptors release via the axon reflex various peptides (substance P, calcitonin-gene-related peptides) inducing vasodilatation, plasma extravasation and activation of mast cells – which is called neurogenic inflammation [53, 54].

Enteric Nervous System

The enteric nervous system, the intrinsic innervation of the gastrointestinal tract, consists of two main ganglionated and interconnecting plexi, the Meissner plexus in the submucosal layer and the Auerbach plexus between the inner circular and the outer longitudinal muscle layer [55]. Functionally the enteric nervous system is more or less independent of the central nervous system with intrinsic primary afferent neurons, interneurons and motor neurons [56, 57]. It regulates complex peristalsis, exocrine and endocrine secretions, microcirculation, immune- and inflammatory processes [58–61].

Due to the lack of perineurium and endoneurium the histological organization of enteric ganglia resembles the organization of the central nervous system. It is composed of neurons, glial cells, unmyelinated axons and synapses. A 20 nm intercellular space separates neural and glial cells. Enteric ganglia are avascular (fig. 4). A thin continuous basement lamina and fibroblast like cells are structural elements separating the enteric ganglia from the microenvironment. Enteric glial cells have much in common with astroglial cell as they exhibit glial fibrillary acidic protein (GFAP) immunoreactivity [62, 63]. Enteric nerve fibers nets are composed of enteric glial cells carrying multiple thin axons (up to 40) lying close together. The enteric glia cover is incomplete and finally absent thus exposing the axonal membrane to the tissue micromilieu. Recent experiments stress the diversity of the enteric glial cells with respect to their GFAP expression and their reaction to proinflammatory cytokines and lipopolysaccharides [64]. The enteric glial cell network is able to take up and degrade neuroligands and to store neurotransmitter precursors thus modulating and supporting enteric neuronal activities. The

competence in antigen presentation and potential to produce cytokines enables enteric glial cells to play a crucial role in various defense and proinflammatory mechanisms [65, 66]. Additionally, the enteric glia cells seem to be involved in maintaining the integrity of the mucosal barrier and vascular permeability in the gastrointestinal tract [67].

Extrinsic nerve fiber bundles entering the walls of the gastrointestinal tract have the typical compartments of the peripheral nerve with endoneurium and perineurium and intermingle at their target site with the enteric nervous plexus.

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Neurotransmitter Receptor-Mediated Signaling Pathways as Modulators of Carcinogenesis

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Abstract

The autonomic nervous system with its two antagonistic branches, the sympathicus and the parasympathicus, regulates the activities of all body functions that are not under voluntary control. While the autonomic regulation of organ functions has been extensively studied, little attention has been given to the potential role of neurohumoral transmission at the cellular level in the development of cancer. Studies conducted by our laboratory first showed that binding of the parasympathetic neurotransmitter, acetylcholine, as well as nicotine or its nitrosated cancer-causing derivative, NNK, to nicotinic acetylcholine receptors comprised of α_7 subunits activated a mitogenic signal transduction pathway in normal and neoplastic pulmonary neuroendocrine cells. On the other hand, beta-adrenergic receptors (β-ARs), which transmit signals initiated by binding of the catecholamine neurotransmitters of the sympathicus, were identified by our laboratory as important regulators of cell proliferation in cell lines derived from human adenocarcinomas of the lungs, pancreas, and breast. The tobacco-specific carcinogen NNK bound with high affinity to β_1- and β_2-ARs, thus activating cAMP, protein kinase A, and the transcription factor CREB. Collectively, neurotransmitter receptors of the nicotinic and β-adrenergic families appear to regulate cellular functions essential for the development and survival of the most common human cancers.

The autonomic nervous system with its two branches, the vagus and sympathicus, regulates functions of the mammalian body that are not under voluntary control. The effects of the vagus and sympathicus are often antagonistic, with one stimulating and the other one inhibiting a given organ or cell function. Maintenance of a physiological balance between these two systems is an essential prerequisite for mammalian health. The neurotransmitter for the vagus,

acetylcholine, transmits signals from vagal nerve endings to the families of nicotinic and muscarinic acetylcholine receptors at the cell membrane. The catecholamines epinephrine and norepinephrine are the neurotransmitters for the sympathicus and transmit signals from nerve endings of the sympathicus to the families of α- and β-adrenergic cell membrane receptors. Hyperfunction of the sympathicus is a well-documented factor in the genesis of cardiovascular disease while malfunctioning of the vagus has been implicated in a number of neurological disorders. Studies into the role of autonomic regulation in the development and progression of cancer are only recently emerging following observations that neurotransmitters and their receptors regulate the proliferation, apoptosis and metastatic spread of numerous cancers as well as cancer-related angiogenesis and neoneurogenesis.

Cancer of the mammary glands, colon, prostate, lung, and pancreas are among the most common human malignancies. Thanks to the development of methods for their early detection, mortality rates of breast cancer, colon cancer and prostate cancer have dramatically decreased over the past two decades. Unfortunately, 5-year survivals for lung cancer patients have remained at $<$15% while the mortality from pancreatic cancer is near 100% within 1 year of diagnosis. Conventional cancer treatment employs combinations of radio- and chemotherapy aimed at killing the cancer cells. In an effort to avoid the non-selective cytotoxicity of these agents for cancer cells as well as normal cells, recent efforts have focused on the development of agents that selectively block regulatory signal transduction pathways in cancer cells. Members of the epidermal growth factor receptor (EGFR) pathway such as tyrosine kinases, farnesyl transferases, or extracellular signal regulated kinases (ERKs) are thus targeted by selective pharmacological inhibitors currently in clinical trials [1, 2]. The EGFR is over-expressed in the majority of adenocarcinomas and squamous cell carcinomas of the lungs [3], and in adenocarcinomas of the breast [4], colon [5], prostate [6], and pancreas [7], leading to the identification of its downstream effectors as promising targets for selective therapy of these cancers. However, despite of impressive efficacies in preclinical test systems, the majority of these agents have disappointed in clinical trials. Recent studies indicate that the EGFR is transactivated by signaling proteins downstream of β-adrenergic receptors (β-ARs) in adenocarcinomas of the lungs [8], pancreas [9, 10], and breast [11], identifying its signaling pathway as only one of several effectors of neurotransmitter receptors in these cancers. In addition, it has been shown that the membrane estrogen receptors can transactivate the EGFR [12, 13]. Selective inhibition of EGFR signaling alone is therefore unlikely to yield a complete response in these cancers.

Nicotinic acetylcholine receptors comprised of homomeric α_7 subunits (α_7nAChR) have emerged as important regulators of cell proliferation and

apoptosis in small cell lung carcinomas (SCLCs) and their cells of origin, pulmonary neuroendocrine cells (PNEC), airway epithelial cells [14–17]. Again, currently available data suggest that agonist-induced stimulation of this neurotransmitter receptor simultaneously activates several signaling cascades involved in the growth regulation of these cancers and their cells of origin, rendering efforts to selectively inhibit one of these pathways ineffective.

This review summarizes current knowledge on the role of signaling via neurotransmitter receptors of the nicotinic acetylcholine and β-adrenergic families in the development of SCLC and adenocarcinoma of the lungs, pancreas, and breast and the implications of these findings for the prevention and treatment of these cancers.

β-Adrenergic Receptor Signaling and Lung Adenocarcinoma

Adenocarcinoma of the lung is the leading histological type of lung cancer in men and women. Risk factors for the development of this cancer are strikingly similar to risk factors for cardiovascular disease, namely smoking and a high fat diet [18]. The carcinogenic nitrosamines n-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are formed from nicotine (fig. 1) in the presence of nitrosating agents during the processing of tobacco and in the mammalian organism. Each of these nitrosamines acts systemically, causing the development of lung adenocarcinoma in laboratory rodents regardless of the route of administration. However, NNK is significantly more potent than NNN, causing the development of adenocarcinomas at a lower cumulative dose and at a higher incidence [19]. NNK is therefore thought to be responsible for the development of most lung adenocarcinomas in smokers. Both nitrosamines are converted by oxidative enzymes in mammalian cells to reactive forms that methylate and pyridyloxobutylate DNA. In turn, this results in activating point mutations in the K-ras gene and in inactivation of the p53 gene [20]. These mutations are found in a significant population of lung adenocarcinomas in humans [21] and in NNK-treated laboratory rodents [22, 23] and are thought to be critically involved in the initiation of this cancer.

Lung adenocarcinoma shares identical risk factors with cardiovascular disease, and over-expression of the arachidonic acid (AA)-metabolizing enzyme COX-2 is also commonly found in both diseases [18]. We therefore hypothesized that β-adrenergic signaling, which is critically involved in the genesis of cardiovascular disease [24], may also play a role in the development of lung adenocarcinoma. In support of this hypothesis, we found that the β-adrenergic agonist, isoproterenol (fig. 1), stimulated the growth of human lung adenocarcinoma cell lines NCI-H322 and NCI-H441 which express the Clara cell-specific CC10

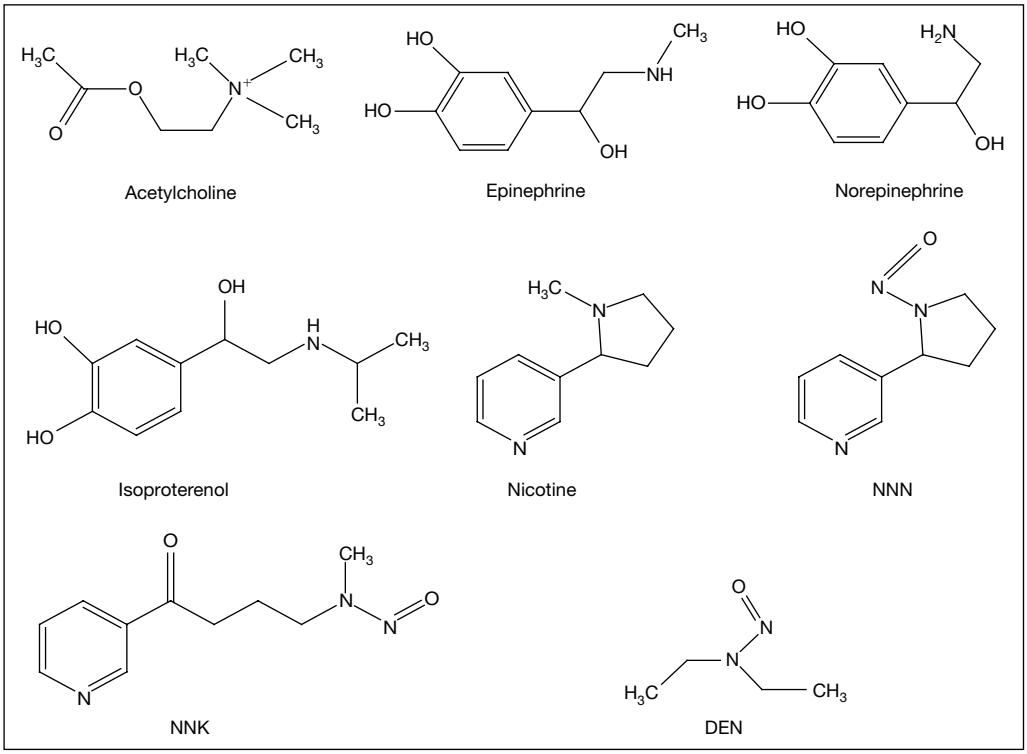

Fig. 1. Structures of physiological and tobacco-associated agonists for nicotinic acetylcholine receptors (acetylcholine, nicotine, NNK, NNN, DEN) and β-adrenergic receptors (epinephrine, norepinephrine, isoproterenol, NNK).

protein, while increasing intracellular cAMP, and that these effects were mimicked by the activator of cAMP, forskolin [25]. Radioreceptor assays with Chinese Hamster ovary cells transfected with the human β_1- or β_2-AR genes and conducted in the presence of inhibitors for the oxidative enzymes required for NNK metabolism, subsequently identified the unmetabolized NNK as a high affinity ligand for both receptors [26]. Similar assays conducted in human lung adenocarcinoma cells NCI-H322 and NCI-H441 identified the presence of β_1- and β_2-AR, with β_1 predominating and NNK binding to both receptor types. NNK as well as isoproterenol stimulated the release of AA from these cells and increased their proliferation by enhancing DNA synthesis. These effects were inhibited by the β-blocker, propranolol. In addition, cell proliferation was partially inhibited by COX-2 inhibitors or by an inhibitor of the mitogen-activated kinases kinase [26]. Subsequent experiments with NCI-H322 cells and their putative cells of origin,

small airway epithelial cells, showed that NNK increased intracellular cAMP, resulting in activation of PKA and the transcription factor CREB while simultaneously transactivating the EGFR and its downstream effectors ERK1/2 in a manner dependent on binding of NNK to β_1-adrenoreceptors and activation of PKA [8]. The resulting stimulation of cell proliferation was completely blocked by PKA inhibitors while equimolar concentrations of EGFR tyrosine kinases yielded partial inhibition. These in vitro data identified the tobacco-specific carcinogenic nitrosamine NNK as a high-affinity agonist for β_1- and β_2-ARs and implicated NNK-induced β-adrenergic signaling in the genesis and/or progression of lung adenocarcinoma. This interpretation was supported by experiments with NNK-induced lung adenocarcinomas in hamsters that showed a strong inhibition of adenocarcinoma development in animals that were given the β-blocker propranolol immediately prior to each NNK injection [27]. Treatment of hamsters with the β-adrenergic agonist, epinephrine, the cAMP activator, forskolin, or the phosphodiesterase inhibitor, theophylline after discontinuation of NNK treatments demonstrated strong tumor promoting effects [27, 28]. Furthermore, the dual signaling of NNK via β-adrenoreceptor and EGFR pathways suggested by the in vitro studies were supported by the simultaneous over-expression of members of signaling proteins of both pathways in NNK-induced adenocarcinomas in hamsters [29]. Our findings on the direct interaction of NNK with β-adrenoreceptors are in accord with recent reports that have shown β-AR-mediated stimulation of colon cancer cell growth [30] as well as multi-site BAD phosphorylation in lung cancer cells via activation of PKCiota downstream of β-adrenoreceptor-dependent c-src induction [31].

Lung adenocarcinoma is more common in women than men, and an association between high expression levels of estrogen receptor β and occurrence of this cancer type has been reported [32]. The classic estrogen pathway involves interaction of estradiol (E2) with nuclear estrogen receptors alpha and beta (ER-α, ER-β), resulting in regulation of gene transcription of specific estrogen responsive elements. However, recent studies have shown rapid activation of signaling pathways in response to estrogen resembling the actions of G-protein-coupled receptor ligands that transactivate the EGFR pathway [33, 34]. These observations lead to the concept that the estrogen receptors are proteins that shuttle between the nucleus and the cell membrane. In light of our findings that NNK is a β-adrenergic agonist and transactivates the EGFR receptor [8], we explored the possibility that NNK-initiated signaling might also transactivate membrane estrogen receptors. In support of this hypothesis, we found that transient over-expression of the ER-β in human small airway epithelial cells significantly enhanced NNK-induced stimulation of cAMP as well as activation of ERK1/2 and cell proliferation. In addition, reverse phase protein microarrays combined with Western blotting showed that NNK rapidly phosphorylated the

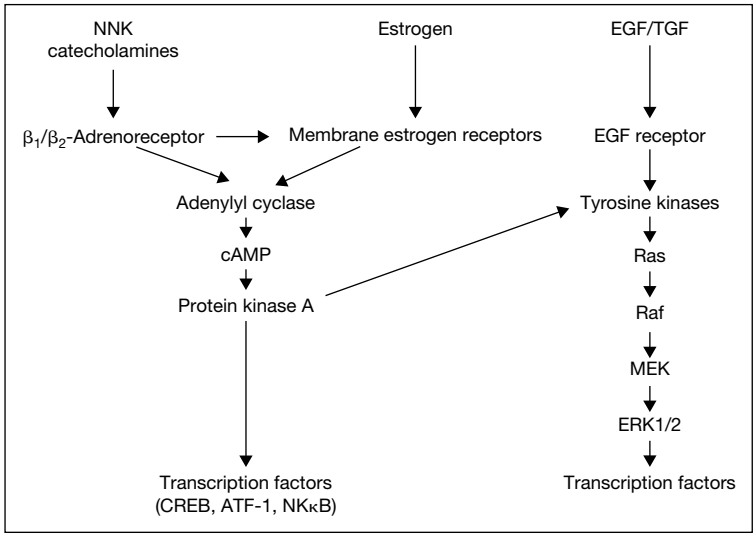

Fig. 2. Simplified scheme of cooperative regulation of adenocarcinomas by β-adrenoreceptors, membrane estrogen receptors, and EGF receptors.

ER-β, an effect completely blocked by the antagonist for β_1-adrenoreceptors, atenolol (unpublished data). These findings suggest direct interactions of NNK with membrane ER-β signaling (fig. 2) and potential tumor promoting effects of estrogen as well as agents that upregulate the ER-β on the development of smoking-associated lung adenocarcinoma. This kind of cross-talk between β-adrenergic, EGFR and ER-β signaling may well contribute to the documented prevalence of lung adenocarcinoma in women.

β-Adrenergic Receptor Signaling and Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma is the most common histological type of pancreatic cancer and demonstrates a mortality of >10% within 1 year of diagnosis [35]. Similar to lung adenocarcinoma, the majority of these tumors harbor activating point mutations in K-ras and over-express the EGFR and COX-2 [18]. Smoking and alcohol consumption have been identified as risk factors for this malignancy [36, 37]. The tobacco carcinogen NNK is a weak pancreatic carcinogen when administered to adult laboratory rodents [38]. However, when hamster females were given 10% ethanol in their drinking water throughout

their pregnancy while receiving one injection of NNK on the last day of their gestation period, about 60% of the offspring developed ductal adenocarcinoma of the pancreas when they were between 8 and 12 months old [39]. The development of these tumors was significantly inhibited when the offspring were subjected to cancer preventive treatments with the COX-2 inhibitor ibuprofen [40], or the β-blocker propranolol (unpublished), suggesting both, β-adrenergic signaling and the AA-cascade as factors in the development and/or progression of this malignancy. In support of this interpretation, studies with cell lines derived from human ductal adenocarcinomas of the pancreas showed that NNK induced the release of AA from these cells as well as DNA synthesis via stimulation of the β_2-adrenoreceptor [41]. Subsequent experiments with human pancreatic duct epithelial cells, the putative origin of this cancer type, further extended these findings to show a concentration-dependent increase in intracellular cAMP in response to NNK or the classic β-adrenergic agonist, isoproterenol [9]. Antagonists for β_1- and β_2-adrenoreceptors inhibited this response. In addition, these studies revealed phosphorylation of EGFR-specific tyrosine kinases and ERK1/2 in response to NNK or isoproterenol, effects completely blocked by the β-blocker propranolol, suggesting transactivation of the EGFR pathway via β-adrenergic signaling. The inhibitor of EGFR-specific tyrosine kinases, AG1478, or the MEK inhibitor, PD98059, also significantly reduced the observed induction of ERK1/2 activation. Treatment of the cells with ethanol caused a concentration-dependent increase in intracellular cAMP and reduced the concentration of NNK from 1 nM to 100 pM required to induce significant activation of PKA, P-CREB, and P-ERK1/2 [10]. This effect is consistent with the concept that agents that increase intracellular cAMP sensitize β-adrenoreceptors, resulting in a requirement of lower concentrations of agonists to elicit responses.

Collectively, these data suggest that β-adrenergic signaling, including transactivation of the EGFR pathway (fig. 2), is critically involved in the development and/or progression of pancreatic ductal adenocarcinoma and that the tobacco carcinogen NNK utilizes these signaling pathways. In addition, ethanol appears to have tumor promoting effects on this malignancy by sensitizing β-adrenoreceptors.

β-Adrenergic Receptor Signaling and Adenocarcinoma of the Mammary Gland

Adenocarcinoma of the mammary gland is the leading cancer in women [42]. Unlike lung cancer, the mortality from this malignancy is relatively low thanks to the availability of effective methods for its early detection. Based on

their responsiveness to anti-estrogen therapy, breast cancers are generally classified into estrogen receptor positive (ER+) or estrogen receptor negative (ER−) tumors. A variety of factors have been reported to increase the risk for the development of breast cancer, including smoking, alcohol consumption, high fat diet, genetic disposition, and estrogen imbalance caused by hormone therapies [43]. Polycyclic aromatic hydrocarbons contained in tobacco products and in the environment cause breast cancer in laboratory animals, an effect thought to be triggered by mutations in the high mutational activity of these agents and by modulation of estrogenic responsiveness via the aryl hydrocarbon receptor signaling [44–46].

Current knowledge on the role of neurotransmitters and their receptors in the regulation of breast cancer is only rudimentary. Studies with three ER+ (ZR-75, MCF-7, MDA-MB-361) and three ER− (MDA-MB-435, MDA-MB-453, MDA-MB-468) human breast cancer cell lines have shown that the β-blocker propranolol significantly inhibited DNA synthesis in all cell lines, suggesting an important role of β-adrenoreceptors in the regulation of cell proliferation regardless of estrogen receptor status [11]. The antagonist for β_1-adrenoreceptors, atenolol, and the antagonist for β_2-adrenoreceptors, ICI118,551 both significantly reduced the proliferation of all six cell lines, with ICI118,551 having the greater effects. Exposure of ER− cell lines MDA-MB-435 or MDA-MB-453 to isoproterenol additionally stimulated the release of AA from these cells and increased DNA synthesis while having no effect on the ER+ cell lines or the ER− cell line MDA-MB-468 [11]. Furthermore, it has been shown that NNK, or the selective agonist for β_2-adrenoreceptors, formoterol, stimulated influx of potassium via inwardly rectifying potassium channels (GIRK), phosphorylation of ERK1/2 and cell proliferation in MDA-MB-453 cells [47]. Studies conducted in Dr. Entschladen's laboratory with the ER− cell line MDA-MB-468 showed that the physiological agonist for β-adrenoreceptors, norepinephrine, stimulated the migration of these cells and that this effect was primarily mediated by β_2-adrenoreceptors. In addition, MDA-MB-468 cells demonstrated positive chemotaxis towards this neurotransmitter [48]. Interestingly, gamma-aminobutyric acid (GABA) potently inhibited the stimulation of cell migration by norepinephrine [48]. GABA is a major inhibitory neurotransmitter in the central nervous system where it counteracts the effects of stimulatory neurotransmitters, such as the catecholamines. GABA and its synthetic analogs may thus be useful in the clinical management of breast cancer by reducing the metastatic spread of the primary cancer.

Human breast cancer xenographs in immunosuppressed mice as well as transplantable rodent breast cancers are stimulated in their growth by diets rich in n–6 polyunsaturated fatty acids (PUFAs) such as linoleic acid while n–3 PUFAs such as eicosapentaenoic acid (EPA) inhibit tumor growth [49]. In

addition, a diet rich in n–6 PUFAs is among the risk factors for the development of breast cancer in humans, while a diet rich in n–3 PUFAs appears to have protective effects [50]. It was initially thought that the cancer preventive effects of n–3 PUFAs is caused by a reduction in the formation of growth promoting prostaglandins from AA due to competition of n–6 vs. n–3 PUFAs during metabolism. However, more recent studies have shown that the growth inhibiting effects of n–3 PUFAs on breast cancer cells were independent of prostaglandin formation and instead involved modulations of a variety of signal transduction pathways [50]. Of particular interest here is a recent report that demonstrated inhibition of MCF7 xenograph growth by EPA via activation of pertussis toxin-sensitive signal transduction, including a reduction in cAMP [49]. These findings suggest inhibitory G-proteins (G_i) as mediators of the inhibitory effects of EPA on the proliferation of these ER+ breast cancer cells. The β_2-adrenoreceptor, which has been identified as an important regulator of cell proliferation [11, 47] and migration [48] of breast cancer cells is coupled to G_s and G_i, with G_s stimulating and G_i inhibiting adenylylcyclase-mediated production of cAMP. The documented cancer preventive effects of n–3 PUFAs on breast cancer may thus involve the G_i-mediated inhibition of β_2-adrenoreceptor signaling.

Nicotinic Acetylcholine Receptor Signaling and Small Cell Lung Carcinoma

SCLC is a highly aggressive type of lung cancer that expresses neuroendocrine markers such as 5-hytroxytryptamine (5-HT, serotonin), mammalian bombesin (MB), calcitonin, neuron specific enolase, and others. SCLC does not harbor activating point mutations in K-ras, but frequently demonstrates amplification of c-myc and mutations in the retinoblastoma and p53 genes [51]. The majority of SCLCs are though to have derived from the epithelial lining cells of large airways while a small population of SCLCs may derive from small airway epithelial cells. SCLC initially responds well to conventional cancer therapy but relapses frequently and progresses rapidly with extensive metastasis to extrapulmonary organs. Of all histological lung cancer types, SCLC shows the closest association with smoking, and a diagnosis of SCLC is very rare in non-smokers [52, 53]. Ionizing radiation and exposure to chloromethyl ethers are additional risk factors [53].

A potential role for nAChRs in smoking-associated lung carcinogenesis was first suggested in 1989 when it was shown that nicotine and NNK stimulated the proliferation of SCLC cells and that this response was blocked by antagonists for nAChRs but not by an antagonist for muscarinic AChRs [14, 54]. These reports were followed by publications in 1990 and 1994 which

documented a nicotine-induced reversal of apoptosis in response to opioids in a large panel of SCLC cell lines as well as non-SCLC cell lines [17, 55]. An additional report in 1993 showed that nicotine stimulated the proliferation of human SCLC cell lines via stimulation of a serotonergic autocrine loop [16]. Collectively, these initial findings suggested that nicotine itself may contribute to the development of smoking-associated lung cancer by interaction with nAChR-mediated proliferative and apoptotic signaling pathways. In addition, the data with NNK indicated that the extreme potency of NNK as a pulmonary carcinogen might be linked to its ability to function as an agonist for nAChRs. Radio receptor assays showed a high Bmax in saturation binding assays with the selective antagonist for nAChRs comprised of homomeric α_7 subunits, α-bungarotoxin (α-BTX), indicative of high levels of expression of this receptor in human SCLC cell lines as opposed to lung adenocarcinoma cell lines which demonstrated low or non-detectable binding of α-BTX [14]. These findings were extended by recent investigations that showed expression of mRNA for the α_7nAChR in a large panel of cell lines derived from different types of human lung cancers and in immortalized human small airway epithelial cells while significant amounts of receptor protein were only detected in SCLC cell lines [56]. Taken together, these findings indicate that the α_7nAChr is expressed in many lung cell types but demonstrates particularly high levels of expression in SCLC cells. Radio receptor assays assessing the relative binding affinities of nicotine and NNK in competition with α-BTX identified NNK as a ligand with unprecedented high affinity for the α_7nAChR. Analysis of these binding data by non-linear regression in fact showed that the affinity of NNK for the α_7nAChR was about 1,300 times greater than that of nicotine [14]. Stimulation of the α_7nAChr by NNK significantly increased cell number and DNA synthesis in SCLC cells, a response blocked by the α_7nAChR antagonist, α-BTX. Flow cytometric analysis showed a significant increase in intracellular Ca^{2+} in response to 1 nM NNK and this effect was blocked by α-BTX [57], thus confirming NNK as an agonist for the α_7nAChR. In conjunction with earlier reports, these findings clearly established an important regulatory role of the α_7nAChr in SCLC. Recent studies have revealed the expression of the α_7nAChRs in a wide variety of cell types in the monkey lung [58, 59]. In vitro studies with immortalized human bronchial epithelial cells and human small airway epithelial cells have additionally shown that stimulation by nicotine or NNK of this receptor activated the serine/threonine kinase AKT, an effect resulting in the attenuation of apoptosis induced by etoposide, radiation or hydrogen peroxide, as well as the induction of a transformed phenotype [60]. However, the concentrations of nicotine (10–100 μM) or NNK (1 μM) required to elicit these effects were significantly higher than those reported in studies with SCLC cell lines (1 μM nicotine, 1 nM NNK). At these high concentrations

nicotine as well as NNK may bind non-selectively to cellular targets other than the α_7nAChR and the reported activation of AKT may have involved non-nAChR receptors. Another laboratory additionally reported the activation of NFκB and up regulation of cyclin D1 in human bronchial epithelial cells NHBE and human small airway epithelial cells exposed to NNK at concentrations ranging from 0.5–10 μM [61]. Again, these concentrations are considerably higher than those required to stimulate mitogenic signaling in SCLC cells and may have involved non-nicotinic receptor types. Convincing support for an important role of α_7nAChR stimulation in the growth regulation of SCLC and the putative cell of origin of this cancer, PNEC, came from a number of in vitro studies which showed that binding of nicotine (1 μM) or NNK (100 pM) to the α_7nAChR resulted in phosphorylation of protein kinase c (PKC), Raf-1, ERK1/2 and c-myc [15, 62]. Another laboratory also reported activation of ERK1/2 in response to nicotine in human SCLC cell lines and showed that this effect was mediated by nicotinic receptor-mediated release of serotonin, which is an autocrine growth factor for these cells [16, 63]. Recent investigations additionally have reported α_7nAChR-mediated NNK-induced functional cooperation between Bcl2 and c-myc that inhibited apoptosis while stimulating cell proliferation of human SCLC cell lines [64]. In addition, NNK phosphorylated μ- and m-calpains in human SCLC cells in an ERK1/2 and Ca^{2+}-dependent manner, resulting in the induction of cell migration and invasion, and these effects were abrogated by the α_7nAChR antagonist α-BTX [65]. It is of note that the concentration of NNK required to elicit these effects reported by all three laboratories were extremely low (100 pM), thus underlining the very high-affinity of NNK for the α_7nAChR which is over expressed in SCLC cells. These findings are in accord with the frequent expression of amplified c-myc in SCLC [53]. In addition, it has been shown that the PKC/Raf-1/ERK1/2 signaling cascade is also stimulated by autocrine growth factors for SCLC, including the neuropeptide growth factors bradykinin, vasopressin, bombesin, neurotensin and galanin as well as serotonin and acetylcholine [63, 66]. In turn, it was shown that the release of some of these growth factors was triggered by the influx of Ca^{2+} caused by stimulation of the α_7nAChR [67]. Interestingly, recent studies in human SCLC cells have shown that the β-adrenergic agonist isoproterenol significantly reduced NNK-induced ERK1/2 activation [68]. These findings suggest that β-adrenoreceptors may have inhibitory function on the growth of this cancer type and that β-adrenergic agonists may be suitable adjuvants to prevent the relapse of SCLC after conventional cancer therapy.

Collectively, these data suggest that the major signaling pathway that regulates SCLC growth, apoptosis and invasiveness includes Ca^{2+} influx, activation of PKC, Raf-1, ERK1/2 , Bcl2, c-myc as well as calpains and that binding of

agonists to the α_7nAChR initiate the activation of these pathways while β-adrenergic signaling may have inhibitory effects.

In addition to the summarized direct effects of nicotinic agonists on lung cancer cells, it has been recently discovered that nicotine stimulates angiogenesis and enhances the neovascularization of lung tumors [69]. In fact, studies in xenographs from Lewis lung cancer cells even showed that exposure to side stream smoke (the experimental equivalent of second hand smoke) increased tumor size and angiogenesis and that this effect was inhibited by the broad-spectrum antagonist for neuronal nAChRs, mecamylamine [70].

It has been shown that PNECs, the putative origin of SCLC, express a receptor protein that senses hypoxia and triggers the release of serotonin (5-hydroxytryptamine, 5-HT) and bombesin (MB) via influx of Ca^{2+} [71]. In addition to modulating bronchial smooth muscle tone and respiration, 5-HT and MB act as autocrine growth factor for PNECs and SCLC. The diseased lung with impaired pulmonary ventilation thus typically demonstrates hyperplasia of PNECs [72]. In addition, uranium mining and other sources of exposure to radon that cause interstitial pulmonary fibrosis are documented risk factors for the development of SCLC [53]. We therefore hypothesized that stimulation of the oxygen sensing receptor in PNECs by impaired pulmonary ventilation would facilitate the development of a neuroendocrine type of lung cancer in animals exposed to the nicotinic agonists nicotine or NNK as well as diethylnitrosamine (DEN) which has structural similarities with acetylcholine (fig. 1). We induced mild pulmonary interstitial fibrosis in Syrian golden hamsters by maintaining the animals in an environment of 60% oxygen. These animals developed multiple foci of hyperplastic PNECs with positive immunoreactivity for 5-HT. Hamsters that were additionally given multiple subcutaneous injections of NNK [73] or DEN [74] developed multiple neuroendocrine lung tumors at a high incidence. Similar to most human SCLCs, these tumors expressed the neuroendocrine markers 5-HT, calcitonin, bombesin and neuron specific enolase, they lacked activating point mutations in K-ras while overexpressing c-myc [75]. Due to their relatively small size and well-differentiated morphological appearance, these experimentally induced tumors were classified as atypical carcinoids even though they demonstrated functional and molecular features of SCLC. Hamsters with hyperoxia-induced pulmonary interstitial fibrosis and treated with multiple subcutaneous injections of nicotine developed a low but significant incidence of lung tumors with focal areas of positive immunoreactivity to the neuroendocrine markers 5-HT and neuron specific enolase [76]. Collectively, these findings support the hypothesis that the diseased lung with impaired pulmonary oxygenation and resulting hyperplasia of PNECs is more susceptible for the development of neuroendocrine lung cancers upon simultaneous exposure to the nAChR agonists nicotine,

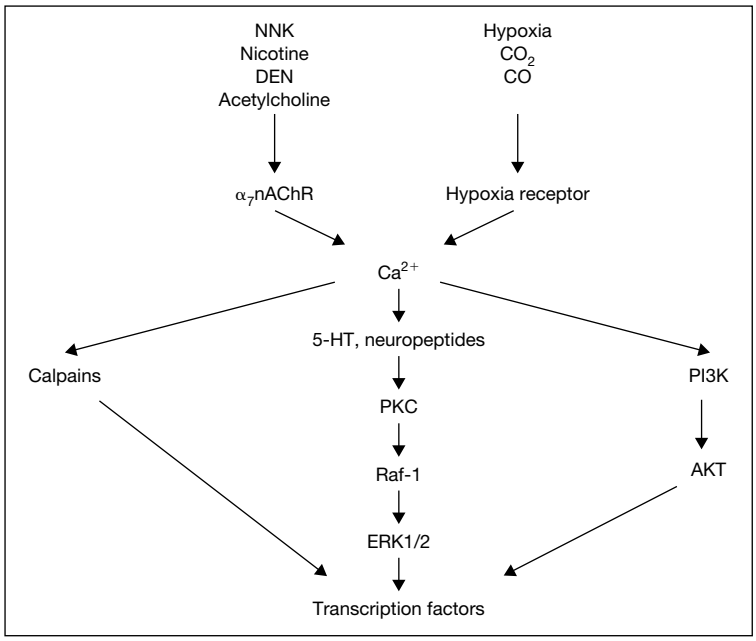

Fig. 3. Simplified scheme of cooperative regulation of SCLC by the α_7nAChR and the hypoxia receptor.

NNK or DEN. In vitro experiments corroborated this interpretation by demonstrating that SCLC or PNEC cells maintained in an environment of high CO_2 at the expense of O_2 showed induction of ERK1/2 activation [77] and enhanced the proliferation response to nicotine or NNK [78], suggesting sensitization of the α_7nAChR. Taken together, these findings suggest that the α_7nAChR and the hypoxia receptor cooperate in the regulation of SCLC (fig. 3).

Conclusions and Future Directions

The data summarized in this review suggest that neurotransmitter receptors of the β-adrenergic, nicotinic, and GABA families are critically involved in the development and progression of some of the most common human cancers and that cross-talk between β-adrenergic, membrane estrogen, and EGF receptors can greatly amplify the resulting signals. Small airway epithelial cell-derived adenocarcinoma of the lungs, duct-derived adenocarcinoma of the pancreas as well as ER+ and ER− adenocarcinomas of the breast all appear to

be under positive growth control by β-adrenoreceptors. In the case of ER− breast cancer cells, GABA has been shown to counteract the β-adrenergic stimulation of cell migration. In light of the prominent inhibitory effects of this neurotransmitter in the central nervous system, it is to be expected that GABA may also inhibit β-adrenergic receptor-mediated stimulation of cell proliferation in adenocarcinomas of the lungs, pancreas, and breast. Studies to test this hypothesis are currently underway in our laboratory. In addition, β-adrenergic signaling counteracted by GABA has been implicated in the migration and metastatic potential of adenocarcinomas of the colon [79]. On the other hand, the α_7nAChR has documented stimulatory effects on the growth of SCLC and this response appears to be counteracted by β-adrenergic receptor signaling. The susceptibility of these neurotransmitter receptors to agonists and antagonists can be greatly modulated by preexisting disposition, environmental factors, life style, preexisting non-neoplastic diseases and the chronic intake of certain medications. The receptors can be up or down-regulated by chronic exposure to ligands and they can be sensitized or desensitized by agents that increase or decrease their second messenger signals. In addition, exposure after cessation of smoking to agents that stimulate or inhibit such receptor-mediated pathways may promote or prevent the progression of premalignant lesions and small tumors into overt cancer. It is particularly worrisome that recent studies have identified agents widely believed to have general cancer preventive effects as stimulators of intracellular cAMP. Among such agents are β-carotene [80], green or black tea which contain significant levels of the phosphodiesterase inhibitors theophylline and caffeine, as well as several soy isoflavones and plant polyphenols [81]. Investigations in human small airway epithelial cells and adenocarcinoma cells that expressed the bronchiolar Clara cell-specific CC10 protein have shown that β-carotene stimulated the proliferation of these cells by increasing intracellular cAMP, leading to activation of PKA, CREB and ERK1/2 [80]. By contrast, increased cAMP and PKA activation in response to β-carotene caused a strong inhibition of cell proliferation and ERK1/2 activation in human large airway epithelial cells [80]. Accordingly, the effects of cAMP and its upstream receptors are highly cell type-specific and may have promoting effects on the most prevalent human adenocarcinomas while inhibiting SCLC and other cancers derived from cells (e.g. large airway epithelial cells) under negative growth control by cAMP. In addition, long-term management of cardiovascular disease by β-blockers, or the chronic use of β_2-AR agonist inhalers in asthmatics may significantly modulate the risk for the development of cancers influenced by β-adrenergic signaling. On the other hand, a host of neurological and psychiatric disorders include malfunctioning nAChR signaling and are clinically managed by nicotinic agonists or stimulators of effectors of these receptors. Tools, such as molecular imaging of receptors and signaling

components need to be developed to identify which signaling pathway(s) are hyperactive in individuals prior to the manifestation of cancer, in order to selectively inhibit those pathways and thus prevent the development of cancer in a custom-tailored way.

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Neuronal Markers in Non-Neuronal Tissues

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Abstract

Many proteins first identified in the nervous system were also found to be expressed elsewhere in the body. The text reviews some of these 'neuronal' markers and delineates intersections between nervous and non-nervous tissues on the structural and functional level. Examples are given for nuclear antigens, cytosolic, cytoskeletal and membrane bound proteins, neurotrophic factors and developmental antigens. Clinical aspects of the expression of neuronal antigens in cancer-like paraneoplastic syndromes of the nervous system and tumor invasion along and within peripheral nerves are discussed. The accumulated data indicates that expression of 'neuronal' protein in tumors may promote proliferation, invasiveness and metastatic spread. The large spectrum of neuronal antigens expressed in cancer including voltage-gated ion channels and numerous neurotrophic factors reflects the continuity from neuronal to non-neuronal differentiation.

Detection of tissue specific antigens by immunohistochemistry plays an important role in histopathological diagnosis. However, tissue-related protein expression patterns become more complicated the more antibodies are available and the overlap of expression patterns increases the more studies are conducted. In the following chapter, some of the physiological and pathological expression patterns of neuronal antigens in non-nervous tissues and tumors are described. Further, some of the interactions of non-nervous tumors and the nervous system which result from expression of neuronal proteins in tumors and nervous tissue will be outlined. The following paragraphs can only exemplify the plethora of intersections between the nervous system and other tissues on the molecular level. Detailed information on some of the molecules mentioned in the following text is provided in other chapters of this book.

Neuronal Antigens

Nuclear Proteins

The expression of neuronal antigens is controlled by different regulators, one factor leading to up-regulation being mammalian achaete-scute homolog-1 (Mash-1 or hAsh-1) which is expressed in neuronal and neuroendocrine tissues. Strict suppression of several neuronal antigens in non-neuronal tissues is realized on the transcriptional level through the zinc-finger transcription factor neuron-restrictive silencer factor (NRSF) and its co-repressors mSin3A and CoREST which bind to the mammalian chromatin remodeling factor SWI/SNF complex. The latter can remodel nucleosomes and increase accessibility of transcription factors to target loci. Watanabe et al. [1] demonstrated that suppression of neuronal antigens was lifted in cell lines of human non-small cell lung carcinoma (SCLC) deficient for components Brm or BRG1 of the SWI/SNF complex. Similar observations were previously reported for various malignancies which were deficient for different factors of the SWI/SNF complex. Malignant human rhabdoid tumors show a bi-allelic loss of the Ini1 gene encoding a subunit of SWI/SNF. Different cell lines of prostate, breast, pancreas and lung carcinoma were found to harbor mutations in the BRG1 gene and a subgroup of SCLC showed a loss of functional NRSF contributing to expression of neuron-specific antigens.

Loss of Brm and BRG1 was found in 10% non-SCLCs and was associated with poorer prognosis. In SCLCs expression of neuronal antigens is found in 30% of the cases due to expression of Mash-1 or elimination of endogenous NRSF. Hence, de-repression of NRSF and concurrent up-regulation of neuronal genes may contribute to an enhanced tumorigenicity [1].

Neuronal nuclear antigen (NeuN) is an established marker for postmitotic neurons. It is found in the cytoplasm and nucleus. Hitherto, the function of NeuN remains unclear. NeuN was demonstrated in non-neuronal tissues in more than 50% of neuroendocrine carcinomas of different grades. There was no correlation between NeuN expression and grade [2].

Cytosolic Proteins

Cytosolic proteins in secretory cells and neurons include neuron specific enolase (NSE) and PGP9.5. NSE is an isoenzyme of the glycolytic enzyme enolase and converts 2-phosphoglycerate to phosphoenolpyruvate. NSE is also found in almost all neuroendocrine tumors. PGP9.5 was first isolated from the brain and belongs to the ubiquitin carboxy-terminal hydrolase family. It is involved in the degradation of cytosolic and nuclear proteins through an ATP- and ubitquitin-dependent mechanism and plays a role in regulation of cell death. In exocrine pancreatic tumors it was found to be associated with poor prognosis [3].

Cytoskeletal Proteins

Neurofilaments are the intermediate filaments of neurons forming 'robes' of 10 nm in diameter which run longitudinally from the cell soma along the axon to the synapses. In neoplasia outside the nervous system neurofilament expression has been described in Merkel cell carcinoma [4] and a number of sarcomas, in particular in Ewing's sarcoma [5].

Microtubule associated proteins (MAPs) are cytoskeleton proteins found in all eukaryotic cells. More than 20 isoforms of MAPs differ in their distribution. MAP-2 is expressed in neurons and glial tumors and is used as neuronal marker. Expression of MAP-2 in malignancies was demonstrated in up to 100% of carcinoid tumors and SCLC, but only in a minority (16%) of adenocarcinomas and squamous carcinomas. For diagnostic purposes MAP-2 seems at least as sensitive as synaptophysin for detection of neuroendocrine differentiation [6].

Cell Membrane Proteins

Cell membrane associated proteins include neural cell adhesion molecule (NCAM, CD56) and leu-7 (CD57). The former is also expressed in regenerating skeletal muscle, whereas the latter is found in a spectrum of different normal tissues. A widespread expression of NCAM was demonstrated in benign and malignant tumors of the skeletal muscle [7] and in various forms of sarcomas [5].

Vesicular Proteins

Exocytosis is an essential mechanism for release of transmitter substances in neurons. In the last decade, the porosome was identified as a supramolecular structure within the plasma membrane through which cell secretion is realized. Vesicles transiently fuse with the porosome and release their content through the porosome into the extracellular space. However, porosomes are not unique to neurons but were found to be universally present in secretory cells, from exocrine pancreas to neurons and endocrine cells like chromaffin cells, growth hormone cells of the pituitary gland, mast cells and β-cells of the endocrine pancreas [8, 9]. Accordingly, neurons share many biochemical properties and ultrastructural features with other secretory cells, i.e. different forms of vesicles which transport the proteins to the porosomes.

Vesicular membrane related proteins comprise synaptophysin and synaptic vesicle protein 2 (SV2), synaptotagmin, vesicular monoamine transporters (VMATs) and synaptobrevin (VAMP2) among others. Synaptophysin is found in membranes of small vesicles of neurons and chromaffin cells in the adrenal medulla as well as in four major neuroendocrine cell types of pancreas. SV2 was primarily observed in the central and peripheral nervous system (CNS, PNS) and later in all neuroendocrine cells. VMATs mediate the transport of amines into the vesicles of neurons and into secretory granules of neuroendocrine cells.

Vesicle docking and fusion is realized via the SNARE complex which comprises N-ethylmaleimide-sensitive factor (NSF), soluble NSF proteins (α/β-SNAP), synaptobrevin (VAMP2), synaptotagmin and two synaptic plasma membrane proteins synaxin 1 and synaptosomal associated protein (SNAP-25). VAMP was first demonstrated in cholinergic vesicles. The three isoforms VAMP-1–3 are anchored to the cytoplasmic part of the vesicular membrane of synaptic vesicles and secretory granules. Synaptotagmins, also referred to as p65, form a large Ca^{2+} binding protein family. The different isoforms are present in vesicular membranes of the nervous system and take part in Ca^{2+} induced exocytosis. In normal pancreas synaptotagmins II, III, VII are co-localized with insulin.

Within the vesicles secretory granule proteins are found in addition to transmitters etc. Dense core vesicles contain chromogranin A. Chromogranins are acidic secretory glycoproteins stored together in vesicles with hormones and neurotransmitters. The granin family consists of chromogranin-A and -B and secretogranin II which all belong to the larger family of regulated secretory proteins. Granins can elecit effects by themselves or serve as precursors to a large number of biologically more active peptides like chromostatin, chromacin I and II, catestatin, parastatin, pancreastatins, vasostatins, chrombacin etc. Chromogranin-A and -B are expressed in several neuroendocrine organs like adrenal medulla, anterior pituitary and endocrine pancreas. Upon infusion chromogranin-A inhibits glucose-stimulated insulin release in rat pancreas [10]. In addition to chromogranins, dense core vesicles contain prohormone convertases. These are endoproteolytic processing enzymes of the trans-Golgi system and in the secretory granules. Eight different enzymes have been identified. PC1/3 and PC2 are present in nearly all neuroendocrine cells and are responsible for most of the post-translational processing of protein precursors.

NSE, synaptophysin and chromogranin expression were demonstrated in small cell carcinoma of the esophagus [11]. In undifferentiated colorectal cancer expression of chromogranin-A, synaptophysin, syntaxin 1, VAMP2, SNAP25 and α/β-SNAP was associated with more aggressive course of the disease [12].

Neurotrophic Factors and Receptors

Neurotrophic factors are defined as target-derived anti-apoptotic molecules maintaining embryonic or adult neurons. However, in the last years neurotrophic factors were demonstrated in numerous non-neuronal tissues rendering this term as a historical misnomer. Indeed, the first neurotrophic factor to be discovered early in the 1950s – nerve growth factor (NGF) – was found in sarcoma. After grafting a murine sarcoma into a chicken embryo an axonal elongation of sensory neurons was seen extending toward the sarcoma. In addition, many sensory and sympathetic nerve fibers grew towards tissues other than the tumor. The same phenomenon was observed in vitro when sensory ganglia were

incubated in the neighborhood of the tumor. Subsequently, the factor was found in snake venom and in salivary gland of male mice [13, 14].

Over the past 50 years a number of additional neurotrophic factors were identified, the group now includes neurotrophins, glial cell line-derived neurotrophic factors (GDNF) and interleukin (IL)-6/ciliary neurotrophic factor.

Neurotrophins are a highly homologous family including brain-derived neurotrophic factor (BDNF), NGF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) [15]. NGF shares a 50% homology with BDNF. In the adult PNS, Schwann cells secrete neurotrophic factors after nerve injury. In addition to BDNF, ciliary neurotrophic factor and GDNF, other molecules are up-regulated that support regeneration of the nerve, like leukemia inhibitory factor, insulin-like growth factor-1 and fibroblast growth factor-5 [16].

Outside the nervous system NGF is expressed under physiological conditions in dermal pigment cells, whereas hepatic cells were found to express BDNF, NT-3 and NT-4/5. Hepatic stellate cells express BDNF, NT-3 and NT-4/5 and cardiac myocytes produce NT-3. Neurotrophic factors also participate in the development and differentiation of non-neuronal tissues. Mice lacking NT-3 suffer from a series of cardiac defects like tetralogy of Fallot, persistent truncus arteriosus or ventricular septal defects all of which are related to abnormal neural crest development. Hair follicles show either precocious or delayed regression depending on NT-3 under- or overexpression. Similar functions have been described for BDNF and NT-4/5. NGF promotes differentiation of B lymphocytes, maintains memory B lymphocytes, neutrophils and peritoneal mast cells.

All neurotrophins bind to the low-affinity receptor $p75^{NTR}$ and selectively to high-affinity receptors which are trans-membrane anchored tyrosine kinases (Trk-A, -B, -C). Receptor $p75^{NTR}$ is found in numerous tissues including hair follicles, hepatic stellate cells, lung, thyroid, kidney etc. The low-affinity receptor may modulate the binding of NGF to its high-affinity receptor. NGF binds to Trk-A, BDNF, NT-3, NT-4/5, NT-6 bind to Trk-B, NT-3 binds to Trk-C.

The signaling pathway which is stimulated by Trk is involved in growth, development and differentiation. Outside the nervous system Trk are mostly expressed as truncated isoforms which may have scavenger function or act as dominant negative receptors. Trk-A and -C are expressed in pancreatic ducts and islets, Trk-B in α-cells of islets and NGF in pancreatic ducts and acinar cells. NT-3 and NT-4 are present in capillary endothelia. Transgenic mice overexpressing Trk-C suffer from dysmorphic defects of the cardiac outflow tract. Early development of the heart was retarded by blocking Trk-C [17].

Neurotrophins were found to be involved in several pathological conditions in non-neuronal tissues. Hemorrhagic damage of rat gastric mucosa due to EtOH was significantly reduced by arterial or venous administration of NGF [18]. Du et al. [17] found NGF family and Trk family mRNA expression

in normal gastric mucosa to be down-regulated in gastric cancer. In their view, Trk probably play a unique role in apoptosis via Ras and Raf signaling. Down-regulation of Trk in gastric cancer may lead to increased survival of tumor cells.

Tsunoda et al. [19] reported evidence for a NGF/Trk-A autocrine loop in esophageal squamous cell carcinoma. NGF expression was observed in 63/109 cases (57.8%) and correlated with formation of metastases, TNM stage, poor differentiation and poor survival as well as low expression of low-affinity neurotrophin receptor $p75^{NTR}$ (but was no independent prognostic factor in multivariate Cox's regression model). Motility of cell lines expressing NGF was significantly decreased by NGF-neutralizing antibody or NGF-siRNA. The authors conclude that NGF assures survival of postmitotic neurons, but may also promote cancer cell proliferation, growth and invasion in breast-, pancreas- and prostate-cancer.

In human lung cancer (30 non-small cell, 8 small cell), Ricci et al. [20] demonstrated expression of neurotrophins NGF, BDNF and NT-3 and their receptors Trk-A–C in vessel walls, immune cells, stromal cells and some cancer cells. Thirty-three percent of bronchoalveolar carcinomas showed strong expression of NGF and Trk-A, 46% of adenocarcinomas highly expressed Trk-A. In addition adenocarcinomas, SCLC and squamous cell carcinomas showed a faint staining for BDNF and Trk-B. NT-3 and its receptor Trk-C was found in a small number of squamous cell carcinomas. No expression of $p75^{NTR}$ receptor was found. Since neurotrophins and their corresponding receptors are not expressed in normal lung tissue the authors hypothesized that factors and receptors may form an autocrine loop in cancer. The expression of factors in non-transformed cells within the tumor was interpreted as a paracrine mechanism modulating tumor growth and invasion. The inverse relation between expression of neurotrophic factors/receptors and proliferation further suggested an effect on tumor differentiation.

Investigation of $p75^{NTR}$ in 1,150 nervous system tumors and non-neuronal tumors demonstrated the low-affinity receptor to be present in a variety of mesenchymal and epithelial malignancies as well as in traumatic neuroma [21]. Hence, $p75^{NTR}$ cannot be regarded to be a specific marker.

Expression of NGF, Trk-A and $p75^{NTR}$ was also found in tumor cell lines from human leiomyosarcoma, rhabdomyosarcoma, prostatic adenocarcinoma, acute promyelocytic leukemia and histiocytic lymphoma [22]. As underlying mechanism of expression of NGF and its receptors, the authors again postulated an autocrine mode promoting proliferation. This assumption was further supported by experiments that showed antibodies against NGF and Trk-A to decrease proliferation. Rende et al. [22] see their in vitro data in line with previous studies that demonstrated NGF/Trk-A/$p75^{NTR}$ expression

in rhabdomyosarcoma, melanoma, prolactinoma, neuroblastoma, breast cancer, lung cancer, prostate cancer, bladder cancer, ovary cancer, pancreas cancer and thyroid cancer.

GDNF, neurturin, artemin and persephin all signal via the high-affinity Ret receptor tyrosine kinase which is part of a larger signaling complex that also includes GDNF family receptor αs (GFRα1–4). GDNF-, Ret- and GFRα1 deficient mice die during the first postnatal day because of a lack of enteric innervation below the stomach and renal aplasia or hypoplasia. Like GDNF neurturin is involved in the development of the urinary system but neurturin deficient mice do not show renal defects. GDNF was also shown to be expressed in Sertoli cells and to be involved in sperm differentiation. In mice with one GDNF-null allele spermatogenic stem cells are depleted, whereas accumulation of undifferentiated spermatogonia is found in overexpression of GDNF. The latter mice were infertile and developed testicular tumors.

Mutations of the Ret gene resulting in constitutive receptor activation lead to two endocrine neoplasia syndromes MEN2A and MEN2B in which thyroid carcinomas, pheochromocytomas and parathyroid carcinomas are encountered. Inactivating mutations of Ret are associated with variable defects in enteric innervation including Hirschsprung's disease. Under physiological conditions binding of the factors to their appropriate receptors GFRα1–3 receptor tyrosine-kinase Ret activates downstream targets Ras/ERK-, P13K/AKT-, p38/MAPK- and JNK-pathways.

Artemin enhances survival, proliferation and regeneration of neurons in vitro and acts as guidance molecule in axonal outgrowth. It also reduces neurotrophic pain and restores neural damage. Artemin protein levels but not mRNA levels were significantly increased in pancreatic cancer. Presence of artemin receptor was histologically demonstrated in vascular smooth muscle, ganglion cells and axons and in cancer cells. In vitro invasion assays with a Matrigel-based system showed an up to 6-fold increase in pancreatic cancer cell invasion depending on cell line and an up to 4-fold increased chemo-attraction ratio. Proliferation was not influenced by artemin [23]. The authors hypothesize that cancer invasion of the nervous system may lead to increased levels of artemin in the axons as an attempt to restore the damage to the nerve fibers. However, this accumulation of artemin in the peripheral nerve may then lead to chemo-attraction and increased peri- and endoneurial invasion by tumor cells.

The family of *neuropoietic cytokines* comprises IL-6, IL-11, LIF, oncostatin M, CNTF and cardiolipin-1 which all bind to the same receptor gp130. The factors exhibit effects on the immune-, hematopoietic- and nervous system. All factors are potent inhibitors of embryonic stem cell differentiation [24].

Developmental Antigens

Doublecortin is a microtubule-associated phosphoprotein involved in neuronal migration and differentiation expressed in migrating neuroblasts in the CNS. In the adult, doublecortin is used as marker of neurogenesis. Doublecortin may be observed in the hippocampus where neural stem cells in the subgranular lining of the dentate gyrus divide and create neuronal precursors integrating into the granule cell layer. Analysis of doublecortin expression in 179 tumors of the CNS and 65 tumors of PNS and in 74 different non-nervous tissues revealed expression in glioneuronal and glial tumors as well as in tumors of the PNS but also in normal epithelia of the kidney, liver, salivary glands and duodenum among others [25].

Neuregulins are a family of structural analogous proteins that regulate fate, differentiation and proliferation of glial cells. In addition, they have been demonstrated to influence expression of numerous neuronal transmitter receptors and synaptic plasticity. Neuregulins bind to type I receptor tyrosine kinases known as ErbBs2–4 [26]. In vitro experiments recently revealed high neuregulin-1 expression and constitutively activated ErbB3 and ErbB4 receptors in 4/8 cell lines of clear cell sarcoma of the musculoskeletal system. Exogenous neuregulin-1 stimulated growth in a subset of cell lines. The growth inhibitory effect of pan-ErbB tyrosine kinase inhibitor CI-1033 correlated with neuregulin-1 expression indicating an autocrine growth stimulation loop [27].

Axonal guidance is of uttermost importance in nervous system development. Several factors have been found to serve as chemoattractants and chemorepellents in axonal growth. All guidance molecules are expressed in adult CNS before and after injury, often in a pattern similar to the developing nervous system.

Slits, semaphorins and netrins are families of axonal guidance molecules which are expressed in the developing nervous system throughout the animal kingdom. More than 30 *semaphorins* have been cloned in mammals. Vertebrate semaphorins are secreted (class 3) or membrane bound (classes 4–7), and have a dual role acting as chemoattractants and chemorepellents in axonal growth [15]. The receptors neuropilin-1 and -2 (NP-1, NP-2), cell adhesion molecule L1 and axonal plexin-A3 transmit the axonal guidance effects of class 3 semaphorins in the developing nervous system. NP-2 deficient mice suffer from developmental defects in axonal pathfinding in the CNS and PNS. In the adult nervous system it was shown that NP-1, NP-2, semaphorin-3F and to a lesser extent semaphorin-3A mRNA levels are increased particularly distal to a crush injury of sciatic nerve in rat. The results suggested that a concentration gradient of semaphorin-3F which results in the injured tissue may serve as guidance in axonal regeneration of the

adult PNS [16]. *Slits* are large extracellular matrix glycoproteins of about 200 kDa. Slits control midline repulsion of axonal growth during development and stimulate axonal elongation and branching [28]. *Netrins* are secreted into the ECM and may act as chemoattractants or repellents. Deleted in colorectal cancer receptor (DCC) and mammalian homologs (UNC5HI-3) are parts of the receptor complex of netrins. DCC exerts chemoattraction and UNC-5 transmits chemorepellent signals in axonal outgrowth – depending on other co-factors like cAMP levels [15].

In addition to directing axonal growth, some of the axonal guidance molecules have been shown to act as angiogenic factors (netrin-1, slit-2) while others may play a role in apoptosis (netrin-1, semaphorin-3B). Outside the nervous system axonal guidance molecules are expressed in various malignancies, in addition to several types of carcinomas they have been found in melanoma (semaphorin-5A and -5D), gliomas (semaphorin-5A, NP-1, NP-2) and non-Hodgkin's lymphoma (semaphorin-4D). Slit-2 up to date seems the most ubiquitous expressed axonal guidance molecule in cancer. It was found in melanoma, bladder squamous carcinoma, neuroblastoma, SCLC, carcinoma of the urinary bladder, colon adenocarcinoma, breast cancer, hepatocellular and salivary gland carcinoma, rhabdomyosarcoma and others. Hitherto the functions of the molecules in cancer are not fully understood, many of them (semaphorins, slit-2, NP-1) seem to play a role in angiogenesis [28].

Clinical Aspects

Cancer Invasion of the Nervous System

As in the previous paragraphs shown, the numerous factors secreted by peripheral nerves, normal non-neuronal tissue and tumors may elicit effects on the secreting cells themselves and on neighboring tissue – presuming the appropriate receptors are expressed. The influence of sarcoma xenografts on nerve growth in chicken embryos which led to the discovery of NGF has already been mentioned. In histopathology tumor invasion along nerve routes with or without invasion of the nerves is observed in different entities, like in pancreatic ductal adenocarcinoma where perineural spread along the extrapancreatic nerve plexus is a characteristic mode of invasion [23]. Perineurial invasion is also a feature of adenocarcinoma of the prostate, adenoid cystic carcinoma of the trachea, infiltrating ductal carcinoma of the breast and is also occasionally observed in benign sclerosing adenosis and papillomatosis of the breast and in otherwise normal pancreas. In the skin perineurial invasion occurs in extensively infiltrating squamous cell carcinoma. Mark [29] described two cases of basal cell carcinoma with tumor growth within peripheral

nerves. Endoneurial tumor growth, often combined with thickening of the perineurium was frequently observed, whereas less frequently perineurial nests of tumor cells were seen which rarely formed a complete ring around the fascicle. Almost all nerves were involved and showed various degrees of degeneration. One tumor showed increasing neural invasion with each successive recurrence.

Tumor growth along and invasion of nerves might be explained by attraction of tumor cells by neurotrophic factors expressed in the nerve. In a study reported by Ketterer et al. [30] carcinoma of the pancreas were found to over express neurotrophins. Laser captured cells of the tumor and surrounding tissue showed these substances to be especially abundant in intratumoral nerves, suggesting that the tumors were attracted by the neurotrophin expression of the nerves. The results were confirmed by in vitro experiments showing that coculture of dorsal root ganglia with human pancreatic cancer cells led to increased tumor cell proliferation.

Lü et al. [31] investigated innervation of 16 squamous esophageal carcinomas and 13 cardiac adenocarcinomas. Numerous scattered fibers immunoreactive for galanin and neuropeptide Y were demonstrated within the tumor stroma, whereas somatostatin and cholecystokinin positive fibers were scarcely found. The fiber composition roughly reflected the distribution of transmitters in the GI tract. In accordance with the experiments of Levi-Montacini and Hamburger [13] chicken ganglions were found to form extensions towards tumor blocks in vitro. A formation of synapses was not observed.

The lack of synapse formation in the latter experiments leads to the question whether axons attracted by neurotrophic factors of malignancies have any function – like innervation of the tumor vasculature. This question was addressed by Ashraf et al. [32], who studied xenografts of human carcinoma HT29 and syngenic MC28 sarcoma in the liver of athymic rats. No innervation of blood vessels by any of the following peptides could be detected: Substance P, vasoactive intestinal polypeptide, calcitonin gene-related peptide, somatostatin and neuropeptide Y. Syngenic and xenogenic tumors yielded identical results. Former investigations focusing on adrenergic innervation of intratumoral vessels also demonstrated a lack of perivascular innervation in an intramuscularly implanted tumor [33] and in a hepatoma and an adenocarcinoma model implanted in rat liver [34].

Concerning the contractility of the newly formed vessels, no smooth muscle cells were observed in the neo-vasculature of the metastases in the animal models. The latter finding contrasts with human colorectal liver metastases in which smooth muscle cells were demonstrated, however, these did not form a complete layer in the walls of the tumor vessels and when present were mostly of the proliferative, rather than the contractile type.

Paraneoplastic Syndromes of the Nervous System

Neuronal tumor antigens may stimulate an immune response which may seldom lead to an autoimmune syndrome of the nervous system. Some of the antibodies associated with these paraneoplastic syndromes have been characterized. In most cases several antibody species directed against various antigens are encountered.

As described above SCLC may express voltage-gated calcium channels (VGCCs). An immune response leading to production of antibodies directed against the VGCCs frequently results in Lambert-Eaton myasthenic syndrome (LEMS). Five different VGCCs have been identified. Of these, the P/Q-type is predominantly expressed in the neuromuscular junction and in SCLC. Like neurons SCLCs are capable of generating calcium spikes. However, LEMS may also occur in patients without cancer. The etiology in these patients is unclear but an association with HLA-B8 was noted suggesting a genetic predisposition in these patients. In some cases LEMS may also be attributed to autoantibodies against synaptotagmin I which is found in vesicles and participates in the calcium dependent release of neurotransmitters [35].

In addition, metastatic prostate and breast cancer cells were recently described to be potentially 'excitable' because of expression of high levels of voltage gated sodium channels [36]. Expression of voltage-gated ion channels in cancer has been linked to the metastatic cascade including process extension, directional motility, secretory membrane activity, adhesion and invasion in vitro [36].

Anti-neuronal nuclear antibodies (ANNA-1, -2, -3) most frequently affect the PNS and CNS. The ANNA-1 (anti-Hu) and ANNA-2 (Anti-Ri) antigens are neuron-specific RNA-binding proteins. ANNA-1 binds to the AU-rich element of mRNAs that regulate cell proliferation in both, neurons of the CNS and PNS. Lung carcinoma was the most frequent neoplasm associated with ANNA-2. In 2001, Chan et al. [37] identified ANNA-3 in 11 patients with suspected paraneoplastic syndrome. The antibody predominantly binds to nuclei of Purkinje cells and renal podocytes. Typical symptoms included subacute multifocal sensory/senorimotor neuropathies, cerebellar ataxia, myelopathy, brain stem and limbic encephalitis. Eight of nine patients in whom the carcinomas were identified suffered from lung tumors (5 small-cell-, 2 adeno- and 1 lung-carcinoma) and one from a carcinoma of the esophagus. Like ANNA-2, ANNA-3 does not recognize peripheral neurons. Chan et al. [37] also detected ANNA-3 in an 8-year old boy with transient cerebellar ataxia who did not suffer from cancer suggesting an association with idiopathic neurological autoimmunity. In line with this observation is a report on ANNA-1 seropositivity observed in children without evidence of neoplasm [38]. In small cell cancer ANNA-1 are detected most commonly, followed by antibodies to collapsin response-mediator protein-5

(CRMP-5), amphiphysin and Purkinje cell cytoplasmic antibody-2 (PCA-2). The latter is found at equal frequencies as ANNA-2 and ANNA-3. In contrast to ANNA-1 to ANNA-3 which are anti-nuclear antibodies, CRMP-5, PCA2 and amphiphysin are cytoplasmic antigens.

In 2003, Pittock et al. [39] published a study in which they screened 75,000 sera of patients with subacute neurological symptoms suspected to be neoplastic for ANNA-2. In 34 cases, ANNA-2 were positive. In 4/28 patients in whom a clinical history was available, diagnosis of carcinoma ante ceded the neurological symptoms. Symptoms included brain stem syndrome, cerebellar syndrome, myelopathy, peripheral neuropathy, cranial neuropathy, movement disorders, encephalopathy, Lambert-Eaton syndrome and seizures. Accompanying antibodies included acetylcholine receptor antibodies, ANNA-1, ANNA-3, CRMP-5-IgG, P/Q-type and N-type Ca-channel antibodies, thyroid peroxidase, thyreoglobulin, GAD65, IA-2 and other anti-nuclear antibodies or mitochondrial antibodies. The female–male ratio was 2:1 just as in ANNA-1 or CRMP-5-IgG. As in previous studies there was a low-distant metastatic spread (12%).

Conclusion and Outlook

The previous paragraphs have shown that many proteins that were first identified in the nervous system later turned out to be expressed in various other tissues. Hence, it is important to be aware that the term ‘neural’ in these cases is only a historical one – a fact that will most likely apply to more and more antigens in the future.

Looking at the function of the proteins mentioned, they fall into (overlapping) categories like (i) maintenance and repair, (ii) directing migration, (iii) secretion and (iv) cell adhesion – fundamental processes of the developing nervous system and vasculature which are switched on again in malign tumors, or as Liotta and Clair [40] commented: ‘. . .cancer invasion in general may be a deregulated form of a physiological invasion process required for neuronal wiring in the embryo, tissue remodeling of blood vessels, and healing’.

The expression of ‘neuronal’ proteins in the tumor may stimulate axonal outgrowth of free nerve endings, thereby enabling the tumor host to ‘feel’ the cancer as in NGF expression in pancreatic cancer or skeletal cancer which are frequently associated with chronic pain. Expression of ‘neuronal’ proteins in the nervous tissue on the other hand seems to play an important role in ‘unwillingly’ guiding tumor growth along nerve routes and may additionally stimulate tumor growth via paracrine mechanisms.

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Chemotropic Axon Guidance Molecules in Tumorigenesis

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Abstract

Recent studies suggest that secreted proteins in several families of axon guidance molecules, the slits, the semaphorins and the netrins may play important roles in cancers. The expression of many of these proteins is either down-regulated or up-regulated in cancer cell lines and tumors. Several of the corresponding genes are localized on chromosomal regions associated with frequent loss-of-heterozygosity and their promoters are hypermethylated, suggesting that they may act as tumor suppressors. Moreover, many axon guidance proteins were also shown to control the development of the vasculature and may thus control angiogenesis in the tumors. These axon guidance molecules may also control the migration and invasion potential of cancer cells. Lastly, they could stimulate their proliferation and regulate cell death. Thus, axon guidance molecules appear as good targets for the development of novel therapeutic agents for the treatment of malignancy.

Soon after becoming post-mitotic, developing neurons migrate away from the proliferative neuroepithelium to their final position in the brain and extend processes, the axons, over long distances. Studies conducted over the last 20 years have led to the identification of many families of so-called axon guidance molecules that can orient growing axons and migrating neurons during their journey [1]. Many of these evolutionary conserved molecules are secreted, form diffusion gradients and act from a distance on neurons and axons. Their activity can be chemoattractive or chemorepulsive depending on the neurons or its activation state [2]. Their receptors and their signaling pathways have started to be identified and it was also discovered that they are expressed in many organs and tissues outside the nervous system. Although their normal function in mature cells is largely unknown, an increasing number of studies suggest that

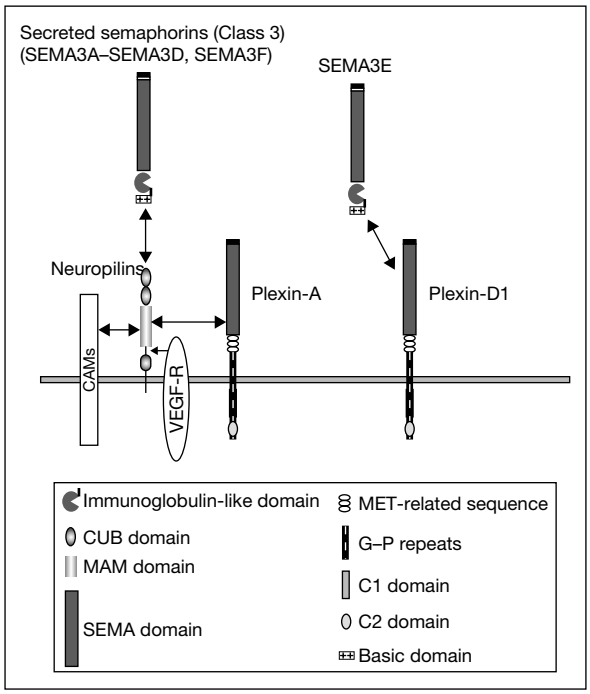

Fig. 1. Secreted semaphorins and their receptors. Most class 3 semaphorins bind to neuropilins and use plexin-As as signaling subunits. Cell adhesion molecules are also part of the receptor complex for class 3 semaphorins. SEMA3E directly signals through plexin-D1. Neuropilin-1 can also bind VEGF.

chemotropic axon guidance molecules are involved in many pathological processes such as cancers [3]. I will review here recent studies on the function in cancer of diffusible axon guidance molecules from three major families of the netrins, the slits and the semaphorins.

The Semaphorins and Their Receptors

The semaphorins are secreted or membrane bound proteins that can inhibit the growth of most axons in vertebrates and invertebrates [4]. More than 30 distinct semaphorins have been identified and are distributed in eight classes based on structural features. All semaphorins share a highly conserved 500 amino acid motif, the SEMA domain, that also exists in other proteins. Semaphorin classes 1 and 2 are found in invertebrate species and classes 3–4, 6 and 7 in vertebrates (fig. 1). Class 5 semaphorins exist both in vertebrate and invertebrate

species. A variety of semaphorin receptors have been identified over the last 10 years. Most semaphorins directly bind and signal through large membrane spanning proteins called plexins [5]. The extracellular domain of plexins contains a divergent semaphorin domain and also has sequence homology with the receptor tyrosine kinases MET (the receptor for scatter factor-1/hepatocyte growth factor receptor) and Ron (the receptor for macrophage stimulating protein). Nine plexins were identified and regrouped in four subclasses (plexin-A–plexin-D). Most secreted semaphorins (class 3) signal through a receptor complex composed of neuropilins as binding moieties and plexins as signaling moieties, although some may directly bind to plexins [6]. Two main neuropilin subtypes (neuropilin-1 and neuropilin-2) have been described and have distinct semaphorin/ligand affinities. The immunoglobulins L1-CAM and NrCAM are also part of the receptor complex for several secreted semaphorins [7]. Additional receptors for transmembrane semaphorins have been identified in non-neuronal cells, in particular in the immune system and bones [8, 9]. Although soluble forms of the extracellular domain of some transmembrane semaphorins have been described, I will focus here on secreted semaphorins, whose chemotropic activity has been well-described.

The Slits and Robos

Slits are diffusible chemorepulsive proteins that play a major role in controlling axon guidance at the midline of the central nervous system [10]. In flies and mice lacking slits, axons converge and stay at the midline. In mammals, three *slit* genes (*SLIT1–SLIT3*) have been cloned. All encode large ECM glycoproteins of about 200 kDa (fig. 2), comprising, from their N-terminus to their C-terminus, a long stretch of leucine rich repeats, seven to nine EGF repeats, and a domain, named ALPS, LNS or LG module. Slits repel developing axons and migrating neurons, but also migrating muscle precursors and mesodermal cells. The roundabout (robo) proteins, a small subgroup within the immunoglobulin superfamily (fig. 2), are the only known slit receptors [10]. Three *robo* genes have been found in flies and mammals. A fourth putative *robo* gene, called *magic roundabout* or *ROBO4* was recently cloned, but it lacks some of the domains found in other robo proteins and its capacity to bind slits is still debated [11].

Netrins and Their Receptors

Netrin-1, a laminin related protein, was the first axonal chemoattractant ever identified [12]. Netrin-1 was later shown to control neuronal migration in

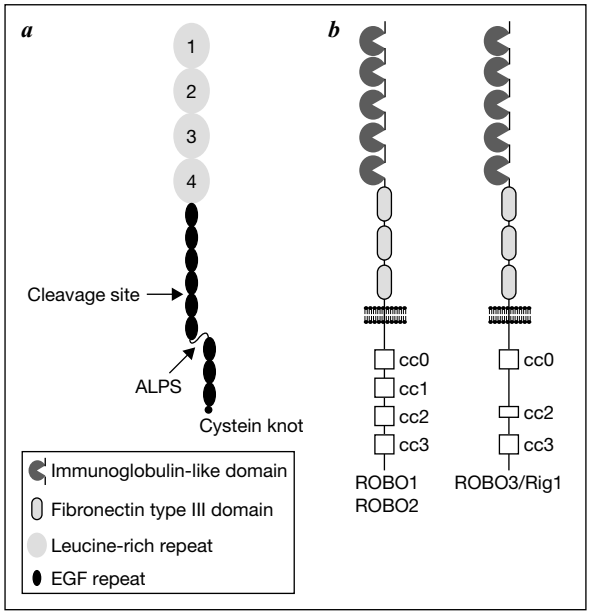

Fig. 2. Slits and their receptors. ***a*** Structure of slit proteins. Slits are proteolytically processed into a large N-terminal and shorter C-terminal fragments. ***b*** Structure of roundabout receptors (ROBO1–ROBO3). Robos define a small subgroup within the immunoglobulin superfamily characterized by the presence of five Ig-like followed by three fibronectin type III repeats, a transmembrane portion and a long cytoplasmic tail containing robo-specific motifs (cc0–cc3). ALPS = domain found in Agrin, Laminin, Perlecan and Slit.

the developing and adult brain and the migration of pancreatic progenitors, neural crest cells, oligodendrocyte progenitors and endothelial cells [3]. There are at least three netrin genes in mammals (netrin-1, netrin-3/NTN2L and netrin-4). In neurons, netrin-1 has several known receptors (fig. 3), deleted in colorectal cancer (DCC), UNC5A, UNC5B, UNC5C and UNC5D and the adenosine receptor A2b [3]. DCC mediates the attractive activity of netrin-1, while UNC5s seem required for its repulsive activity [13]. However, UNC5 may also signal independently of DCC and netrin-1 [14].

Expression of Chemotropic Axon Guidance Molecules in Cancer Cells

Over the last few years, the expression of chemotropic axon guidance molecules in a variety of cancer cell lines and tumors has been thoroughly

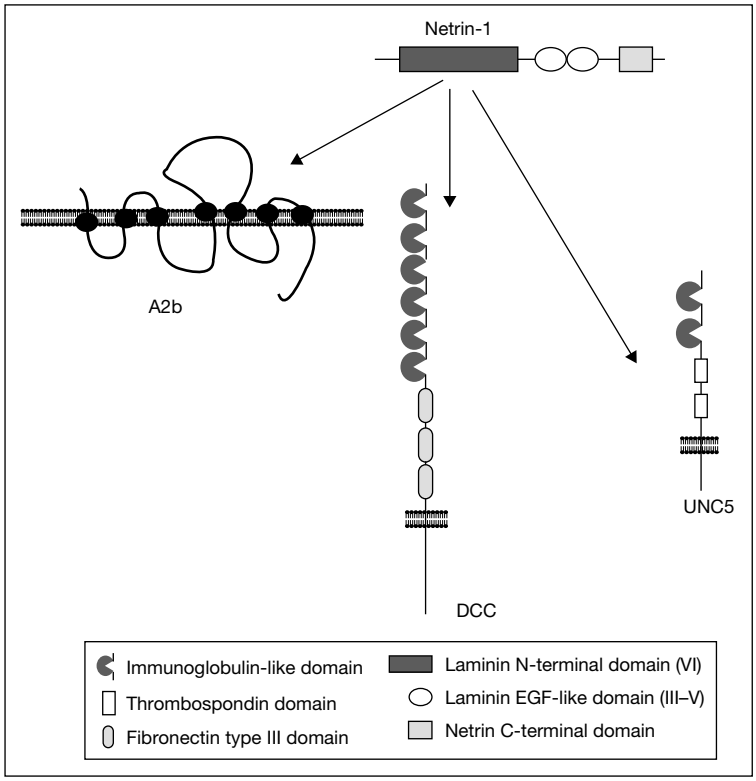

Fig. 3. Netrin-1 and its receptors. Netrin-1 contains a laminin N-terminal domain, two laminin EGF-like domain and a netrin C-terminal domain. Several transmembrane netrin-1 receptors are known. DCC, UNC5A–UNC5D and the adenosine receptor A2b, a seven membrane domain receptor.

investigated [3]. These studies revealed that many of these molecules are either up- or down-regulated in tumor cells.

Axon Guidance Molecules Highly Expressed in Cancer Cells

SEMA3C was the first secreted semaphorin proposed to be involved in tumorigenesis, as it is overexpressed in non-MDR (multidrug resistance) drug resistant ovarian and lung cancer cell lines [15, 16]. Several glioma cell lines also express high levels of SEMA3C. Likewise, SEMA3E expression was correlated positively with tumor progression in mouse mammary carcinoma and is overexpressed in metastatic human lung adenocarcinoma cell. SEMA3A, SEMA3F are also overexpressed several cancer cell lines and tumors [17]. Last, SLIT2 is

strongly expressed in many tumor cell lines (melanoma, neuroblastoma, colon adenocarcinoma …) and primary tumors, while SLIT1–3 expression and ROBO1 expression are upregulated in prostate tumors and colorectal cancer respectively [18, 19]. Although the function of the overexpressed semaphorins and slits in cancer cell lines is unclear, they could via an autocrine/paracrine action modify cell survival and migration, inhibit the immune response [17], but also regulate tumor vasculature.

Down-Regulation of Axon Guidance Molecules in Cancer Cells: Putative Tumor Suppressors?

Most studies point to a down-regulation of the expression of secreted axon guidance proteins in cancers. Thus, the netrin-1 receptor DCC was first characterized as a gene of frequently DCCs and its expression is also down-regulated in many tumor cell lines [20]. DCC is at 18q21.2, a locus with frequent loss of heterozygosity (LOH) in gastrointestinal cancers, suggesting that DCC is a tumor suppressor gene. Interestingly, the UNC5 genes, that encode the other netrin-1 receptors, are frequently down-regulated in many primary tumors in association with significant LOH [20].

SEMA3B and SEMA3F, were mapped to the 3p21.3 locus, a region that is thought to contain putative tumor suppressor genes [21]. Many studies suggest that SEMA3B may be a candidate tumor suppressor. First, SEMA3B is down-regulated in lung cancer cells and is also often mutated, suggesting that it may play a suppressive role in tumorigenesis. Morevover, SEMA3B promoter is hypermethylated in multiple cancer cell lines, or in tumor samples and there is a significant LOH and hypermethylation of its promoter [22]. Ovarian adenocarcinoma cells also express 25-fold less SEMA3B than in normal human ovary and have decreased tumorigenic properties in xenograft model [23]. SEMA3F expression is also down-regulated in several cancer cell lines and tumors and its promoter methylated [16]. As mentioned above, deletions and heterozygous loss in regions 3p12, 3p14 and 3p21 occur frequently in lung cancer. Interestingly, ROBO1/Dutt1 was mapped within the deletion and its promoter region is hypermethylated in primary lung, renal breast and cervical cancer [24]. Although, no somatic point mutation of ROBO1 (or of its ligands slits) was reported in tumors, ROBO1 may be a tumor suppressor gene [25]. Likewise, SLIT2 is mapped to 4p15.2 a region associated with frequent LOH in many tumors. Accordingly, the inactivation of SLIT2 in tumors was shown to be epigenetic and caused by the hypermethylation of the promoter region. SLIT1–3 expression is decreased in breast, lung cancer cell lines, gliomas or tumors and epigenetic inactivation of slit genes was demonstrated [18, 24]. The observation that exogenous SLIT2 suppresses colony growth in breast cancer cell lines further supports a possible tumor suppressor function of SLIT2.

The development and growth of tumors require the simultaneous formation and sprouting of new blood vessels from pre-existing capillaries and veins [26]. Surprisingly, mounting evidence suggests that diffusible axon guidance molecules are very potent angiogenic or anti-angiogenic factors [26] and that blood vessels that irrigate tumors express receptors for several secreted axon guidance proteins, in particular ROBO1, neuropilin-1, plexins-D1 and UNC5B.

The first direct evidence for a link between axon guidance molecules and angiogenesis came from studies focused on neuropilin receptors [16]. Binding experiments revealed that in addition to binding most class 3 semaphorins, neuropilin-1 is a receptor for VEGF-A (the VEGF165 but not the VEGF121 isoform), VEGF-B, VEGF-E and placental-derived growth factor-2. Neuropilin-1 is expressed by tumor cells and endothelial cells, where it is a co-receptor for VEGFR-2 mediating VEGF function in angiogenesis [16]. The analysis of neuropilin-1 knockout mice has confirmed that neuropilin-1/VEGF interaction is required for normal development of the vasculature [27]. A soluble neuropilin-1 isoform was identified and found to have anti-tumor activity. Recently, two other soluble forms of neuropilin-1, sIIINRP1 and sIVNRP1, generated by alternative splicing, were discovered and both are expressed in human cancerous tissue. These soluble neuropilins also bind VEGF165 and SEMA3A. Likewise, neuropilin-2 is a receptor for VEGF165, VEGF145 and placental-derived growth factor-2. Interestingly, SEMA3A binding to neuropilin-1 and SEMA3F binding to neuropilin-2 block the migration of endothelial cells [28]. SEMA3F also inhibits endothelial cell survival [29]. Several class 3 semaphorins are also expressed by endothelial cells and could have an autocrine action. Accordingly, SEMA3A seems to exert a permissive role on angiogenesis by inhibiting integrin-mediated adhesion of endothelial cells allowing their deadhesion. The ratio of SEMA3A/VEGF165 expression in patients with multiple myeloma was also proposed to be critical to the angiogenic potential of bone marrow endothelial cells [30]. More recent studies have shown that in contrast with other secreted semaphorins, SEMA3E directly binds and signals via the plexin-D1 receptor [6]. Interestingly, plexin-D1 controls angiogenesis during development and both molecules could exert a similar function in tumors.

As mentioned above, many tumors express high levels of SLIT2 and several studies suggest that SLIT2 and its receptors have a potent angiogenic activity. ROBO1 is expressed on human umbilical vein endothelial cells and SLIT2 increases their migration [31]. This chemotactic activity of SLIT2 requires phosphatidylinositol-3 kinase (PI-3K) activation and can be inhibited by recombinant ectodomain (RoboN). There is also an in vivo evidence for a role of slit/robo in angiogenesis.

Recent studies suggested that netrins could also regulate angiogenesis. Thus, endothelial cells express UNC5B and A2b receptors, respond to netrin-1 and vascular defects were detected in UNC5B knockouts [32]. However, contradictory data have just appeared [33]. Thereby, the exact mechanism of action of netrin-1 in endothelial cells, and its pro and anti-angiogenic activity are still debated.

Overall these studies suggest that in tumors, some axon guidance proteins (SLIT2, netrin-1) are up-regulated and may increase angiogenesis upon binding their receptors on endothelial cells. Other axon guidance molecules (SEMA3E, SEMA3A and SEMA3C …), may act as inhibitors of angiogenesis in normal condition for instance by interfering with VEGF function.

Control of Cancer Cell Survival and Proliferation by Axon Guidance Molecules

In addition to controlling indirectly tumor cell survival by regulating angiogenesis, chemotropic axon guidance molecules may also exert a direct action on proliferation and apoptosis [3]. Thus, SEMA3B transfection in lung cancer cell lines or application of exogenous soluble SEMA3B ectodomain decrease colony formation and induces apoptosis. An anti-proliferative activity of SEMA3B has been shown for breast cancer cell lines. Likewise, VEGF binding to neuropilin-1 is required for the survival of breast carcinoma cells. In addition, after stable transfection with SEMA3B expression constructs, their proliferation rate is decreased. SEMA3B may also act as a mediator of p53-suppressor activity in glioblastoma cell lines [34]. The emerging model suggests that in premalignant cells, the activation of the p53 pathway leads to a decrease of SEMA3B expression and/or an overexpression of its antagonist VEGF, therefore allowing cancer cells to survive and proliferate. As HEY cells and lung cancer cells express neuropilins, it was proposed that in tumor cells, SEMA3B signals through these receptors and competes with neuropilin-mediated VEGF signaling. SEMA3F overexpression in mouse fibrosarcoma or ovarian cancer cells was also shown to block proliferation.

This new apoptotic/anti-apoptotic function for axon guidance proteins is mostly supported by studies on the netrin-1 and its receptors UNC5 and DCC, that were demonstrated to be dependence receptors: in absence of their ligand netrin-1, their cytoplasmic domain is cleaved by caspases and massive cell death occurs when they are overexpressed in cultured cells [20]. Moreover, there is a death domain at the C-terminus end of UNC5 proteins. The exact mechanism by which DCC and UNC5 receptors trigger apoptosis is still largely unknown, but it in the case of UNC5, a p53-dependent pathway may be

involved [35]. It was proposed that DCC and UNC5s act as tumor suppressors only when their ligand netrin-1 is not present. According to this model, the normal function of DCC and UNC5s could be to induce the death of tumor cells that have migrated away from their normal location, in territories where the ligand netrin-1 is absent. Therefore, in tumor cells, the lack of functional DCC or/and UNC5 would make the tumor cells resistant to apoptosis. Likewise, an excess or abnormal expression of netrin-1, or a still unknown ligands [14], would protect tumor cells still expressing DCC and UNC5 from death. This also suggests that netrin-1 function in normal tissues would be to interfere with DCC and UNC5s-dependent apoptosis.

Control of Cancer Cell Invasion and Migration by Axon Guidance Molecules

The analysis of neuropilin expression in tumors and tumor cell lines showed that there is a differential expression of neuropilin-1 in two rat prostate carcinoma cell lines (AT2.1 and AT3.1), that have a differential motility in Boyden chambers [36]. AT3.1 cells are more motile and express higher level of neuropilin-1 than AT2.1 cells. Upon transfection with neuropilin-1 AT2.1 cells increase their level of migration. They also form larger tumors when grafted in rats, possibly through an enhancement of angiogenesis involving VEGF signaling. Breast carcinoma cells were also shown to express SEMA3A (and plexin-A1, a neuropilin-1 co-receptor) and lowering SEMA3A expression stimulate their migration. Likewise, SEMA3A expression is decreased in mesothelioma [37]. In these cells, SEMA3A expression is transcriptionally induced by VEGF, through a p38 MAPK-dependent pathway. As for SEMA3B, it is thought that a deregulation of the VEGF/SEMA3A ratio occurs in tumor cells, increasing their invasive potential.

In breast cancer cells (MCF7) SEMA3F inhibits the attachment and spreading of apparently through interaction with neuropilin-1 and not neuropilin-2. SEMA3F is also able to antagonize VEGF action on these cells. More recently it was shown that SEMA3F overexpression in highly metastatic melanoma cells (that only express neuropilin-2 and not neuropilin-1, VEGFR-1 or VEGFR-2) inhibits adhesion and migration but not proliferation [28].

Tumor cells often migrate to distant organs leading to secondary tumor formation and chemokines play a role in this process. Recently, SLIT2 was shown to be a potent inhibitor of stromal-derived factor-1 induced leukocyte chemotaxis [38]. This effect requires the interaction of CXCR4 with ROBO1 that are both expressed by leukocytes. Breast cancer cells and human melanoma also express CXCR4, ROBO1 and ROBO2 and chemokines such as CXCL12

stimulate the migration of cancer cells. It has been shown that slit inhibits CXCL12/CXCR4-induced breast cancer cell (DU4475) chemotaxis, chemoinvasion and adhesion. Slit inhibits CXCL12-induced phosphorylation of the focal adhesion component FAK and RAFTK/Pyk2 and paxillin. It also inhibits CXCL12-induced Src kinase and PI3-kinase activities, p44/42 MAP kinase and activity of the matrix metalloproteinase MMP-2 and MMP-9 two proteolytic enzymes which play a role in tumor invasion through degradation of the extracellular matrix [3]. More recently, SLIT2 was also shown to inhibit the invasion of medulloblastoma cells [39].

In Vivo Evidence for a Role for Axon Guidance Molecules in Cancers

To data, most studies on axon guidance molecules in tumorigenesis have focused on expression profiles and in vitro assays and only a few in vivo evidence has been obtained. SEMA3F-transfected melanoma cells injected into nude mice do not become metastatic [28]. This could be explained by an inhibitory action of SEMA3F on endothelial cell invasion and/or tumor cell migration.

A targeted mutation of mouse *ROBO1* was generated by deletion of exon 2, mimicking a deletion that naturally occurs in human small cell lung cancer cell line NIH-H219X, and resulted in the removal of ROBO1 Ig1 [25]. A majority of ROBO1$^{-/-}$ homozygous die at birth due to abnormal lung development. A few homozygous survive up to 1 year and show bronchial hyperplasia, but no spontaneous tumor formation was detected. Recently, the tumor susceptibility of ROBO1 heterozygous mice was analyzed [25]. During their second year of life, ROBO1 heterozygotes develop lymphoma and carcinomas. In malignant tumor samples from ROBO1$^{+/-}$ mice, the expression of ROBO1 is undetectable. Moreover, the study of the remaining allele showed that its promoter is hypermethylated. Overall, these studies support a role for ROBO1 as a tumor suppressor gene, at least in the mouse. In addition, in vivo inhibition of ROBO1 function has been shown to reduce tumor microvessel densities and tumor size [31]. However, the expression of Robo proteins by endothelial cells in normal or metastatic tissue has not been reported yet and SLIT1/SLIT2 knockouts have an apparent normal vasculature. Therefore, the physiological relevance of these results is still unclear.

Although DCC and netrin knockout mice die at birth, precluding from providing direct evidence for their involvement in cancers, it has recently been shown that transgenic mice overexpressing netrin-1 in the intestine develop spontaneous intestinal tumors [40]. However, an overexpression of netrin in or around human tumors has not been reported yet.

Conclusion Perspectives

Overall, these experiments suggest that agonists and antagonists of axon guidance molecules could be used to block tumor growth. These antagonists could be soluble neuropilin-1 recombinant proteins that could sequester VEGF, or SEMA3A and netrin-1 proteins and peptides that could block tumor progression and endothelial cell migration. Other possible therapeutic agents are sIIINRP1 and sIVNRP1 that can block breast cancer cell migration [41]. Axon guidance proteins such as netrin-1, SEMA3B and SEMA3A may also be used to kill premalignant cells by blocking their migration and proliferation. Likewise, the signaling cascades activated by many axon guidance proteins have started to be identified in neurons [1, 4] and they may be similar in tumor cells. Interestingly, other secreted proteins, the morphogens, that have been long known to be involved in tumorigenesis, were recently shown to control axon guidance [42]. In conclusion, these studies suggest that the molecular machinery used by neurons to migrate and grow processes during development is also used in mature tissues by cancer cells.

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Neoneurogenesis and the Neuro-Neoplastic Synapse

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Abstract

A tumor is not an isolated entity within an organism, but tissue that strongly interacts with its environment. This interaction is however not restricted to direct cell-to-cell interactions, but generally comprises the susceptibility of tumor cells for chemokines and cytokines, as well as neurotransmitters and hormones by the expression of the according receptors. These signal substances have influences on tumor cell functions such as proliferation and migration. The other way round, tumor cells themselves release a broad range of these signal substances, which influence the cells of the environment. One of the first and most important interactions in this respect is the angiogenesis, which was discovered about 30 years ago. Tumor cells release angiogenic factors, i.e. the vascular endothelial growth factor as well as angiogenic chemokines among others. These factors initiate the vascularization of the tumor. Recently, a similar process was found for the development of lymphatic vessels in tumors. We herein seize these observations and combine them with arguments provided in the previous chapter, which leads us to the hypothesis that tumor cells may also be able to stimulate their own innervation; a process that we have termed neoneurogenesis.

The previous chapters of this book have shown several important aspects of the neuronal activity in tumor tissues. With regard to the topic of this chapter, we would like to highlight again two of the previous contents. Firstly, the peripheral nervous system of an adult organism is, in contrast to the central nervous system, cytologically dynamic and able to respond to growth signals and guidance signals after injury, tissue regeneration or after physiological events such as training stimuli. Secondly, neurotransmitters play a role in tumor diseases, which is evident on a molecular basis with regard to the modulation of carcinogenesis, and on a clinical basis with regard to the prognostic consideration of nerve cell markers for the course of a cancer disease. We now combine

these two facts. As a consequence of the regenerative potential, peripheral nerve cells are principally able to ingrow into tumor tissues, which would deliver an explanation for the detection of nerve cell markers in tumors. The consecutive questions are then, how might tumor cells be able to regulate this innervation and in turn, what effects do the thereby supplied neurotransmitters provoke in the tumor cell? May this explain the poor outcome in patients with nerve cell marker positive tumors? In the following two chapters, we elaborate on the interaction of peripheral nerve cells and tumor cells, which we call the neuro-neoplastic synapse. We provide arguments, that the tumor cells regulate their own innervation, and that neurotransmitters supply a regulatory matrix for tumor cell migration and metastasis development.

In the previous chapter, Chedotal [1] has provided excellent insight into axon guidance molecules, their role in cancer and in angiogenesis. He elaborates on his recently published review on the expression of these guidance molecules, i.e. semaphorins, slits, and netrins, in tumor cells and their paracrine and autocrine effects on tumor cell migration. We would now like to switch the focus and ask, how these guidance molecules may initiate tumor tissue innervation by nerve cells.

Neoangiogenesis

Over 30 years ago, the term neoangiogenesis was coined for the vascularization of tumors. This field of research initially started by a publication of Folkman et al. [2], who described a tumor-angiogenesis factor. Today, we know that this vascularization is initiated by the tumors for their own nourishment and that this process is regulated by a multitude of signal substances. Without sustained angiogenesis, a tumor cannot grow over a certain size due to the lack of nutrition. Angiogenesis is regarded as one of the six capabilities, which collectively accomplish malignant growth [3]. The vascular endothelial growth factor (VEGF) is considered to be one of the most important angiogenic factors. VEGF is secreted by tumor cells in response to cellular stress such as hypoxia [4]. However, Strieter [5] has recently discussed that an inhibition of the VEGF function alone is not sufficient to inhibit angiogenesis. His work shows that CXC chemokines play an important role in the regulation of angiogenesis, too [6]: neoangiogenesis is balanced by proangiogenic Glu-Leu-Arg motif positive (ELR^+) CXC chemokines and anti-angiogenic ELR^- CXC chemokines, which are induced by interferon. In light of this complex regulation of neoangiogenesis in tumors, a more promising approach for its inhibition might be the blockade of endothelial cell proliferation, e.g. by cyclopentane analogs of fumagillol [7], instead of neutralizing angiogenic factors or blocking their respective receptors.

Lymphangiogenesis

More recently, a mechanism analog to the neoangiogenesis was postulated for the development of new lymph vessels, called lymphangiogenesis. Similar to the neoangiogenesis, this process seems to be controlled by VEGF, too [8]. The VEGF receptor-3 is especially expressed on lymphatic endothelium, and its ligands VEGF-C and -D are sufficient lymphangiogenic factors [9]. Both neoangiogenesis and lymphangiogenesis are supposed to support the development of metastases [10, 11], which is most reasonable, since a direct connection of the tumor to the blood stream or lymph drainage facilitates the passive dissemination of the tumor cells. A study with patients suffering from cutaneous melanoma showed, that the lymphatic microvessel density is correlated with sentinel lymph node metastasis development and with a shorter survival of the patients [12]. Consequently, in parallel to the inhibition of angiogenesis, the inhibition of lymphangiogenesis is under investigation to block tumor progression towards metastasis development [13, 14].

Neoneurogenesis

It has turned out that tumors are also able to release factors that regulate the survival, growth and differentiation of nerve cells. For example, the nerve growth factor was detected in breast and prostate carcinoma cells [15, 16], in pancreatic cancer [17], and in bronchioloalveolar carcinomas as well as lung adenocarcinomas [18]. The brain-derived growth factor was detected in prostate carcinoma cells [19], and the neurotrophic factor 3 was detected in lung squamous cell carcinomas [18]. NGF is also known to have angiogenic effects on endothelial cells [20], and in turn VEGF receptors have been detected in the hippcampus of mice [21], and on neural progenitor cells in Xenopus laevis and mouse embryos [22]. Furthermore, VEGF is under certain conditions a chemoattractant for rat neural progenitors [23]. In conclusion, VEGF seems to play a role in neurogenesis, too [24], showing that angiogenesis, lymphangiogenesis, and neurogenesis are processes that have, at least in part, a common regulation. In addition, with regard to neurogenesis, axon guidance molecules are expressed and frequently dysregulated in tumor tissues, as has been introduced above referring to the work of Chedotal [1]. Most interestingly, a loss or downregulation of the semaphorins SEMA3B and F has been detected in lung and breast cancer cells [25, 26]. Semaphorins are ligands for neuropilins and act as repellents for the axon growth. These neuropilins interact with the VEGF receptor, and the effects of SEMA3B and SEMA3F are antagonized by VEGF [25, 26]. We assume that these

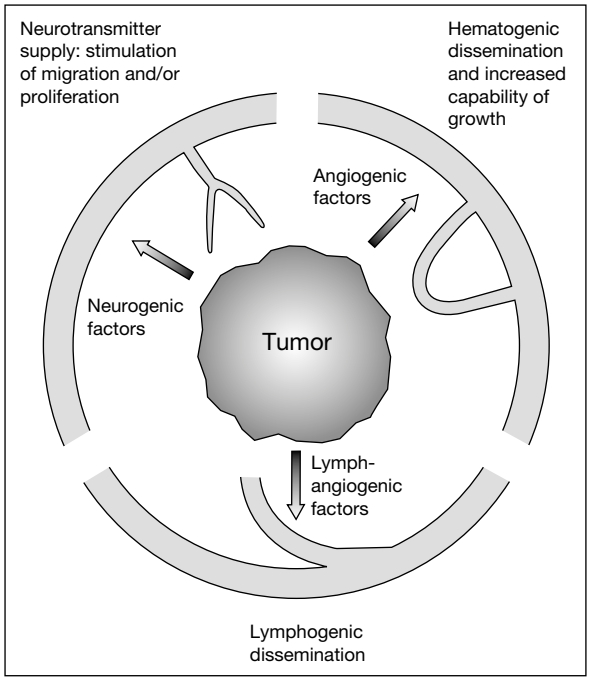

Fig. 1. Release of neurogenic, angiogenic and lymphangiogenic factors by tumor cells and potential consequences of their effects for cancer progression.

substances, which are released by the tumors, protect the nerve cells from degeneration during and after destruction of the normal physiological environment. In addition, the tumors facilitate their own innervation in analogy to neoangiogenesis and lymphangiogenesis (fig. 1). We have named this neoneurogenesis. These two processes – neuroprotection and neoneurogenesis – would explain, why some tumors are positive for nerve cell markers, as discussed in chapter 'Neuronal Markers in Non-Neuronal Tissues' of this book. By the preservation and ingrowth of nerves or nerve endings in tumors, the nervous system provides neurotransmitters directly to the tumor cells, which can be termed as a neuro-neoplastic synapse. What consequences might such synapses cause for the tumor progression and why is the occurrence of nerve cells in tumors correlated with a poorer course of the disease?

Epidemiological Aspects

Since 1926, it has been assumed that psychosocial factors influence the cancer incidence and tumor progression [27]. This argument is difficult to verify scientifically, as the individual perception of environmental influences widely varies. However, it seems that negative emotions such as stress, anxiety or depression facilitate tumor progression. This has been shown largely by epidemiological studies in two directions. The first direction aims at pharmacological evidences. Stress, anxiety, or depression are translated into the release of neurotransmitters. These are predominantly catecholamines (epinephrine and norepinephrine), besides others (e.g., dopamine and substance P). Catecholamines have a strong increasing effect on the blood pressure, and thus inhibitors for their respective receptors are in clinical use for the treatment of hypertension. These drugs block the β-adrenergic receptors (therefore called β-blockers). A french study by Algazi et al. [28] showed a reduced risk of various types of cancer by the use of such β-blockers. In a canadian study by Perron et al. [29], a reduction of the prostate cancer risk was correlated to the duration of the β-blocker use. After 4 years, the prostate cancer risk was reduced by 18%. The second line of argumentation regarding the role of psychosocial influences on cancer aims on the life habits and sentiments of the patients. These studies are very contrary and data are difficult to evaluate, as of course the individual perception on certain events differ. This line of argumentation is thus not as hard and convincing as the pharmacological approach, but overall the majority of such epidemiological studies seem to support the view, that negative stress and depression have a negative influence on the course of a cancer disease. For example, Lillberg et al. [30] published studies on Finnish women, that there was no role of daily activities' stress or the personality [31] in the etiology of cancer. But the same group reported, that strong stressful live events (e.g., death of a relative) do actually increase the breast cancer risk [32]. Furthermore, it was shown in a study in Swedish women, that the subjective, self-reported level of stress predicted the development of breast cancer [33]. It seems worth of note in this context, that stress has an influence on the estrogen metabolism, too [34]. As high concentrations or prolonged exposure to estrogen are known risk factors for breast cancer [35], an impaired estrogen production due to chronic stress might under certain conditions have a cancer-protective function [36].

These epidemiological observations allow an interpretation in two ways, which not exclude but rather complement each other. On the one hand, stress and depression are known to lower the activity of the immune system. Such a suppression of the immune surveillance and anti-cancer action of the immune system might support the manifestation and progress of a cancer disease [37]. On the other hand, a direct interaction of a tumor with nerve cells via a

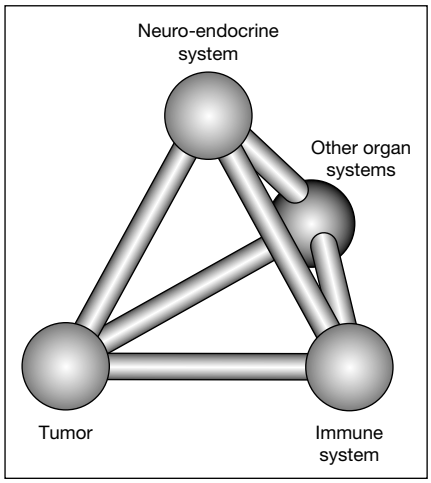

Fig. 2. Tetrahedron of the mutual interaction of a tumor with the neuro-endocrine system, the immune system, and other organ systems as well as their interaction likewise.

neuro-neoplastic synapse would deliver a more direct molecular explanation for the above-described observations on emotional influences. Furthermore, the tumor cells interact with the cells of the immune system, which sums up to a complex interaction triangle of a multi-directional regulation (fig. 2). In the following chapter, we will review the current knowledge on the function of tumor cells and leukocytes in response to neurotransmitters.

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Neurotransmitter Effects on Tumor Cells and Leukocytes

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Abstract

During the last 10 years new evidence has come to light which shows that the biology of neurotransmitters has expanded beyond their traditional role as chemical messengers, which is the release from a neuron, diffusion across a synaptic cleft, binding to and stimulation of a post-synaptic cell. These external signaling substances of the nervous system have been found to exert a strong influence on cells of the immune system and tumor cells. The latter express neurotransmitter receptors and several studies demonstrate the involvement of neurotransmitters in tumor cell progression and metastasis development. Besides their impact on the migration of lymphocytes, which is of primary importance for an anti-tumor response, neurotransmitters comprise a multitude of other immunomodulatory properties, which differ depending on the cell type and cell function. To illuminate the interplay between the nervous system, the immune system and tumor cells, we herein summarize in vitro and in vivo experiments on the effects of neurotransmitters on the migratory activity, proliferation and survival of tumor cells, as well as on the function of leukocytes.

The neuro-endocrine system is a superordinate organ in every higher animal, which regulates the function of the cells by the release of neurotransmitters and hormones, and it is also supposed to be the supreme regulator of immune and inflammatory reactions [1]. It is likely that tumor cells are susceptible to the same signal substances of the neuro-endocrine system as the normal cells of the tissue they descend from. In addition, tumor cells might acquire susceptibility to more of these substances or forfeit a certain sensitivity by genetic dysregulation in the course of the cancer disease.

Neurotransmitters are usually short-lived ligands to G protein-coupled receptors (GPCRs) with a wide structural variation. Groups of neurotransmitters are biogenic amines, peptides, normal and unusual amino acids, as well as

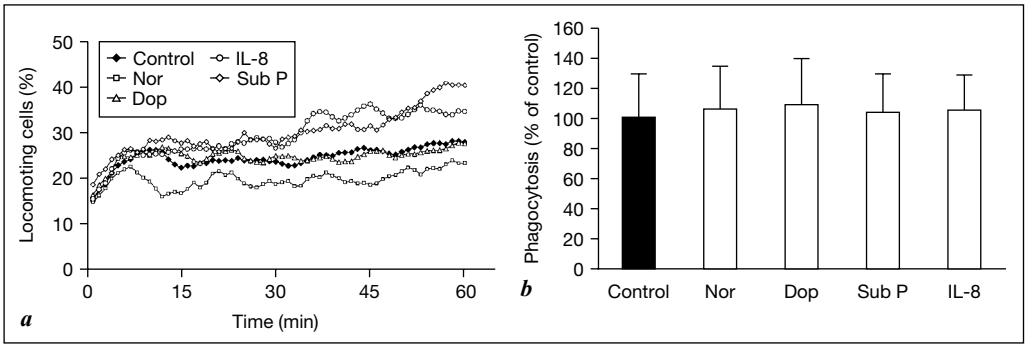

Fig. 1. Influence of chemokines and neurotransmitters on the migration (***a***) and phagocytosis (***b***) of macrophages. Dop = Dopamine; IL-8 = interleukin-8; Nor = norepinephrine; Sub P = substance P.

non-related molecules (e.g. acetylcholine or anandamide). The biogenic amines are metabolites of amino acids. They comprise the catecholamines norepinephrine and epinephrine as well as their metabolic precursor dopamine, which originates from the amino acid tyrosine via the intermediate dopa. Serotonin, also named 5-hydroxytryptamine, descends from the amino acid tryptophan, and histamine is a derivate of the amino acid histidine.

Biogenic Amines

Epinephrine, Norepinephrine and Dopamine

Catecholamines have strong, however, diverse impacts on the function of various leukocytes, and lymphatic organs are directly innervated by noradrenergic nerve fibers [2]. On the one hand, the migratory activity of cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells is enhanced by norepinephrine [3]. Furthermore, mice that are deficient in the dopamine β-hydroxylase, an enzyme which converts dopamine to norepinephrine, have an impaired T-cell function [4]. On the other hand, the cytotoxicity of NK cells [3, 5], the function of dendritic cells [6], as well as the formyl-methionyl-leucyl-phenylalanine-induced migration of neutrophil granulocytes [7] are strongly impaired. Norepinephrine reduces the migratory activity of macrophages, whereas dopamine has no effect, and interleukin-8 as well as substance P induce migratory activity (fig. 1a). Interestingly, neither the phagocytic activity nor the secretion of cytokines by macrophages were influenced by any of these substances (fig. 1b). As mentioned above, dopamine is the metabolic precursor of norepinephrine. Dopamine receptors are present on leukocytes, too [8]. T-lymphocyte migration is induced

by dopamine [9], whereas it attenuates the promigratory effect of interleukin-8 in neutrophil granulocytes [10]. In conclusion, the effects of dopamine occur to be qualitatively the same as of its metabolite norepinephrine.

Norepinephrine and dopamine are the strongest inducers of breast carcinoma cell migration [11], and norepinephrine stimulates the migration of human colon [12] and prostate carcinoma cells [11] as well. In athymic BALB/c nude mice, norepinephrine enhances the development of lumbar lymph node metastases from prostate carcinoma cells injected in the thighs [13]. In combination with the above-mentioned fact, that noradrenergic nerve fibers are present in the lymph organs, i.e. spleen, thymus, bone marrow, and lymph nodes [2], one might even argue that the localization of metastases can be driven by neurotransmitters. This view is supported by the clinical observation that certain tumors, such as the small cell lung carcinoma, frequently develop metastases in the relatively small, catecholamine-producing adrenal glands [14, 15], and in the brain [16, 17]. Interestingly, in both the immune system and in tumor cells, the effects of norepinephrine are mediated via β_2-adrenoceptors [18, 19]. Thus non-heart active, β_2-specific β-blockers might be pharmacological tools to inhibit metastasis formation of certain tumors or to modulate the function of leukocytes, whereas it seems a general tendency that the function of leukocytes from the lymphoid lineage is enhanced and of cells from the myloid lineage is reduced by catecholamines.

Histamine

This neurotransmitter is especially interesting, as it is found in the brain of vertebrates and invertebrates, and is released locally in inflammatory responses by mast cells. It is therefore released by cells of both the nervous system and the immune system. Histamine plays a central role in the symptoms of allergy and asthma. There are four receptors known for histamine (H1–4R), which are all GPCRs [20]. Several aspects of the distribution of these receptors and of the function of histamine in the adaptive immune response have recently been summarized in an article by Gutzmer et al. [21]: in brief, H1R and H2R are expressed on T-cells and dendritic cells, whereas they have different effects on Th1- and Th2-responses and dendritic cell functions. The H3R is predominantly expressed in the central nervous system, where it has an auto-inhibitory function on the release of histamine by the inhibition of pre-synaptic calcium channels [22]. Furthermore, H3R regulates the neurotransmitter release of cholinergic [23, 24], as well as of serotoninergic, dopaminergic and noradrenergic neurones [22, 25]. In contrast to H3R, H4R is mainly expressed in peripheral tissue, immune cells and lymph organs, respectively [26].

The role of histamine in cancer is still not clear, and several observed effects have been ascribed more to a dysregulation of the immune system than to a direct action on tumor cells. However, several histamine receptors are expressed

on tumor cells and it now seems clear that histamine indeed has a direct influence on tumor cell function. The balance of the expression of the four histamine receptors seems to be important for the mode of action therein [27]. Especially, the H1R is under investigation in tumor biology; engagement of the H1R leads to an increased proliferation in melanoma and carcinoma cells, and histamine acts as a chemoattractant via this receptor [28]. In contrast, in another study, H1R activation inhibited the proliferation of a prostate carcinoma cell line [29]. Thus, the role of histamine and the cellular main source – mast cells – in cancer is in controversial discussion, e.g. its function in neoangiogenesis, too [30].

Serotonin

Serotonin, also termed 5-hydroxytryptamine, is a metabolite of the amino acid tryptophan. Serotonin has an important role in the development and function of the central nervous system [31], and in the pathogenesis of neurological diseases [32], as well as in the innate and adaptive immune response [33]. Mast cells are a major source of serotonin at sites of inflammation, where it has impact on the maturation and cytokine release of dendritic cells [34]. Activated T-lymphocytes are able to synthesize and release serotonin, too. In turn, dendritic cells uptake and store serotonin and provide it to naïve T-cells [35]. Thus, serotonin is supposed to play a role in the communication of the immunological synapse. Furthermore, serotonin is a chemoattractant for eosinophil granulocytes [36].

Under certain conditions, prostate cancer cells can differentiate to neuroendocrine cells [37], which then produce a bunch of neurotransmitters including serotonin [38]. Already in 1994 Abdul et al. [39] described the growth-stimulating role of serotonin and suggested serotonin receptor antagonists as targets in prostatic carcinoma. Ten years later, Dizeyi et al. [40] elaborated on this topic, analyzed the receptor expression in prostate cancer tissue, and identified potential target receptors for the growth inhibition of such tumors [41]. However, serotonin seems to play a role not only in prostate cancer, but also in tumors of the bladder, colon, and lung [42].

It is noteworthy that several invertebrate venoms contain high amounts of serotonin, besides histamine and some peptides that mimic neuropeptides of the kinin family, e.g. bradykinin [43]. The latter will be discussed below in the chapter on inflammatory neuropeptides.

Amino Acids

Glutamate

Similar to serotonin, glutamate is an important neurotransmitter of the central nervous system. It plays a role in learning and memory, but also in

neuropsychiatric disorders [44], and drug addiction [45]. There is an amazing list of publications characterizing the role of glutamate in malignancies of the brain, but a potential role of glutamate in cancer outside the central nervous system has been recognized only recently [46]. Likewise, there are some reports that describe a modulating function of glutamate in the immune response of the central nervous system [47], but reports on the function of immune cells, especially in peripheral tissue, are rare. However, glutamate has been detected in macrophages, and its concentration increases upon activation of these cells [48].

γ-Aminobutyric Acid

γ-Aminobutyric Acid (GABA) is synthesized from glutamate by decarboxylation [49]. In contrast to glutamate, GABA is the major inhibitory neurotransmitter of the central nervous system: engagement of the chloride channels $GABA_A$ and $GABA_C$ increases the chloride conductance that inhibits neuronal firing [50, 51]. However, effects of GABA in non-neuronal tissue seem to be mainly mediated by the $GABA_B$ receptor, which is a G protein-coupled receptor. The norepinephrine-induced migratory activity of colon and breast carcinoma cells is inhibited by the engagement of this receptor [52, 53]. In the immune system, GABA functions as an inhibitor for the locomotor activity of the chemokine-induced migration of CTLs, whereas migratory activity of neutrophil granulocytes was not affected [54], and the cytotoxicity of NK cells seems to be slightly increased [55]. GABA has been found in macrophages too, but in contrast to glutamate, its concentration decreases upon activation of these cells [48].

Peptides

Peptides constitute a large group of neurotransmitters, which can be subgrouped by their function, localization, or source. However, other peptides are solitary in each of these aspects or could be put in more than one group; they are therefore discussed individually herein.

Substance P

Substance P is a peptide of the neurokinin family, localized in the central and peripheral nervous system; it plays a role in the regulation of affective behavior, in stress reactions, and in anxiety and depression. The pharmacological inhibition of the neurokinin-1 receptor, the preferential receptor for substance P, is an effective tool for the treatment of depressive disorders [56]. Substance P is involved in inflammatory processes, too [57]. The lung is richly supplied with nerves that secrete substance P [58], and this neurotransmitter might contribute

to the pathogenesis of asthma, because of its inflammatory effects on the airways [59]. Eosinophil granulocytes, macrophages, lymphocytes, and dendritic cells are able to produce substance P and neurokinin A, which binds to neurokinin receptors, too [60]. Furthermore, substance P influences the migration of neutrophil granulocytes across endothelial and subendothelial barriers towards inflammatory sites of the lung, thereby regulating their interstitial accumulation and traffic to the alveolar space [61, 62], and it stimulates the migratory activity of macrophages with the same potency as interleukin-8 (fig. 1a).

Substance P is expressed in tumor tissue of several types of tumors [63]. Furthermore, substance P induces the migration of colon [54] and breast [53] carcinoma cells and is a chemoattractant for small cell lung carcinoma [64]. In breast carcinoma cells, the promigratory effect is mediated via the neurokinin (NK)-1 receptor, as we have shown by the use of the specific receptor blocker L-733,060 [11]. The role of substance P in carcinogenesis and tumor progression has recently been reviewed by Esteban et al. [65], and in accordance with our results, the authors suggest the use of NK-1 receptor blockers for the treatment of cancer.

Angiotensin

The decapeptide angiotensin I is cleaved from angiotensinogen by renin. In a second step, the biologically most active form angiotensin II is then generated by the cleavage of two further amino acids by the angiotensin-converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor and has an integral role in the aldosterone regulation, which in turn regulates the renal retention of sodium and water [66, 67]. Angiotensin II then breaks down to angiotensin III – which has less biological activity – by the loss of the aminoterminal asparagic acid. Inhibitors of the ACE represent a well-established group of pharmacological substances in use for the regulation of blood pressure in hypertension and congestive heart failure. Captopril, one of these ACE inhibitors has also been shown to be a potent inhibitor of neovascularization [68]. Furthermore, direct blockade of the angiotensin II type 1 receptor inhibited tumor angiogenesis and led to a reduced growth of melanoma cells engrafted in mice [69]. These results are supported by the epidemiological evidence, that the long-term use of ACE inhibitors protect against cancer [70]. These and other functions of ACE in cancer, e.g. the role in metalloproteinase-regulation, have been reviewed by Lindberg et al. [71].

ACE is also expressed in leukocytes, namely dendritic cells and activated macrophages [72], and it is discussed to be expressed in T-lymphocytes [73, 74]. The latter would explain, why angiotensin I – which is generally regarded as physiologically almost inactive – inhibits the chemokine-induced migration of CTLs with the same potency as angiotensin II [75]. In contrast, angiotensin II is an inducer for the migration of monocytes [76], which supports the

above-described observation that neurotransmitter-effects may be opposite in the lymphoid and the myeloid lineage.

Inflammatory Neuropeptides

More than 100 years ago, Rudolf Virchow deduced from his observations the hypothesis, that cancer is linked with inflammatory processes. Balkwill and Mantovani [77] have elaborated on this functional interconnection and described the role of cytokines and chemokines derived from the immune system in tumor growth and progression, also referring to the idea of Dvorak [78] that tumors are wounds that do not heal. With the aforementioned examples on histamine and substance P, we have already described two neurotransmitters, which are involved in inflammatory processes and have effects on tumor cell functions. Besides these substances, there are further neurotransmitters, which are involved in inflammation, therefore collectively termed as inflammatory neuropeptides, i.e. bradykinin and the calcitonin gene-related peptide (CGRP). Bradykinin leads to increased vascular permeability, vasodilation, and contraction of various smooth muscles [79]. The B2 receptor is constitutively expressed, predominantly in smooth muscle cells [79], whereas the B1 receptor is underexpressed in normal tissues and upregulated during inflammatory responses [80]. The B1 receptor has a higher affinity for the metabolite des-Arg9-bradykinin than for bradykinin [81]. Bradykinin chemotactically recruits neutrophil granulocytes to sites of inflammation [82], and neutrophil granulocytes can themselves produce kinins from plasma kininogens [83]. Furthermore, bradykinin increases pain sensitivity by reducing the activation threshold of nociceptor neurons [84]. Interestingly, wasp and hornet venoms contain peptides, which have some properties of bradykinin [43]. This might contribute to the certain pain of these insect stings.

The B2 receptor is expressed in human benign and malignant prostate specimens, but the B1 receptor is detected only in prostatic intraepithelial neoplasia and malignant lesions and not in benign prostate tissues [85]. Specific stimulation of endogenous B1 receptor promotes growth, migration, and invasion of PC-3 prostate cancer cells [85]. Furthermore, bradykinin stimulates the proliferation of normal breast epithelial cells and their malignant counterparts [86].

CGRP is a vasodilating peptide that occurs in the central and peripheral nervous system; this neurotransmitter is present in nerve endings in lymphoid organs, too [87, 88]. CGRP directly regulates the differentiation and function of B- and T-lymphocytes by the engagement of specific receptors on these cells [87, 89], e.g. it is a chemotactic mediator for T-lymphocytes [90]. In macrophages and dendritic cells CGRP modulates the antigen presentation [91], and it has a promigratory effect on neutrophil granulocytes [92].

CGRP stimulates the migration of the F9 teratocarcinoma cells, and is therefore supposed to play a role in embryonic development [93]. Likewise, CGRP

stimulates the migration of cells of the prostate cancer cell line PC-3 [94]. Furthermore, CGRP might play a role in tumor-induced hyperalgesia, as was shown by the blockade of the specific CGRP-receptors [95]. Adrenomedullin is structurally related to CGRP and has a broad range of physiological and pathological effects. It has various effects on vascular endothelial cells [96], and functions as an anti-microbial peptide [97]. Besides this, adrenomedullin has been implicated in several diseases including cancer [98], where it plays a role in angiogenesis [96, 99].

Opioid Peptides

Pro-opiomelanocortin (POMC) is the 91 amino acid precursor of several neuropeptides, such as adreno corticotropic hormone (ACTH), alpha-melanocyte-stimulating hormone (α-MSH), and the opioid peptides, which are the endorphins, enkephalins and dynorphins. All these peptides are generated from POMC by post-translational cleavage, e.g. β-endorphin – the most potent analgetic peptide – consists of the amino acids 61–91. One source of opioid peptides are circulating leukocytes, which migrate to inflamed tissues. Under stressful conditions or in response to releasing agents (e.g. corticotropin-releasing factor, cytokines, norepinephrine), leukocytes can secrete the opioid peptides β-endorphin [100], as well as met-enkephalin and dynorphin A [101]. Thereby, they activate peripheral opioid receptors and cause analgesia by inhibiting the excitability of sensory nerves and/or inhibiting the release of excitatory neuropeptides. Thus, the immune system takes part in the balanced control of pain [100, 102, 103]. In turn, endogenous and exogenous opioids are known to exert direct effects on the immune system and the expression of functional opioid receptors has been reported for several immune cell types. Met-enkephalin has a stimulatory effect on neutrophil function by increasing their oxidative burst activity [104]. Dynorphin A suppresses the capacity of dendritic cells to induce T-cell proliferation, however antigen uptake as well as phenotypic maturation of the dendritic cells are not influenced [105]. Furthermore, in mouse peritoneal macrophages dynorphin A enhances phagocytosis, whereas other opioid peptides have not such an effect [106]. Opioid peptides may also play a role in the negative selection process during T-cell development, since the delta-opioid receptor-1 as well as the respective enkephalins are expressed during maturation of T-cells [107].

Besides the opioid peptides, other cleavage products of POMC have effects on leukocytes. α-MSH induces cell death in mast cells [108], and suppresses antigen-induced lymphocyte proliferation [109]. ACTH was one of the first neuropeptides shown to bind to receptors on leukocytes and modulate immune responses. Generally, ACTH inhibits immune responses, but for example enhances the T-lymphocyte cytotoxic response [110].

Met-enkephalin, but not β-endorphin, is a strong stimulator for the migration of MDA-MB-468 breast carcinoma cells [53]. Both of these substances bind to the δ- as well as the μ-opioid receptors, however, met-enkephalin has a higher affinity for the δ-opioid receptor, and β-endorphin binds to both receptors with similar affinity. This might explain the differences of the observed effects of these two substances [75]. In contrast to its stimulatory effect on tumor cell migration, met-enkephalin inhibits tumor cell proliferation by an arrest in G0/G1 phase [111].

In melanoma cells, α-MSH reduces cell migration and invasion, which are essential for metastasis formation [112]. Furthermore, α-MSH inhibits the TNF-α-stimulated migration of the human melanoma cell line HBL [113], and reduces uveal melanoma invasion through fibronectin [114].

Gastrointestinal Peptides

The gastrointestinal peptides cholecystokinin (CCK) and gastrin are released from certain parts of the brain (i.e. the neocortex and the hypothalamus, respectively), as well as from gastrointestinal endocrine cells of the stomach and upper small bowl. They are involved in the peristalsis and secretion of digestion enzymes as well as of gastric acid [115]. Histamine via the H2 receptor, and acetylcholine via the muscarinic-3 acetylcholine receptor, are able to stimulate acid secretion of gastric parietal cells directly, whereas histamine has a permissive effect for gastrin [115]. Gastrin and CCK bind to two related receptors, which have different expression patterns: the CCK-A receptor is expressed in pancreatic acinar cells, and the CCK-B receptor is mainly expressed in the stomach [116]. About 50% of all gastric carcinoma tissues show a high expression of gastrin and gastrin receptors. Those patients with diffuse-type gastric carcinoma tissues expressing both gastrin and gastrin receptor have a poorer prognosis than those negative for both, which suggests that gastrin acts as an autocrine growth factor in a subgroup of gastric carcinomas [117]. Furthermore, CCK has been demonstrated to regulate the invasiveness of human pancreatic cancer cell lines [118], and it is able to induce the chemotaxis of monocytes [119].

The gastrin-releasing peptide (GRP) has, besides its name-giving ability to stimulate the release of gastrin, several other functions, including not only the secretion of gastrointestinal neurotransmitters, but also effects on the smooth muscles of the stomach and gallbladder as well as on the intestinal transit [120]. GRP receptors are expressed and GRP functions as a growth factor in a wide range of cancers not only from gastrointestinal sites, e.g. lung, prostate and breast cancer as well as melanoma [54, 121]. Besides its effect as a growth factor, GRP has also been shown to act on angiogenesis, cell migration and cell adhesion [122]. For example, GRP induces angiogenesis and the specific GRP blocker 77,427 inhibits tumor growth in lung cancer in vitro and in vivo [123].

Furthermore, bombesin, a homolog of GRP derived from the skin of the frog *Bombina bombina*, was shown to stimulate the expression of proangiogenic factors in prostate cancer cells [124], and in human experimental breast cancers bombesin antagonists inhibit the expression of these factors [125]. Bombesin stimulates the migration of colon carcinoma cells [126] and prostate cancer cells in a Rho dependent manner [127]. With regard to the immune system, bombesin and GRP dose-dependently inhibited maturation of dendritic cells and inhibited interleukin-12 production by dendritic cells and their ability to activate T-lymphocytes [128]. In mice, bombesin and GRP have a chemoattractive effect on macrophages and lymphocytes [129], and stimulate the cytotoxicity of CTLs and NK cells [130].

The vasoactive intestinal polypeptide (VIP) retards the gastric emptying [131], and is thus a functional antagonist for GRP and gastrin. Serum levels of VIP are frequently increased in patients with pancreatic and colon cancer [132], and the receptor $VPAC_1$ for VIP is highly expressed in various tumors, i.e. breast, prostate, colon, lung, and bladder carcinomas [133]. Thus, VIP is supposed to act as a potential growth factor in these tumors. VIP is an autocrine growth factor in lung cancer [134], and stimulates the proliferation of human H9 lymphoblastoma cells [135]. VIP can protect prostate cancer cells from apoptosis by phosphorylating the pro-apoptotic protein Bad [136]. In turn, VIP receptor antagonists inhibit the growth of glioblastoma cells [137], and $VPAC_1$ receptor antagonists reduce the mammary tumor burden in C3(1)SV40Tag mice [138].

The VIP receptors $VPAC_1$ and $VPAC_2$ are both expressed on T-lymphocytes, and VIP has in general an immunosuppressive, anti-inflammatory function, whereas effects on T helper1 and T helper2 cells differ [139]. T helper2 cells produce VIP after activation and have the greatest functional responses to VIP [140, 141]. VIP promotes T helper2 cell responses and reduces T helper1 cell responses [142, 143]. In the myeloid part of the immune system, VIP inhibits the production of inflammatory cytokines and chemokines from macrophages and dendritic cells [142], and impairs the chemotaxis of monocytes [144].

Somatostatin is a further gastrointestinal neurotransmitter with a predominantly inhibitory function. Like VIP, this neurotransmitter inhibits gastric emptying [145], the release of gastrin [146], and acid secretion [147]. The five receptors for somatostatin are widely expressed in normal and tumor tissues [148, 149], as well as on leukocytes [150]. The expression of somatostatin receptors on human T-lymphocytes depends on their differentiation and activation status, but they produce no somatostatin themselves. Somatostatin regulates lymphocyte functions, e.g. adhesion to extracellular matrix components via distinct somatostatin receptor subtypes [151]. Somatostatin is an inhibitor for the chemokine-induced migration of T-lymphocytes [152], but stimulates the migration of neutrophil granulocytes [153]. In tumor cells, somatostatin

seems to have over all a growth-inhibitory effect [149], although the expression of somatostatin receptors in breast cancer is positively correlated with the tumor size [154]. In pancreatic cancer, the expression of proangiogenic factors is inhibited by engagement of the somatostatin receptor subtype 2 [155].

Endothelins are a group of peptides with vasoconstrictive effects, which are released by endothelial cells as well as by smooth muscle cells and leukocytes [156]. Endothelins are thus not especially gastrointestinal neuropeptides, however, they play an important role in the regulation of contraction and relaxation of the esophagus, stomach, ileum and colon [157]. Furthermore, endothelins regulate growth in several normal cell types and various kinds of cancer [158]. For example, an (over)expression of endothelins and the according receptor promotes the invasive potential in ovarian [159], breast [160], and prostate cancer cells [127].

The endothelins-1 and -4 stimulate the production of cytokines (e.g. interleukin-1 and -6, TNF-α, and GM-CSF) in monocytes [161], and endothelin-2 is a chemoattractant for macrophages, but not for freshly isolated monocytes [162]. Endothelin-1 is an important autocrine or paracrine factor for the normal maturation and function of human dendritic cells [163].

Non-Related Neurotransmitters

Acetylcholine

Acetylcholine is found in animals, plants and bacteria [164]. In humans, acetylcholine is an important neurotransmitter, but it also plays a role in non-neural tissues including epithelial and endothelial cells as well as leukocytes [164]. Receptors for acetylcholine are divided in two groups according to their binding of the plant alkaloids nicotine and muscarine [165]. Nicotinic acetylcholine receptors (nAChR) are pentameric cation channels, and muscarinic acetylcholine receptors (mAChR) are GPCRs. Lymphocytes express both of these receptor subtypes, contain and release acetylcholine, and express the choline acetyltransferase which is the enzyme that generates acetylcholine [166, 167]. Acetylcholine regulates the function of NK cells and lymphocytes [168]. Engagement of nAChR leads to the release of the proinflammatory chemokine interleukin-8 from neutrophil granulocytes [169], and leads to proinflammatory responses in macrophages [170], although the cholinergic system in leukocytes is in general supposed to have an anti-inflammatory function [171], and blocks endothelial cell activation [172].

Hildegard Schuller has in the chapter ‘Neurotransmitter Receptor-Mediated Signaling Pathways as Modulators of Carcinogenesis’ of this book provided detailed insight into the role of nAChRs, especially the α_7nAChR, in lung cancer. mAChRs are present on lung cancer cells, too [173], and in contrast

to the mitogenic signaling of the nAChR, the m3AChR has an anti-proliferative function in lung cancer cells, and furthermore increases E-cadherin-mediated adhesion [173]. However, acetylcholine is also expressed in lung cancers and functions as an autocrine growth factor [174]. Thus, if the results are validated that the acetylcholine receptors subtypes have opposite effects on lung cancer cell proliferation, the balanced signaling seems to privilege the pro-mitogenic function of the nicotinic receptors. In contrast, the m3AChR promotes the growth of colon cancer cells [175]. Interestingly, the m3AChR is expressed in normal prostate tissue and well-differentiated tumors, whereas less-differentiated tumors show a loss of m3AChR expression [176], and the expression of mAChR in melanoma plays a role in the tumor progression towards infiltration and metastasis formation [172].

Anandamide

Cannabinoids are compounds of the plant *Cannabis sativa*, of which the δ_9-tetrahydrocannabinol is the most psychoactive substance upon intake. The two cannabinoid receptors CB_1-R and CB_2-R have been identified in 1988 [177], and 1993 [178], respectively, and the natural ligand for the CB-Rs, termed anandamide from the Sanskrit word 'ananda' for bliss and the amide-containing structure, has been isolated in 1992 by the same group, which has identified the CB_1-R before [179]. Anandamide is a derivate of the arachidonic acid and binds to CB_2-R with higher affinity than to CB_1-R [180]. The CB_2-R was initially identified in macrophages in the margin zone of the spleen [178]. Since then, both CB-Rs have been found in several lymph organs and leukocyte subpopulations, and cannabinoids are modulators for the immune response, especially with regard to the balance between T helper1 and T helper2 lymphocytes [181, 182]. The migration of CTLs is inhibited by CB_2-R engagement, whereas the migration of SW 480 colon carcinoma cells is inhibited via the CB_1-R [183]. Likewise, anandamide inhibits the proliferation of human breast, colon and prostate cancer cells via the CB_1-R [184, 185], suggesting the use of cannabinoids, or in special CB_1-R agonists as anti-cancer agents.

Conclusion

In conclusion, nerve cells as well as tumor and immune cells are able to produce various neurotransmitters which then differentially regulate a plethora of immune cell functions and e.g. the migratory activity and proliferation rate of tumor cells (see table 1). The understanding of this expanded role of neurotransmitters in tumor biology and during the immune response may illuminate new avenues for novel therapeutic interventions of cancerous disease.

Table 1. Effects of neurotransmitters on leukocytes and tumor cells

Neurotransmitter	Structure	Leukocytes	Tumor cells
Norepinephrine and epinephrine	Biogenic amine	Modulates T-cell and NK cell function [5, 55], affects macrophage migration	Stimulates migration of carcinoma cells [11, 12] and lymph node metastases development
Dopamine	Biogenic amine	Stimulates T-cell migration attenuates chemoattractant effect of IL-8 in neutrophils	Increases breast carcinoma cell migration
Histamine	Biogenic amine	Different effects on T-cell and DC function	Induces proliferation and chemotaxis of carcinoma and melanoma
Serotonin	Biogenic amine	Role in the communication of the immunological synapse [34, 35]	Tumor growth stimulation [39, 41]
γ-Aminobutyric acid	Amino acid	Inhibits chemokine-induced migration of CTLs	Inhibits norepinephrine-induced locomotion of colon and breast carcinoma cells [52, 53]
Substance P	Peptide	Modulates migration of neutrophils and macrophages	Role in cancer promotion and progression
Angiotensin	Peptide	Inhibits chemokine-induced locomotion of CTLs stimulates migration of monocytes	Increases tumor growth and angiogenesis
Bradykinin	Inflammatory neuropeptide	Neutrophil chemotaxis	Promotes proliferation and invasion of prostate cancer cells
Calcitonin gene-related peptide	Inflammatory neuropeptide	Regulates B- and T-cell function [87, 89], modulates antigen presentation by macrophages and DCs [91]	Stimulates motility of prostate cancer cells [94]
α-Melanocyte-stimulating hormone	Opioid peptide	Cell death in mast cells suppress lymphocyte proliferation [109]	Reduces migration and invasion of melanoma [112–114]

Table 1. (continued)

Neurotransmitter	Structure	Leukocytes	Tumor cells
Endorphins, enkephalins and dynorphin	Opioid peptide	Stimulate phagocytotic activity of neutrophils and macrophages [104, 106]	Inhibit cell cycle progression induce breast carcinoma motility [53]
Cholecystokinin and gastrin	Gastrointestinal peptide	Induce monocyte chemotaxis [119]	Autocrine growth factor in gastric carcinoma [117]
Gastrin-releasing peptide (bombesin)	Gastrointestinal peptide	Inhibits maturation of DCs stimulates cytotoxicity of CTLs and NK cells [130]	Stimulates tumor growth [54, 121]
Vasoactive intestinal peptide	Gastrointestinal peptide	Immunosuppressive [142, 144]	Growth factor in tumors [134, 135]
Somatostatin	Gastrointestinal peptide	Inhibits chemokine-induced migration of T-cells induces neutrophil locomotion [153]	Inhibitor of tumor growth [148, 149]
Endothelins	Gastrointestinal peptide	Stimulates production of cytokines in monocytes function and survival of DCs [163]	Promote invasive potential of cancer cells [159, 160]
Acetylcholine	Non-related neurotransmitter	Autocrine/paracrine factor for regulating immune function [168, 170, 171]	Opposite effects on cancer cell proliferation [173–175]
Cannabinoids and anandamide	Non-related neurotransmitter	Modulators for immune response [181, 182, 186]	Anti-proliferative effects [184, 185, 187]

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Stem Cells and Neurogenesis in Tumors

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Abstract

Bone-marrow-derived and tissue-resident stem cells promote repair of injured tissues by contributing to new blood vessel, muscle and nerve formation. These same stem cells may contribute to tumor growth and spread. Tumors express numerous growth factors that induce both angiogenesis and neurogenesis; these factors may also induce tissue-resident stem cell recruitment and differentiation. Tumors also recruit circulating bone-marrow-derived stem or progenitor cells, which play roles in promoting tumor growth and spread. As innervation of tumors promote cancer pain and can contribute to tumor spread, an understanding of the roles of stem cells in tumor innervation will assist in the development of new cancer therapies.

Tumors are comprised of aberrantly proliferating aneuploid cells that are surrounded by normal diploid cells from the local microenvironment. These immune cells, fibroblasts, endothelial cells, vascular smooth muscle cells and neuronal cells contribute to angiogenesis, lymphangiogenesis and neurogenesis within the tumor microenvironment. A number of factors present in tumors promote the growth and guidance of both endothelium and neuronal cells, including basic fibroblast growth factor (bFGF) [1], brain-derived neurotrophic factor (BDNF) [2], vascular endothelial growth factor (VEGF) [3], nerve growth factor (NGF) [4], neuropilins [5], and others [1–5]. These factors promote the development of new blood vessels (angiogenesis) and nerves. Circulating bone-marrow-derived stem cells also contribute to tumor neovascularization and neurogenesis [6].

Tumors may initiate own innervation through release of neurotrophic factors [7]. Neuronal cell growth from neighboring tissues or upon recruitment of circulating bone-marrow-derived stem cells contributes to the innervation of tumor tissue. This chapter will review current literature on the roles of stem cells in tumor innervation and the similarities between neurogenesis and angiogenesis.

Innervation of Tumors

Tumors express a number of neurotrophic factors that stimulate their own innervation [1–5]. A key consequence of tumor innervation is cancer pain. Another consequence is tumor spread, or metastasis, along neural networks. Key neurotrophic factors such as NGF [4], artemin [8], netrin [9–10], BDNF [2] and even VEGF [3] play important roles in this process. For example, NGF promotes cancer pain; an anti-NGF function-blocking antibody suppresses skeletal pain induced by prostate tumor cells growing in bone [11]. Tumor necrosis factor alpha, a key factor produced by most tumor cells, also stimulates neuropathic pain [12].

Neuronal innervation also promotes tumor spread along axons. A number of neurotrophic factors promote tumor invasion and metastasis. For example, netrin promotes mammary epithelial cell invasion and migration [9]. Netrin-1 also promotes tumor cell survival through its receptor Deleted in Colon Carcinoma [10]. Another neurotrophic factor, artemin, promotes pancreatic cancer cell invasion along pancreatic nerves [8]. BDNF may also stimulate spread of tumors along nerves [2, 13, 14]. Increased BDNF is observed in several tumors, including orthotopic hepatocellular carcinoma, multiple myeloma, and neuroblastoma, where it is a marker of a poor prognosis [13]. This neurotrophic factor promotes migration and growth of multiple myeloma cells [14]. It also activates TrkB, which stimulates VEGF expression in neuroblastoma cells [2]. Thus, neurogenesis within tumors contributes significantly to cancer pathology.

Similarities between Tumor Angiogenesis and Innervation

Neovascularization, the formation of blood vessels, plays important roles in development, inflammation, and wound repair. Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100–200 μm of blood vessels, which is the diffusion limit of oxygen. New blood vessels typically arise from pre-existing vessels by activation, proliferation and migration of endothelial cells through a process named ‘angiogenesis’ [15]. Specific growth factors, such as VEGF and bFGF stimulate the proliferation and migration of quiescent endothelial cells in pre-existing blood vessels, resulting in the formation of new vessels during embryonic development and tumor growth [15]. Vasculogenesis, or the coalescence of new blood vessels from individual endothelial progenitor cells, also occurs in tumors [16]. Additionally, myeloid lineage cells such as monocytes and macrophages can modulate tumor angiogenesis and vasculogenesis.

Importantly, a number of similarities between the development of vascular networks and neural networks have been recently characterized. Both tissues form branching networks that are regulated by guidance factors and cytokines such as semaphorins [17], plexins [17], neuropilins [5] and VEGF [3]. Both can arise from nearby tissues or by the homing of circulating bone-marrow-derived stem cells. In some tissues, neuronal cells guide endothelial cells so that newly forming vessels coordinately track along recently migrated neuronal cells. This is especially clear in the developing retina in which astrocytes guide the newly migrating endothelial cells [18].

Neurotrophic Factors that Promote Angiogenesis

A number of neurotrophic growth factors promote both neurogenesis and angiogenesis. These factors include NGF [4], BNDF [2], semaphorins [17], plexins [17] and neuropilins [5]. Besides stimulating neurite outgrowth, NGF promotes angiogenesis in a quail chorioallantoic membrane model of angiogenesis [4]. Semaphorins and their receptors, the plexins and neuropilins, regulate guidance of neurons as well as guidance of new blood vessels [17]. The NGF receptor (tropomyosin related kinase – TrkA) has been shown to play a key role in angiogenesis [16]. VEGF has been shown to promote neuronal survival [3]. Semaphorin D provides a link between axon guidance and angiogenesis in tumors. It is expressed by invading cells of head and neck squamous cell carcinomas, breast carcinomas, prostate, and lung and stimulates endothelial cell migration as well as neurite outgrowth. Knockdown of Semaphorin D expression inhibits tumor vascularization [19].

Angiogenic Factors that Promote Neuronal Outgrowth

A number of clinical observations suggest that angiogenesis as well as angiogenic factors promote neurogenesis. For example, brain injury due to seizures or cerebral ischemia stimulates angiogenesis, but it also stimulates neurogenesis [20, 21]. Neurogenesis is also observed in patients with Huntington's disease (HD) [22], Alzheimer's [23], and Parkinson's [24] and in animal models of HD, Alzheimer's and Parkinson's [25]. Angiogenic factors promote neurogenesis; when HD transgenic R6/2 mice and wild-type mice were treated by subcutaneous administration of bFGF, 5-fold more proliferating cells were observed in the subventricular zone in HD mice than in wild-type mice. bFGF also induced the recruitment of new neurons from the subventricular zone into the

neostriatum and cerebral cortex of HD mice and blocked cell death in primary striatal cultures [1].

VEGF also promotes neuronal survival. This key angiogenic factor promoted neuronal survival in a model of diabetic sensory neuropathy [26]. VEGF also protects neurons from hypoxia-induced apoptosis by activating Akt and ERK [27]. Reduced cerebrospinal fluid levels of VEGF have been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS), suggesting a possible role for VEGF gene regulation in the pathogenesis of ALS [28]. Intracerebroventricular delivery of VEGF suppressed motor neuron degeneration in a rat model of ALS [29]. Additionally, intracerebroventricular delivery of recombinant VEGF in a SOD1(G93A) rat model of ALS delays onset of paralysis by 17 days, improves motor performance and prolongs survival by 22 day [30]. Similar delivery of VEGF improves sensory and cognitive neural functions after focal cerebral ischemia [31].

Additional factors that promote both neurogenesis and angiogenesis include sphingosine-1-phosphate. Mice lacking S-1-P receptor exhibited failure to close neural tube and defective embryonic angiogenesis [32]. Together these studies show that the regulation of angiogenesis and neurogenesis are closely intertwined.

Bone-Marrow-Derived Stem Cells in Tumors

Bone-marrow-derived, $CD34^+$ stem or progenitor cells have been shown to promote the repair of damaged tissues, offering promise for the treatment of hereditary and acquired human diseases. These cells differentiate into endothelium, hematopoietic cells, and some studies report, into neurons, fibroblasts and muscle [16]. $CD34^+CD133^+$ progenitor cells participate in neovascularization by differentiating into endothelial cells [33–35]. Neovascularization stimulates healing of injured tissues, but also promotes tumor growth and inflammatory disease [15]. A number of studies indicate that bone-marrow-derived cells infiltrate tumors and directly participate in neovascularization [33–35], giving rise to approximately 15% of the neovasculature [37].

Other studies have shown that bone-marrow-derived cells of the myeloid lineage cell also home extensively to tumors and other neovascular or repairing tissues [38]. Macrophages express growth factors such as VEGF that stimulate angiogenesis [38].

Analyses of human sex-mismatched bone-marrow transplantation patients provided evidence that endothelial cells do arise from bone-marrow in humans. In one study, a small percentage of vasculature of sex mismatched transplant patients was derived from the transplanted bone-marrow. When patients were analyzed on

average 1 year after transplantation, 2% of all endothelial cells arose from the donor bone-marrow [39]. In another study of human sex-mismatched bone-marrow transplant recipients who later developed tumors, fluorescence in situ hybridization analysis showed that approximately 5% of endothelial cells infiltrating tumors were derived from bone-marrow [40]. Thus, experimental and clinical data confirm the existence of bone-marrow-derived endothelial progenitors.

Recent studies indicate that bone-marrow-derived stem cells also promote neurogenesis. Mesenchymal stem cells transfected with glial-derived neurotrophic factor promoted recovery from ischemia after cerebral artery occlusion [41]. Granulocyte colony stimulating factor and stem cell factor both promoted neurogenesis after focal cerebral artery occlusion in mice in part by mobilizing bone-marrow-derived stem cells into the brain where they appeared to differentiate into neuronal cells [42]. In additional studies, damaged skeletal muscle recovered function through synchronized vasculogenesis, myogenesis and neurogenesis after transplantation of $CD34^+CD45^-$ cells [43]. In one key study, $CD34^+$ stem cells were used to promote neurogenesis after stroke in animal models. Surprisingly, rather than directly stimulating neurogenesis, $CD34^+$ cells promoted angiogenesis, indirectly improving neuronal function [44].

Additional studies support a common lineage of precursors for endothelial cells, neuronal cells and hematopoietic cells. The Zebrafish 5′ stem cell leukemia (scl) gene encodes a basic helix loop helix transcription factor that is essential for angiogenesis and hematopoietic cell specification in the zebrafish embryo. In studies by Jin et al. [45], an upstream genomic DNA fragment containing the scl promoter was sufficient to drive expression of EGFP in endothelial cells, hematopoietic cells and in the brain and spinal cord, suggesting the existence of common precursor cells for these distinct cell types.

Our lab has recently identified a molecular mechanism that promotes the homing and recruitment of bone-marrow-derived progenitor cells to remodeling tissues. We found that integrin $\alpha_4\beta_1$ promotes the homing of circulating bone-marrow-derived progenitor cells to the $\alpha_4\beta_1$ ligands, vascular cell adhesion molecule and cellular fibronectin, which are expressed on neovasculature of tumors and other repairing tissues [46]. By regulating the homing of these cells, this integrin also promotes their participation in angiogenesis and tumor growth. In addition, our studies have shown that integrin $\alpha_4\beta_1$ also promotes the homing of myeloid lineage cells to tumors [38].

Conclusions

Tumors express growth factors that induce both angiogenesis and neurogenesis, leading to tumor growth, tumor invasion and tumor pain. Tumors also

recruit circulating bone-marrow-derived stem or progenitor cells, which can differentiate into endothelial cells or neuronal cells, thereby participating in cancer pathogenesis. Further investigation into the roles of stem cells in tumor innervation will assist in the development of new cancer therapies.

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Potential Inhibition of the Neuro-Neoplastic Interactions: The Clue of a GPCR-Targeted Therapy

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Abstract

Other sections of this monograph, dedicated to neuronal activities in tumor tissue, have highlight the chief influence of neurotrophins, neurotransmitters, adhesion, guidance molecules and different nerve cell markers in the progression, but also for the prognostic, therapy and survey of cancers. The G-protein-coupled receptors (GPCR) are among the most successful and promising target proteins for drug discovery and therapeutic research. GPCR are frequently overexpressed in cancer cells, an interesting property for tumor imaging or for a targeted radiotherapy, using radiolabeled ligand derivatives. The tumor microenvironment contains a number of GPCR ligands (e.g., bioactive peptides, biogenic amines, purins, chemokines), known to regulate the proliferation, migration or survival of both tumoral and neural cells and that may be key actors of the neuro-neoplastic interactions. Here will be reviewed the potential utilization of substances that target a selected choice of GPCR, especially neuropeptide receptors, for a novel concept of therapy, concerning the numerous types of cancers where neurons infiltrate the tumoral mass or those where the malignant cells invade nerve branches (perineural invasion). Some molecular mechanisms linked to these GPCR (or linking GPCR to other types of membrane receptors or co-receptors), involved in these processes, will also be considered.

Introduction

GPCR and Their Ligands in the Tumor Microenvironment

Cancer progression is highly dependent on tumor microenvironment cell and molecular factors: stromal fibroblasts, infiltrating immune cells, vascular and perivascular tissues and the extracellular matrix. Neoplastic tissue also

release growth and differentiation factors that cause vascularization (neoangiogenesis) and development of lymph vessels (lymphangiogenesis) in the tumor. These phenomena are indispensable for the progression and spreading of virtually all types of cancers. As a matter of fact, a majority of drugs presently developed or approved to target the tumor microenvironment are designed to cut down vascularization or inflammation [1]. More novel is the hypothesis that some tumors may express soluble or cell-to-cell signaling components that could initiate their own innervation, a process called neoneurogenesis [2]. Furthermore, in a number of peripheral cancers, malignant cells have been described to invade nerve branches, a process called perineural invasion (PNI) [3, 4]. These situations indicate the importance of neuro-neoplastic interactions for tumor progression. Other sections in this book dedicated to neuronal activities in tumor tissue, have highlighted the chief influence of neurotrophins, neurotransmitters, adhesion, guidance molecules and different nerve cell markers in the progression, but also for the prognostic, therapy and survey of cancers. A number of signaling components and mechanisms link the interdependent fate of the various tissues cooperating within the tumoral mass, which renders the etiology of cancer so complex and multifactorial. G-protein-coupled receptors (GPCRs) are frequently overexpressed in cancer cells, an interesting property for tumor imaging or for a targeted radiopharmaceutical therapy, using radiolabeled ligand derivatives [5–7]. The tumor microenvironment undoubtedly contains a number of GPCR ligands, such as bioactive peptides, biogenic amines and other neurotransmitters, purins, chemokines, potentially involved in the regulation of cancer cells behavior (fig. 1). The action of these molecules on tumor cell migration and metastasis has been extensively reviewed by Entschladen et al. [8–10]. These compounds can be released by the tumor cells themselves, especially those expressing a neuroendocrine phenotype, but also by numerous other cell types, such as fibroblasts, nerves, neuroendocrine, endothelial cells, and different actors of the immune system, such as macrophages, dendritic cells or granulocytes. GPCR are among the most successful target proteins for drug discovery research to date and there is a number of marketed drugs that target these receptors [11, 12]. Here will be reviewed potential therapeutic utilization of some of these drugs to target specific GPCR that are expressed in tumor cells, but sometimes also in the surrounding nerves. Such compounds could inhibit the related false or exacerbated intercellular communication mechanisms involved in neuro-neoplastic interaction, and cut-down both the tumor and nerve progression, with more limited interference with the normal cell functions and machinery. This type of targeted-therapy could lead to novel concepts of molecular medicine, for the numerous types of cancers where neurons infiltrate the tumoral mass or conversely, those where the malignant cells invade nerve branches. Since modulation of carcinogenesis by

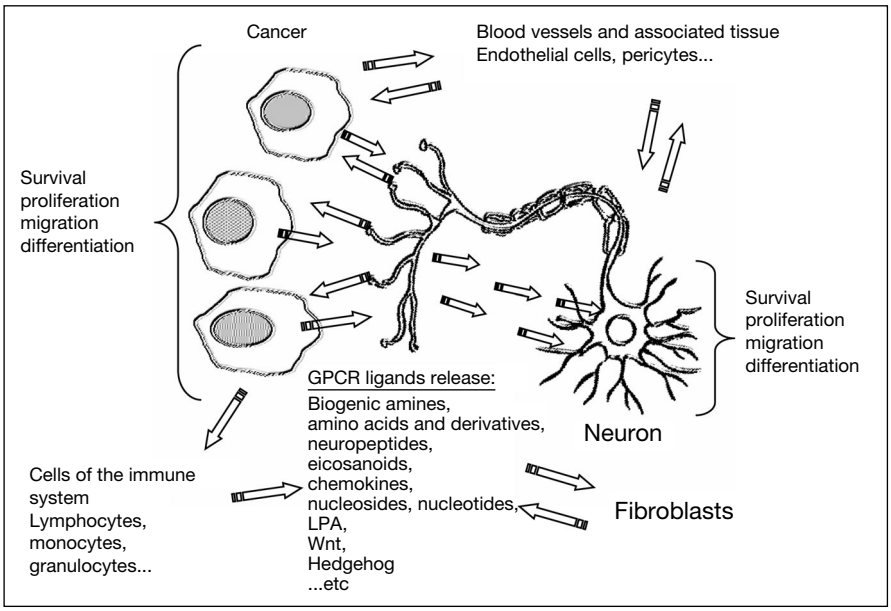

Fig. 1. The neuro-neoplastic synapse. This figure describes the different types of tissues and cells that cooperate in the neuro-neoplastic synapse and the GPCR ligands that may play important functions in the neoneurogenic process. For more detailed comments and bibliographic references, see the sections 'GPCR and Their Ligands in the Tumor Microenvironment' and 'Why and How do Cancer Cells Interact with Neurons?'

classical neurotransmitters (biogenic amines, amino-acids and derivatives), will be reviewed by others in this volume, the present section will be mostly focused on other types of ligands: neuropeptides, development regulators and lipid derivatives.

Why and How do Cancer Cells Interact with Neurons?

Virtually all types of cancers have been observed to contract interactions with neuronal structures, at least at some generally advanced stages of the disease. Numerous types of cancers from epithelial (lung, breast, gut) or mesenchymal (bone) tissues give frequently rise to intracranial metastases, infiltrating the nervous tissues [13]. Another obvious example is the case of non-neuronal intracranial tumors, like gliomas. These astrocyte-derived tumors, diffusely infiltrate the normal brain. From a clinical point of view, the diffuse infiltration of these cancer cells into the healthy brain parenchyma makes

complete surgical resection nearly impossible and focal radiation therapy difficult [14]. Neuro-neoplastic interactions have also been particularly well-described for a number of peripheral cancers that infiltrate the surrounding nerve arborescences, wrapping around these structures. This process called PNI, as cited above, has been described long ago by Ernst [3]. It has been particularly well-studied for prostate, bile duct, and pancreatic carcinomas as well as head and neck cancers [15–19]. For a long time, it was believed that in PNI, cancer cells where escaping the initial site of tumor, along lymphatic vessels following the perineural spaces, until Rodin et al. [4], demonstrated that these locations are frequently devoid of such vessels. The cellular and molecular events of PNI that lead cancer cells to interact and migrate along nerve trails remain however poorly documented.

One can speculate that the cooperation of cancer cells with nerve structures may be an indispensable adaptative process that ensures the survival and proliferation of selected cells. This could lead to the emergence of cancer cells presenting a particular phenotype that allow them to contract tight interactions with nerves, through the so-called neuro-neoplastic synapse (fig. 1). An attractive hypothesis should be that these cancer cells may have acquired several molecular components and mechanisms of peripheral neurons or more generally cells of neuroectodermal origin. An intriguing observation is the expression of synaptophysin, a marker of differentiated neurons involved in synaptogenesis, in non-neural cancers [5]. This could reflect the establishment of a novel cancerous phenotype in cells displaying a high-predilection for nerves and perineural spaces, a concept which is particularly well-illustrated by small cell lung carcinoma (SCLC) which display several features of neuronal cells [20]. Neuroendocrine tumoral cells that release neurotransmitter, neuropeptides as well as other growth and migratory-promoting factors, are frequently observed in the course of cancer progression. This process is particularly well-illustrated in SCLC, but also in some types of prostate cancers [20, 21]. Generally, this type of cells that display strong migratory properties and invasiveness, is considered of quite bad prognosis. Some compounds released by neuroendocrine cells are strong inducers of neoneurogenesis, but also of angiogenesis and lymphangiogenesis. Conversely, cells in the perineural microenvironment may be influenced by the nerve to evolve a growth and survival advantage (fig. 1). This is the case for prostate cancer, for instance. The consequence is increased tumor volume around the nerve, a more aggressive phenotype and a poor survival score for patients [19]. Another dramatic consequence for cancer progression is that substances delivered by these cells, such as chemokines, excitatory neurotransmitters, kininogen and tachykinin derivatives, may lead to an exacerbated development and activation of peripheral sensory nerves, leading to chronic and intractable neuroinflammation and pain.

Targeting Neuropeptide Receptors to Inhibit the Neuro-Neoplastic Interaction

High levels of expression of neuropeptides receptors have been reported in several types of human cancers, which represents a molecular basis for a novel concept of peptide receptor targeting of tumors with potential clinical applications in oncology. Such paradigm is well-illustrated for somatostatin (SST) and SST receptors, that have been extensively studied in the context of in vivo targeting of neuroendocrine tumors and for the development of a receptor-targeted radiotherapy, using for instance indium-111 or yttrium-90 radiolabeled derivatives of octreotide, a synthetic octapeptide SST analog [7]. Receptors for other promising neuropeptides have also been proposed for such targeted therapy: vasoactive intestinal peptide (VIP), gastrin-releasing peptide, cholecystokinin/gastrin, neurotensin, substance P (SP), and neuropeptide Y [7, 22–24]. Here the potential utilization of peptide receptors agonists or antagonists that may be efficient to inhibit the neuro-neoplastic interaction will be briefly reviewed.

Somatostatin Receptors Trace the Paradigm Towards Future Anti-cancer Therapies Targeting Polypeptide GPCRs

Hypothalamic SST is the major negative regulator of growth hormone (GH) secretion from the anterior pituitary. More generally, SST inhibits the secretion of pituitary, pancreatic, and gastrointestinal hormones but also intestinal motility, absorption of nutrients and ions, vascular contractility, and cell proliferation [25]. The 14 or N-terminal extended 28-amino acid natural forms of SST, contain a cyclic domain through an intrachain disulfide bridge. They derive from the pre-pro-SST precursor and interact with five identified GPCR subtypes (SST1–5), in human tissues. All five subtypes bind SST14 or SST28 with the same high-affinity. Several SST synthetic analogs have been generated such as the pseudo-octapeptides octreotide and lanreotide, which behave like more selective, potent and stable agonists towards the SST2 and SST5 receptors. Later on, other compounds with high-selectivity for other SST subtypes have been developed (table 1). High-expression of SST receptors has been observed in different types of neoplasia, with a predominance of SST2, particularly in neuroendocrine tumors (table 1). These receptor-selective compounds have been widely utilized to demonstrate that their anti-proliferative action can lead to cytostasis or apoptosis depending on the receptor subtype expressed on target cells, as extensively reviewed elsewhere [26, 27]. SST2 primarily mediates the anti-proliferative effect of SST analogs in vitro, by activation of the phosphotyrosine phosphatase SHP-1 or inhibition of tyrosine kinase activities. SST1, 2, 4, and 5 can induce G1 arrest by down-regulating the phosphorylation

Table 1. Structure of some synthetic SST receptor agonist displaying a high selectivity for certain subtypes of SST receptors

SST receptor subtype	Tumor type	Selective SST analog	Analog structure
SST1	Prostate carcinoma	CH-275	C[Cys-Lys-Phe-Phe-Trp-IAmp-Thr-Phe-Thr-Ser-Cys]-OH
		TT2-32	D-Phe-c(Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH_2
SST2	GH-producing pituitary adenoma, gur carcinoid, gastrinoma, paraganglioma, pheochromocytoma, SCLC, meningioma, neuroblastoma, medulloblastoma	Octreotide (Sandostatin)	D-Phe-c(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Thr(ol)
		RC-160	D-Phe-c(Cys-Tyr-D-Trp-Lys-Val-Cys)-Trp-NH_2
		BIM23014 (Lanreotide)	D-Nal-c(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Thr-NH_2 c[N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]
		MK-678	D-Tyr-D-Tyr-D-Tyr-D-Tyr-c(Cys-Phe-D-Trp-Lys-Thr-Cys)-Thr-NH_2
		WOC 4D	
		BIM23066	NH_2-D-Phe-p-NO_2-Phe-Tyr-D-Trp-Lys-Val-Phe-Thr-NH_2
SST3	Non-functioning pituitary adenoma	BIM23056	NH_2-D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-Nal-NH_2
SST5	GH-producing adenoma, gut carcinoid	Octreotide (Sandostatin)	D-Phe-c(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Thr(ol)
		RC-160	D-Phe-c(Cys-Tyr-D-Trp-Lys-Val-Cys)-Trp-NH_2
		BIM23014 (Lanreotide)	D-Nal-c(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Thr-NH_2 c[N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe]
		MK-678	

Tumors types that generally express high levels of the indicated subtypes of SST receptor are also precised in this table. For futher informations concerning these molecules and bibliographic references, see 'Somatostatin Receptors Trace the Paradigm Towards Future Anti-cancer Therapies Targeting Polypeptide GPCRs'.

of retinoblastoma oncogenic proteins. SST1 can also cause cell cycle arrest by induction of cdk inhibitor p21Waf-1/Cip-1. SST5 inhibits cell growth by down-regulating the MAPK pathway via a guanylyl cyclase sensitive mechanism. The apoptosis of cancer cells seems to be mediated via the SST3 subtype, through a mechanism involving induction of p53. SST2 has been found to mediate apoptosis via a p53 independent pathway and SST1, through sustained activation of JNK and p38 kinase cascade with concomitant blockade of the extracellular-regulated kinase 2 signaling pathway. Anti-neoplastic actions of SST and

analogs also involve inhibition of the synthesis of mitogenic hormones, growth factors, and cytokines mediated by inhibition of cAMP and calcium production. Additionally, SST can interfere with the exocytotic machinery by down-regulating the protein phosphatase calcineurin. This could account for the potent inhibition exerted by SST on exocrine and endocrine secretion of a number of growth factors from tissues of the tumor environment that play a major role in the etiology, growth and pathogenesis of several carcinomas. Growth factors like epidermal growth factor, insulin-like growth factor-1, basic fibroblast growth factor, and platelet-derived growth factor appear to be implicated in the proliferation of many types of cancer cells such as pancreatic, prostate, mammary, colorectal carcinoma [28].

Another therapeutic ability of SST and its analogs in vitro and in vivo also depends, at least in part, on its effect on the development of blood vessels [26, 29]. The anti-angiogenic activity of a panel of SST analogs has been studied years ago in the chicken chorioallantoic membrane model [30]. The cyclo-octapeptide analog RC-160 and octreotide were the most potent inhibitors of neovascularization, suggesting that SST2 receptors were involved in this effect. As a matter of fact, SST2 gene expression is generally low in the quiescent vascular endothelium, while it is strongly induced in proliferating angiogenic sprouts of human endothelial cells [31]. Anti-angiogenic activity of SST and SST analogs includes direct inhibition of endothelial cell proliferation, adhesion, migration, and invasion, but also blockade of the release of pro-angiogenic growth factors (vascular endothelial growth factor [VEGF], basic fibroblast growth factor, insulin-like growth factor-1, platelet-derived growth factor) of monocyte migration [26].

The growth of new blood vessels is a crucial event not only for tumor growth but also for shaping the nervous system and protecting it from disease. The understanding of the processes that allow the brain and other tissues to grow new blood vessels under normal and pathological conditions, has greatly improved during the last decade. Angiogenesis factors, especially VEGF, are also known for their role in neurogenesis and neuroprotection, but also in the pathogenesis of neurodegenerative diseases and in neuron destruction upon ischemia or trauma [32]. Hence, blockade of their release and effects by selective SST analogs, also appears like a promising strategy to inhibit nerve development in the tumor microenvironment.

Furthermore, it has been demonstrated that SST is in primary afferent neurons and reduces vascular and nociceptive components of inflammation. SST2 receptors are expressed in a significant number of peripheral afferent sensory fibers. It has been proposed that targeting peripheral SST receptors would provide effective analgesia. As a matter of fact, it has been demonstrated that local injection of octreotide reduces formalin-induced nociceptive behaviors,

responses to thermal stimulation in C-mechanoheat sensitive fibers suppress; and responses of C-mechanoheat fibers to bradykinin-induced excitation and sensitization to heat. Each of these actions can be reversed following co-injection of octreotide with the antagonist cyclo-SST. Thus, activation of peripheral SST receptors reduces both inflammatory pain and the activity of sensitized nociceptors, and may be clinically useful in the treatment of pain of peripheral origin associated with cancer progression [33].

Vasoactive Intestinal Peptide and Pituitary Adenylate-Cyclase Activating Polypeptide Receptors

VIP and pituitary adenylate-cyclase activating polypeptide (PACAP) represent another typical example of neuropeptides that may play a major role in neoneurogenesis, especially in some types of cancers known to release these peptides in their microenvironement. The reason is that besides their growth-factor properties in cancer cells, these polypeptides also display strong growth-promoting and neuroprotective functions on nerve cells, that have been extensively described in several excellent reviews. VIP-induced neuroprotection involves, among other phenomena, an increased secretion from astroglial cells of a potent survival protein called activity-dependent neurotrophic protein (ADNP) [34, 35]. VIP and PACAP are also recognized as potent modulators of cancer cell proliferation, acting in some cases, through an autocrine/paracrine process, as reported for lung cancers (SCLC or non-SCLC), androgen-independent prostate cancers, neuroblastoma and generally neuroendocrine tumors [36–40]. Hence, they may represent crucial regulators of the neuro-neoplastic interactions.

The 28-amino acid VIP neuropeptide, isolated from the small intestine, derives from a propeptide which gives also rise to a VIP analog, the 27-amino acid peptide histidine isoleucine or its human counterpart peptide histidine methionine. It is a member of the secretin-like peptides family. As well as PACAP, the structurally similar 27- or 38-amino acid long peptide, VIP displays a very large spectrum of biological activities and these peptides modulate virtually all the vital functions in the body. They are main neurotransmitters in the gut and both play a neuromodulatory role in the central and peripheral nervous systems, at the neuronal and glial levels. A most prominent signaling pathway of VIP/PACAP is the stimulation of the adenylate cyclase activity [41, 42]. There are two VIP receptors, VPAC1 and VPAC2 , both with high-affinity for VIP and PACAP [43, 44]. A third receptor type, named PAC1, has been characterized for its high-affinity for PACAP but a low-affinity for VIP. Numerous isoforms of this receptor, corresponding to at least 17 splice variants of the same gene, have been identified. These isoforms display distinct pharmacological

profiles and coupling to intracellular effectors. Most of them behave like specific PACAP receptors but some of them like the newly discovered δ 5–6 splice variants, appear to be efficiently activated by both VIP and PACAP [45].

The expression pattern of VIP and PACAP receptors in tumors has been described in several well-documented reviews. Briefly, expression of the VPAC1 receptor subtype has been described in the most frequently occurring malignant epithelial neoplasms, such as cancers of the lung, stomach, colon, rectum, breast, prostate, pancreatic ducts, liver, urinary bladder and in neuroblastoma [39, 46]. A predominance of VPAC2 receptors is found in only few tumors, such as leiomyoma, a benign smooth muscle tumor. In contrast, several different human tumor types express predominantly PAC1 receptors, such as endometrial carcinomas and tumors originating from the neuronal and endocrine systems [39, 46]. This includes glial tumors (astrocytoma, glioblastoma, oligodendroglioma), neuroblastoma, as well as various pituitary adenomas (especially GH-secreting and non-secreting adenomas, but not prolactinomas), most catecholamine-secreting tumors, including both pheochromocytoma and paraganglioma [39, 46]. This short overview underlines that PAC1 receptor is a common denominator, in neural, glial or neuroendocrine tumors of neuroectodermal origin. Interestingly, the in vitro effects of VIP and PACAP in neuroblastoma cell lines could be mostly triggered through the δ 5–6 PAC1 receptor variant, which is highly expressed in some of these cell lines, while VPAC1 or VPAC2 receptors appear to be generally poorly represented [45].

Generally, the action of VIP or PACAP on cancer cells results in an increased proliferation of target cells. For these reasons, synthetic derivatives of these peptides displaying antagonist properties have been developed and their efficiency has been demonstrated on in vivo and in vitro models of cancer [47, 48]. Among these compounds, the neurotensin(6–11)VIP(7–28), also termed VIPhyb has been particularly well-studied. It was shown to inhibit glioblastoma growth in a concentration-dependent manner. A dose of 10 mM VIPhyb significantly inhibited the proliferation of different human glial cancer cell lines. In vivo, 0.4 mg/kg VIPhyb inhibited U87 glioblastoma cells xenograft proliferation in nude mice. The results suggested that the VIPhyb displays antagonist properties towards VIP and PACAP receptors in glioblastoma cells and inhibits their proliferation. The data also indicate that these cells may also release VIP or VIP-related peptides acting as autocrine/paracrine growth factors [49]. VIPhyb was also daily administered (20 mg, corresponding to 10 nM) to rats having chemically-induced colon cancer. This caused a significant regression in tumor dimensions and incidence of carcinoma. The antagonist treatment reduced the tumor volume, staging, lymphocyte infiltrate and number of dysplastic crypt [50]. As well, subcutaneous administration of 10 mg VIPhyb in C3(1)SV40TAg transgenic mice developing mammary tumors that are histologically similar to

human breast cancer and predominantly express the VPAC1 receptors, significantly increased the survival of the mice and reduced the tumor development, in comparison with control animals [51].

Addition of a stearyl N-terminal and the exchange of the methionine in position 17 to the VIPhyb, resulted in a novel antagonist for VPAC1, VPAC2 and PAC1 receptors, called SNH with a 10-fold higher-affinity for VPAC1 than VIPhyb [52, 53]. In lung cancer cells, it was shown that SNH inhibited VIP-induced elevation of cyclic AMP and increase of c-Fos gene expression [37]. SNH also inhibited the growth of 51 of 56 cancer cell lines tested, including leukemia, lung cancer, colon cancer, central nervous system cancer, melanoma, ovarian cancer, renal cancer, breast cancer and prostate cancer [54]. SNH was also shown to potentiate the action of classical chemotherapeutic agents, such as taxol in nude mice bearing MDA-MB231 breast cancer xenografts [55]. SNH also enhanced the anti-proliferative activity of the diverse chemotherapeutic agents: doxorubicin, vinorelbine, paclitaxel, gemcitabine, irinotecan and cisplatin [56]. Other remarkable VIP or PACAP antagonists are GH Releasing Hormone (GHRH) derivatives. GRF, a structural analog of VIP and PACAP, interacts with specific high-affinity GRF receptors and with a lower-affinity (with Kd values in the 10 nM range) with the VPAC1 and VPAC2 receptors. Some of the side-effects of GRF are recognized to be a consequence of interaction of this polypeptide with the VIP and PACAP receptors. Several groups have developed GHRH derivatives that display antagonist properties towards the GHRH receptors but also for the VPAC1 and VPAC2 receptor subtypes. These antagonists have been shown to inhibit the growth of androgen-independent prostate cancers by inhibiting the autocrine–paracrine action of endogeneous VIP. Examples of these molecules are:

- JV-1–52, a non-selective VIP/GHRH antagonist (Ac-His1 D-Phe2 Phe(4-Cl)6 Har9 Tyr(Me)10 Abu15Nle27 D-Arg28 Har29)hGHRH(1–29) NH_2
- JV-1–53 (Ac-His1 D-Phe2 Phe(4-Cl)6 Lys15 Arg16 Lys20 Tyr22 Nle27 D-Arg28 Har29)hGHRH(1–29)NH_2, a VIP antagonist devoid of GHRH antagonistic effect.

Both antagonists (20 mg/day, subcutaneously) produced a similar reduction in tumor volume (about 65%) and tumor weight (about 60%) in nude mices bearing the PC-3 human androgen-independent prostate carcinoma [57].

In our own group, two GHRH derivatives and the VIP antagonist VIPhyb have also been checked on C6 glioblastoma cell growth. These compounds were able to inhibit VIP-induced cell growth stimulation, even at very low concentrations of the picomolar range. Binding experiments carried out on intact cultured C6 cells, using ^{125}I-labeled VIP and PACAP as tracers, revealed that the effects of the peptides on cell growth were correlated with the expression on

C6 cells of polyvalent high-affinity VIP-PACAP binding sites and of a second subtype corresponding to very high-affinity VIP-selective binding species. The latter subtype, which interacted poorly with PACAP with a 10,000-fold lower-affinity than VIP, might mediate the antagonist effects of VIPhyb and of both GHRH derivatives on VIP-induced cell growth stimulation [58].

Other interesting compounds are VIP derivatives coupled to anti-mitogenic substances such as ellipticine, a DNA-intercalating natural plant product. The ellipticine derivative 9-methoxy-1-chloro-5,11-dimethyl-6Hpyrido[4,3-b]carbazole was attached to VIP C-terminal end using a peptide spacer. The VIP-E derivatives (LALA-E or ALALA-E) bound with high-affinity to VPAC1 receptors expressed in breast cancer cells and displayed cytostatic or cytotoxic effects, probably due to the release of the ellipticine inside the cells [59].

However, main disadvantages limit the utilization of the VIP and PACAP antagonists so far developed for clinical trials:

- Their relative lack of selectivity, which may cause several side effects of these molecules, such as diarrhea or cardiovascular troubles, due to the large distribution of VIP and PACAP receptors in virtually all tissues.
- Their short lifetime in the body, due to their peptidic nature and rapid proteolysis by plasma and tissue proteases.

An important function of VIP relevant to neoneurogenesis is its potent stimulatory effects on VEGF production, particularly in neuroendocrine prostate cancers which are submitted to a paracrine/autocrine action of this neuropeptide. VIP-induced neuroendocrine differentiation of human prostate cancer LNCaP cells was associated with an up-regulation of the expression of the three forms of VEGF mRNAs and VEGF(165) protein expression. This effect mediated by VPAC1 receptor, was cAMP/protein kinase A (PKA) and Ca^{++} dependent and was associated with increased c-Fos expression. The promoter region of the VEGF gene possesses AP-1 (i.e. c-Fos/c-Jun heterodimer) response elements. Phosphoinositide 3-kinase (PI3-K) and mitogen-activated protein kinase Erk1/2 systems were also be involved as shown with specific kinase inhibitors. Recent studies demonstrate that hypoxia-mimicking agent Ni^{++}, known to promote VEGF gene expression by activating the hypoxia-inducible factor-1 alpha (HIF-1α), also induced VIP expression at both mRNA and peptide levels, in the LNCaP cancer cells. Interestingly, in this cell line, VIP did not stimulate HIF-1α mRNA expression but increased the translocation of HIF-1α from the cytosolic compartment to the cell nucleus [60–62]. Knowing the potent proangiogenic and proneurogenic functions of VEGF, increased release of this substance in the tumor microenvironment, in response to VIP produced by the prostate cancer cells themselves, may play a major role in neoneurogenesis.

To complete this section on VIP and PACAP potential involvement in neoneurogenesis, one has to keep in mind the potent neurotrophic and neuroprotective

actions of VIP and PACAP. VIP also displays strong anti-inflammatory properties, that have been found to be protective in several inflammatory disorders [63]. The neuroprotective effects of VIP are generally indirect and requires an induction by this peptide of the release of neurotrophic soluble factors by astrocytes, such as ADNP [34, 35]. The mice model of neuroprotection by VIP of white matter excitotoxic lesions caused by ibotenate (a glutamate analog), showed that protein kinase C (PKC) and mitogen-associated protein kinase (MAPK) pathways were critical for neuroprotection. The combination of in vitro and in vivo studies suggested that VIP activates PKC in astrocytes, which release soluble factors; these released factors activate neuronal MAPK and PKC, which will permit axonal regrowth. VIP-treated cultured astrocytes release growth and survival factors, including ADNP and the 14-amino acid peptide ADNP derivative, which has been shown to protect the developing white matter against ibotenate-induced lesions [64]. In neuron-glia co-cultures, PACAP38 also induced ADNP mRNA expression in a bimodal fashion at subpico- and nanomolar concentrations. The response was attenuated by a PAC1-R antagonist at both concentrations and by a VPAC1-R antagonist at nanomolar concentration only. An IP3/PLC inhibitor attenuated the response at both concentrations of PACAP38 while a PKA inhibitor suppressed the response at nanomolar concentration only, suggesting that PACAP-induced ADNP expression is mediated through multiple receptors and signaling pathways [65].

Another potential mode of induction of nerve cells development in the tumor environment relies on the process of transactivation of Trk receptors tyrosine kinases for neurotrophins, such as nerve growth factor (NGF) or brain-derived neurotrophic factor, through a GPCR-coupled mechanism. In the PC12–615 rat pheochromocytoma cell line, TrkA and TrkB receptors can be activated in the absence of brain-derived neurotrophic factor or NGF by PACAP acting on PAC1 receptors but also by the nucleoside adenosine or CGS 21680, an adenosine agonist, acting on the A_{2a} adenosine GPCR subtype. This phenomenon promotes the phosphorylation of the canonical effectors of the Trk signaling cascade: Shc adaptator or phospholipase C gamma. Transactivation of the Trk receptors occurs in an intracellular compartment where they colocalize with membrane markers of the Golgi apparatus. The resulting activation of PI3-K, Akt, Mek and Erk1/2 kinases cascade accounted for PACAP or adenosine neuroprotective effects in this cell line [66]. However, it has also been demonstrated that the MAPK cascade could also be directly triggered in PC12 cells as a consequence of PACAP binding to PAC1 receptor resulting in G-protein dependent activation of PKA and PKC [67].

To conclude this section, blockade of the complex effects of VIP and related peptides by efficient and selective antagonists, really deserves to be checked on neoneurogenesis, for the numerous types of neoplasia which have

been demonstrated to be submitted to an autocrine/paracrine action of VIP, such as neuroendocrine prostate cancers, SCLC or non-SCLC lung cancers, pancreatic carcinoma, neuroblastoma, ganglioneuroma and pheochromocytoma [68]. This means that there is also an urgent need for novel peptide-like or non-peptide stable molecules which could target one single VIP/PACAP receptor subtype and consequently antagonize selectively the direct or indirect effects of VIP and related peptides on tumor cells and on surrounding tissues, such as nerves and blood vessels (fig. 2).

Antagonizing Multiple Neuropeptide Receptors and Related Signaling Pathways to Inhibit Neoneurogenesis

The examples of SST or VIP/PACAP receptors define a novel paradigm for future potential therapeutic applications to inhibit neoneurogenesis, using either selective agonists (for SST receptors) or antagonists (for VIP/PACAP receptors) of these neuropeptides. SST receptors agonists may suppress tumor growth directly, and indirectly through a blockade of the release by the tumor and surrounding tissues, of growth, anti-apoptic or proangiogenic and proneurogenic factors, such as VEGF. For VIP, PACAP and related peptides, blockade of their effects by selective antagonists may lead to similar phenomena, particularly for the neuroendocrine tumor types that are submitted to an autocrine/paracrine action of these neuropeptides. Accumulating evidence supports the autocrine and paracrine involvement of a number of other neuropeptides acting on GPCR in lung, gastric, colorectal, pancreatic and prostatic cancers: bombesin-like peptides (such as gastrin-releasing peptide or neuromedin B), SP, neurotensin, gastrin, cholecystokinin and arginine vasopressin. The growth factor properties of these neuropeptides and the autocrine or paracrine signaling pathways regulated through their receptors in cancer cells have been extensively reviewed in excellent reports [69, 70] (fig. 2). Interestingly, these neuropeptide autocrine/paracrine systems may regulate each other in some types of cancer. For example, In SCLC cells, VIP increased the secretion rate of bombesin-like peptides [71]. VIP and corticotrophin releasing factor may cause phosphorylation of proteins such as synapsin1, causing exocytosis of granules which contain bombesin-like peptides [72]. These peptides may then bind to cell surface receptors stimulating the proliferation of SCLC and the VIP/autocrine system operating in this type of cancer.

A major conclusion of these different reports on neuropeptide autocrine/paracrine systems in cancers, is that the strategy of interrupting one single neuropeptide autocrine system to block the progression of a given type of cancer appears quite illusory. Lung SCLC that possess multiple, redundant neuropeptide autocrine/paracrine systems is a good example to illustrate the concept that tumor growth may not be suppressed with a single specific antagonist but by

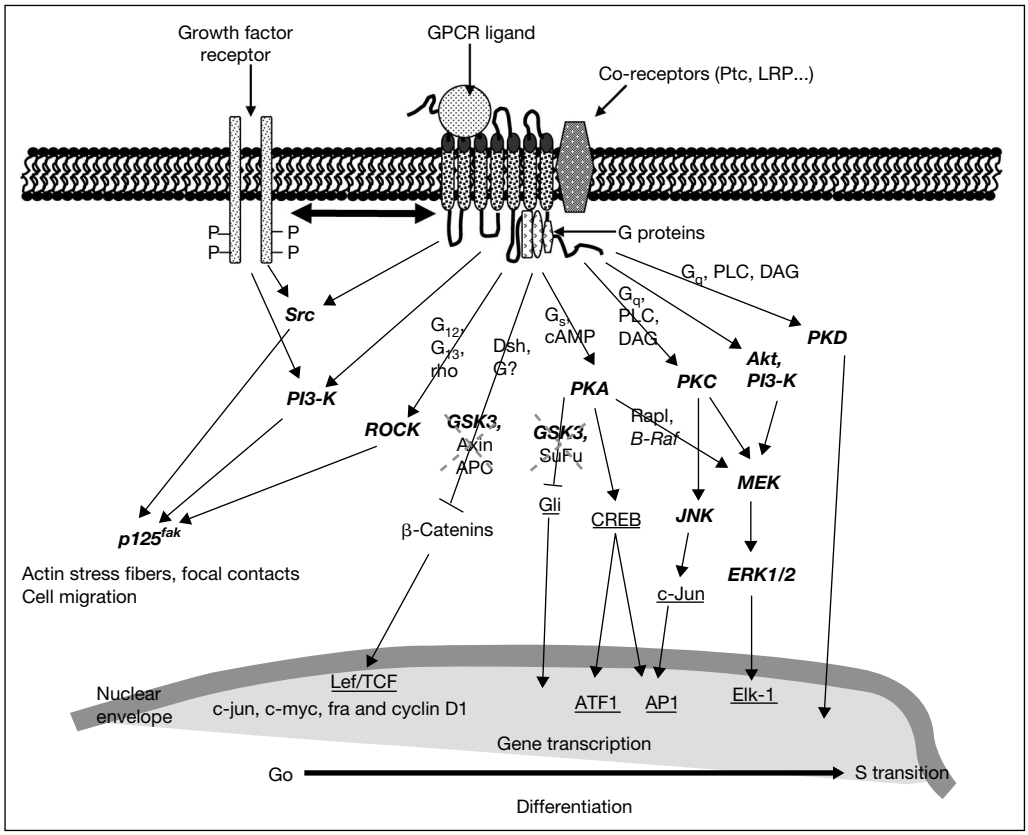

Fig. 2. A simplified view of the multiple signaling pathways linked to GPCR, illustrating different sections of this review. Binding of the ligands to GPCR lead to activation (or inhibition for SST receptor agonists) of multiple protein kinases (in bold italics), through G-protein dependent processes (see 'Vasoactive Intestinal Peptide and Pituitary Adenylate-Cyclase Activating Polypeptide Receptors' and 'Antagonizing Multiple Neuropeptide Receptors and Related Signaling Pathways to Inhibit Neuroneogenesis'). Alternatively, GPCR/co-receptor activation leads to the dissociation and degradation of multiple protein complexes, leading to the liberation of transcriptional regulators, such as β-catenins (Wnt canonical pathway) or Gli proteins (Hh pathway), as discussed in 'Developmental GPCR and Neoneurogenesis'. These compounds are translocated inside the nucleus where they modulate gene expression. A third possibility is represented by transactivation by GPCR of receptors tyrosine kinases, which can occur in the absence of the corresponding growth factors ligands (an example, is the transactivation of NGF receptors upon stimulation of PACAP GPCR, see 'Vasoactive Intestinal Peptide and Pituitary Adenylate-Cyclase Activating Polypeptide Receptors'). More complete information and bibliographic references on these pathways and the role of their molecular actors, will be found in the indicated sections.

a combination of molecules antagonizing their corresponding GPCR. This principle may lead to a novel concept of polytherapy targeting multiple polypeptide GPCR, which may be efficient to inhibit neoneurogenesis and tumor progression, in certain types of peptide-secreting neuroendocrine tumors. However, such mixture of compounds displaying individually numerous side-effects, may also lead to unexpected physiological reactions in the body.

An exciting promise as novel therapeutics is the principle of a neuropeptide receptor-mediated induction of apoptosis in cancer cells. Among the most advanced studies in this respect, are those conducted with SP derivatives, including [D-Arg(1),D-Phe(5),D-Trp(7,9),Leu(11)]SP or [D-Arg(1),D-Trp(5,7,9),Leu(11)] SP are broad-spectrum GPCR antagonists that have potential anti-tumorigenic activities. Their mechanisms of action which are not fully understood involve apoptosis in tumor cells. These molecules possess the remarkable ability to selectively stimulate JNK activity and cytoskeletal changes, possibly through G_{12} and G_{13} proteins, but not Ca^{++} mobilization and subsequent ERK activation through G_q proteins [69, 73]. According to this ability to trigger certain signaling pathways while they antagonize other ones, they are considered by some investigators like a novel class of agonist, referred to as biased agonists, because they inhibited proliferation signals while they stimulated apoptosis. It has been proposed that the ligand–receptor complexes formed in the presence of these molecules, may correspond to a conformational state that allows activation of G_{12} and G_{13} proteins, while G_q proteins are locked in an inactive state [74].

Very interestingly, the effects of these SP derivatives appear to be heterologous, since they have been demonstrated to suppress the growth promoting effects of SP but also those of other neuropeptides, including arginin-vasopressin, cholecystokinin, galanin or neurotensin, as demonstrated in SCLC cells. Significantly, the SP derivatives also inhibited SCLC growth as xenografts in nude mice with little or no apparent toxicity [75]. Hence, through their peculiar mode of action, these atypical antagonists, or biased agonists, able to block certain signaling pathways but not others, provide a promising approach and an alternative to a GPCR-oriented polytherapy, to disrupt the multiple neuropeptides autocrine/paracrine systems operating in some types of cancers.

A compound represented by a dimer of two molecules of a bradykinin antagonist, called CU201, has been demonstrated to inhibit the growth of SCLC and non-SCLC cell lines with a 10-fold higher-efficacy and a longer plasma half-life than SP biased agonists. In the same studies, bradykinin agonists in either monomeric or dimeric form and the monomeric unit of CU201 had no effect on lung cancer cell growth. CU201 inhibited intracellular Ca^{++} release in response to bradykinin, indicating blockade of G_q-dependent signals, while JNK was stimulated, indicating stimulation of the G_{12} and G_{13} pathway. CU201-induced apoptosis was preceded by unique changes in apparent nuclear

DNA binding and by JNK and caspase-3 activation. In brief, CU201 behave like a biased agonist, suggesting that this concept could be generalized to a variety of neuropeptide receptors [76].

Before closing this section, it is worth reminding here the potent effects of neuropeptides on cell migration and metastasis [8–10]. Binding of several polypeptides to their respective GPCR, such as bombesin-like peptides, cholecystokinin, gastrin and neurotensin leads to phosphorylation and assembly of a number of proteins, such as $P130^{cas}$, the focal adhesion kinase $p125^{fak}$ or paxillin, which are involved in focal adhesion and actin stress fibers formation. This phenomenon is a consequence of G_{12}-dependent activation of small G-proteins of the Rho family and subsequently of Rho kinases (ROCK). Furthermore, activated Rho protein members, such as cdc42, allow the recruitment and assembly of WASP and Arp2/3 proteins to actin fibers, which increases their branching and leads to growth of pseudopodia. Neuropeptides also promote $p125^{fak}$ activation through a process depending on the PI3-K and on the Src family of kinase. These different signaling processes and components, which are also triggered downstream integrin activation, are leading components of the molecular machinery that governs intracellular remodeling, neurite outgrowth and cell migration not only in cancer cells [70, 77, 78], but also in neurons [79, 80] (fig. 2). Hence they represent a major common link between cancer progression and neurogenesis.

Other GPCR Relevant to Neuro-Neoplastic Interaction

Lysophosphatidic Acid Receptors

The bioactive phospholipid lysophosphatidic acid (LPA) is produced by cancer cells and stimulate cell proliferation, migration and survival by acting on its cognate GPCRs. Aberrant LPA production, receptor expression and signaling probably contribute to cancer initiation, progression and metastasis [81–84]. A growing set of evidences also demonstrate LPA involvement in neurogenesis, neuronal migration, neuritogenesis, and myelination [85, 86]. Such properties suggest that this bioactive lipid derivative may represent a key chemical messenger in the neuro-neoplastic synapse. The bioactivity of LPA is mediated by a set of specific GPCRs (especially LPA1, LPA2, and LPA3) leading to the activation of a number of intracellular effectors, particularly glycogen synthase kinase 3 beta (GSK3β) an conventional PKC, which results in nuclear translocation of β-catenin and subsequent transcriptional activation of target genes. Remarkably, this transduction pathway is identical to the so-called canonical Wnt/frizzled (fzd) signaling cascade, that will be presented in the next section. Furthermore, ecto-enzymes mediate the production, degradation

or chemical conversion of LPA by target cells, as well as the generation of LPA derivatives behaving like receptor-selective analogs. Hence, LPA receptors, as well as the enzymes involved in LPA metabolism, appear like promising pharmacological targets to investigate the relevance of bioactive LPA in neoneurogenesis. In this respect, novel lipidic synthetic LPA antagonists may be of great interest for such studies [87–89].

'Developmental' GPCR and Neoneurogenesis

In this section, will be briefly discussed the potential utilization of compounds that antagonize two subtypes of GPCR which have been initially discovered as key regulators of cell differentiation, polarity and of tissue patterning during development, particularly in the nervous system:

- The fzd family of GPCR are associated with membrane co-receptors, the low-density-lipoprotein-receptor related protein (LRP) family. Fzd receptors interact with a family of secreted lipid-modified glycoproteins called Wnt, homologous to the drosophila 'wingless' developmental factor [90].
- The smoothened (Smo) GPCRs, which are activated after binding of a class of proteic ligands called Hedgehog (Hh) to a Smo co-receptor called Patched (Ptc) [91].

Activation of fzd–LRP complexes promotes the stability and nuclear localization of β-catenin by compromising the ability of a multiprotein complex containing axin, adenomatosis polyposis coli and GSK3 to target it for degradation and block its nuclear import. This process involves activation of Dishevelled proteins, possibly through heterotrimeric G proteins and LRP-mediated axin binding and/or degradation. This leads to the nuclear translocation of β-catenins, subsequent activation of transcriptional regulators such as the Lef/TCF (T-cells factor) complexes and expression of target genes such as c-jun, c-myc, fra and cyclin D1. This signaling cascade is the so-called canonical Wnt pathway. However, Wnt ligands can also activate non-canonical β-catenin-independent processes. A number of mammalians cancers (colonic adenocarcinoma, breast cancers, hepatocellular carcinoma, melanoma) release Wnt ligands and act as paracrine/autocrine ligands [90]. These ligands are also potent activators of neurite outgrowth through the canonical pathway [92]. Furthermore, several components of the Wnt canonical pathway are known to be mutated during carcinogenesis, such as adenomatosis polyposis coli, axin or β-catenins themselves [93].

Secreted proteins such as Dickkopf-1 [94], Wnt inhibitory factor-1 [95] or secreted fzd-related protein [96], have been demonstrated to act as extracellular antagonists of the Wnt signaling pathways. Expression of the genes encoding these proteins is down-regulated in different types of cancers, such as colonic adenoma and adenocarcinoma or pleural mesothelioma [94–96]. Potential effects of these molecules in the context of neoneurogenesis really deserve to be investigated.

Interestingly, it has been demonstrated that stimulation by neuropeptides like neurotensin [97] or gastrin [98] of colonic or gastric tumor cell growth can be triggered through elements of the canonical Wnt pathway, leading to β-catenin activation. These data demonstrate that multiple GPCRs, including not only Fzd, but also neuropeptides and LPA (see section 'Lysophosphatidic Acid Receptors') receptors lead to a convergent β-catenin-dependent regulation of cell growth and behavior during neurogenesis and carcinogenesis (fig. 2).

The secreted proteins of the Hh family and particularly Sonic Hh (Shh) are crucial for the specification of neuronal subtype identity in the vertebrate neural tube [99]. Binding of Hh ligands to the Smo co-receptor Ptc causes activation of the Smo GPCR, resulting in the dissociation and proteasome-dependent degradation of a multiprotein complex comprising among other components, the SuFu, Cos2, Rab23 and Gli proteins, microtubules, GSK3 and PKA. Upon activation of the Smo/Ptc complex, prevention of degradation of the transcriptional regulator Gli-3 allows translocation of its full-length, fully active form to the nucleus, to induce expression of target genes like Gli-1, encoding another Gli family transcription factor [91, 100]. It has been demonstrated that SuFu and Ptc mutations have been associated to medulloblastoma [91] and that SCLC tumors maintain their malignant phenotype in vitro and in vivo through ligand-dependent Hh pathway activation [101]. Generally, this embryonic pathway is involved in development and progression of several human tumors resembling primitive precursor cells, deriving from the brain, skin, lung and gastrointestinal tract [91, 100–102]. Taken together, these data indicate that the Hh/Smo pathway is a common mechanism in neurogenesis and tumor progression [102]. Effects on neoneurogenesis of signaling inhibitors of this pathway, such as cyclopamine, a small organic compound with potent Hh antagonist properties, or small interfering RNA against Gli-1, deserve to be tested in the future.

Interaction of PACAP and Shh has also been studied in the developing cerebellum, where both PACAP and Shh are known to play major roles. PACAP and the PAC1-specific agonist, maxadilan, were found to potently block the proliferative action of Shh, as well as Gli-1 transactivation, on developing cerebellar granule neurons [103, 104]. These data indicate that multiple GPCRs are involved in a convergent regulation of the Hh pathway, most probably, among other processes, through their role on the modulation of PKA activity.

Conclusion

Here some examples of GPCR have been presented that are known to be frequently up-regulated in cancer cells and to mediate major cellular pathways

involved in tumorigenesis and in nerve development. They have been chosen because their corresponding natural agonists may possess:

- direct suppressive properties on neoneurogenesis, as it is the case for SST;
- autocrine/paracrine growth factors properties in tumor cells that may also promote nerve development in the tumor microenvironment. This is the case for several neuropeptides, LPA, and components of the Hh and Wnt families.

Blockade of the action of the latter chemical messengers using receptor antagonists cited in this review and drugs that target key components of their multiple cognate transduction pathways (fig. 2), should lead future investigations dedicated to inhibition of neoneurogenesis and more generally of tumor progression. A number of antagonists are already available in this purpose but as discussed here, in vivo utilization of these molecules, especially those of polypeptidic nature, may lead to unexpected side effects. Hence, there is a urgent need for the development of small organic molecules with potent and selective antagonist or biased agonist properties towards selected GPCR involved in neoneurogenesis. Detailed anatomical studies of the expression and distribution of specific subtypes or GPCR, co-receptors or components of the cognate transduction pathways, in the tumoral and nervous tissues present in neoplastic formations, also deserve to be conducted. This could be effected through immunohistochemical approaches, using the great variety of commercial antibodies against these different components. The development of novel proteomic approaches, including the most advanced protein array and high-performance mass spectrometry techniques today available, should also complement this precise molecular typing of individual tumor masses in a given patient. A progressive adaptation of these technologies to the clinical practice may lead in the future to a novel concept of anti-cancer polytherapy, using a mixture of compounds properly adapted to the neoplastic formation and to the patient, with maximal effects on the pathological tissues and little repercussions on vital functions.

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The Neuro-Neoplastic Synapse: Does it Exist?

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Abstract

Since the pioneering work of Judah Folkman and colleagues in the 1970s on tumor neoangiogenesis, we learned more and more about the heterogeneity of the cellular, subcellular and stromal architecture within a tumor mass. The research on neoangiogenesis has lead to novel molecular entities (vascular endothelial growth factor, platelet-derived growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor-β, tumor necrosis factor-α, interleukin-8), which can be targeted within the framework of tumor neoangiogenesis inhibition. Accepting the paradigm of anti-angiogenic therapy, a new class of drugs could be developed some of which already obtained clinical approval. As blood vessels and nerves often follow parallel trajectories within a tumor tissue, it was consequent to argue that tumor cells for their growth advantage and survival and metastases formation use common cues that induce vascularization and innervation. Autocrine, paracrine or endocrine interactions between a resident tumor cell type with neurocrine cell types and their signaling molecules can be regarded as a neuro-neoplastic synapse. That cross-talk molecules are equally interesting molecules as selectable anti-tumor targets as it turned out to be in the past for tumor angiogenesis factors. An extended model of human tumor dormancy as well as metastasis formation is provided assuming an angiogenic and neurogenic switch from the non-angiogenic and non-neurogenic phenotype.

Angiogenesis is the formation of blood vessels from the pre-existing vascular architecture and also encompasses the regression of capillary blood vessels by apoptosis, defined by the nature of the perfusion requirements of the tissue. The result of the carefully orchestrated signaling system is an orderly vascular architecture. Soluble factors such as vascular endothelial growth factors (VEGF) are secreted by tissue composing cells and result in vascular

recruitment and stabilization. Other factors influencing angiogenesis include angiopoitins and tie receptors, acidic and basic fibroblast growth factors, platelet-derived growth factor, transforming growth factor-β, interleukin-8 and tumor necrosis factor-α.

In the early days of modern molecular medicine, Algire and Chalkey [1] suggested in 1945 that one feature of tumor cells is their capacity to stimulate continuously the growth of new capillary endothelium in vivo. In the early 70s of the last century, Folkman et al. [2] isolated a tumor factor which was responsible for angiogenesis. During the development of the field of angiogenesis over the past three decades since there, fundamental concepts have been introduced; many of them are now taken for granted, but this was not always the case; however, angiogenesis inhibitors for the treatment of cancer have now been approved by the Food and Drug Administration in the US, and in 28 other countries including China [3]. Cancer cells cannot expand as a tumor past the minuscule size of 1 cbmm because diffusion of oxygen and nutrients as well as clearance of otherwise toxic metabolic substances is insufficient in masses larger than that size. In contrast to the physiological angiogenic signaling system, the tumor generates signals that are the results of the stresses of low oxygen supply, nutrient supply and hyperglycemia; even in the presence of adequate oxygen supply, many tumors metabolize the majority of the glucose they take up through glycolysis [4]. In tumors exposed to hypoxia, the transcription factor HIF (hypoxia-induced factor)-1α is activated and induces the transcription of glycolytic enzymes [5]. The HIF-1 dependent gene products are involved in tumoral angiogenesis and in the metabolic switch of differentially utilizing the glycolysis or oxidative phosphorylation pathways to ATP-generation depending on physiological or pathophysiological glucose levels within the tumor tissue [6]. In tumors, because of the complexity of a broad variety of different growing cells under the command of a plethora of soluble stimuli, the angiogenic response is often disorganized and chaotic. Tumor blood vessels exhibit numerous abnormalities, including dilations, incomplete or absent vascular endothelial linings and basement membranes, leakiness, irregular and tortuous architecture, arteriovenous shunts, blind ends, and lack of contractile wall components and cellular receptors. During cytotoxic chemotherapy, apoptosis of endothelial cells in the vascular bed of tumors proceeds apoptosis of tumor cells, even when the tumor has been made drug resistant. Administration of an angiogenesis inhibitor which is not directly cytotoxic to tumor cells can increase tumor cell apoptosis and inhibit tumor growth by inhibiting endothelial proliferation and migration and/or by inducing endothelial apoptosis [7], as recently shown for members of the ribosome-inactivating protein family [8].

The Multi-Faceted Activators and Inhibitors of Angiogenesis

Recently, it has been shown by Yang et al. [9] that angiostatin decreases cell migration and VEGF to pigment epithelium-derived factor mRNA ratio in vitro and in a murine ocular melanoma model. Angiostatin inhibits the migration of melanoma cells in vitro and decreased hepatic micrometastases in a mouse model of uveal melanoma; within the melanoma micrometastases it increases the pigment epithelium-derived factor mRNA which might be judged as an inducer of a cellular redifferentiation process.

Blood vessels and nerves often follow parallel trajectories, suggesting that distal targets use common cues that induce vascularization and innervation. Netrins are secreted by the floor plate and attract commissural axons toward the midline of the neural tube. Netrin-1 stimulates proliferation, induces migration, and promotes adhesion of endothelial cells and vascular smooth muscle cells with a specific activity comparable to VEGF and platelet-derived growth factor. Netrin-1 stimulates angiogenesis in vivo and augments the response to VEGF [10]. Netrin-1 is a secreted neural guidance cue with the unique capability to attract both blood vessels and axons, as Chédotal A. has described in detail in a previous chapter herein. It is most important to note that a chemokine, like netrin-1, is critical for axonal pathfinding but shares similarities with formation of vascular network. Netrin-1 induction of angiogenesis is mediated by an increase in endothelial nitritic oxide production which occurs via a – Deleted in Colorectal Carcinoma (DCC) – DCC-dependent ERK1/2-eNOS feed forward mechanism [11]. Furthermore, there is evidence that DCC induces apoptosis unless DCC is engaged by its ligand, netrin-1. The inhibition of cell death by enforced expression of netrin-1 in mouse gastrointestinal tract leads to the spontaneous formation of hyperplastic and neoplastic lesions [12].

Molecular Links between Neuronal Structures, Neuronal Signaling Substances, Blood Vessels and Tumor Cells

Since, and because of the continuously pioneering work of Folkman and colleagues, elucidating tumor angiogenesis, we are challenged with the important implications for understanding how new blood vessels are guided around blocked arteries and veins, and how they penetrate and aid the growth of cancer. VEGF can enhance the growth of blood vessel that nourish tumor cells and it may accelerate their spread. Neuropilins (NRP) are one entity of molecules bridging the gap between the vascular architecture of a tumor and the progression of cancer. NRPs are multifunctional non-tyrosine kinase receptors that bind to class 3 semaphorins (SEMA) and VEGF, and regulate two diverse

systems, neuronal guidance and angiogenesis. NRPs prevent nerve cells from taking a wrong turn as they wire-up a brain and nervous system growing in the womb. Finding NRPs on the surface of blood vessels indicates they may also guide their growth. Growing evidence supports a critical role for these ligand/(SEMA)–receptor/(NRP) interactions in tumor progression. NRP expression is up-regulated in multiple tumor types, and correlates with tumor progression and prognosis in specific tumors. NRPs may indirectly mediate effects on tumor progression by supporting angiogenesis or directly through effects on tumor cells [13]. The book on the role of NRPs and SEMAs in tumor progression and angiogenesis has just been opened [14], but the molecular introduction to this book is already written, namely by the description of multiple stimulating or inhibiting interactions of molecules, which are predominantly signal molecules of the nervous and, to a less extent, of the immune system [15, 16]. It is an eye-opener to find that molecules that give blood vessels, nerve cells and axons a sense of direction are involved in the cascade of metastasis; therefore, it is literally and scientifically correct to coin such an operational representation, where neuronal-derived molecules are directly engaged and are interacting with tumor growth and spread, as a neuro-neoplastic synapse. This view of an existing neuro-neoplastic synapse is further substantiated by the description of L1, which is a binding partner for NRP-1. NRP-1 belongs, as already mentioned, to the VEGF receptor family and NRP-1 expression may stimulate tumor growth, e.g. colon carcinoma cells, by enhanced angiogenesis and suppression of tumor cell apoptosis, which lead to metastasis and poor prognosis [17]. Interestingly, the neuronal cell adhesion molecule L1 was detected as a target gene of β-catenin-TCF signaling in colorectal cancer cells. L1 expression was high in spare cultures and co-regulated with ADAM10, a metalloprotease involved in cleaving and shedding L1's extracellular domain. L1 expression conferred increased cell motility, growth in low serum, transformation and tumorigenesis, whereas its suppression in colon cancer cells decreased motility. But what is very impressive, L1 was found exclusively localized in the invasive front of human colorectal tumors together with ADAM10 [18]. L1 is also located on ovarian carcinoma cells and when overexpressed associated with bad prognosis. NRP-1, a receptor for L1, was found only marginally expressed in primary ovarian carcinoma cells, but a strong expression could be observed in mesothelial cells, which form the lining of the peritoneum. Ovarian carcinoma cells expressing L1 can bind to NRP-1 overexpressing cells and mesothelial cells; this ligand (L1)–receptor (NRP-1) interaction suggests that ovarian carcinoma cells can bind mesothelial cells [19] and may therefore contribute to peritoneal carcinomatosis. The constitutive role of SEMAs as regulating proteins of cell motility and attachment in axon guidance, vascular growth, immune cell regulation and tumor progression was reviewed

recently by Kruger et al. [20]. It becomes more and more clear that axon guidance molecules such as netrins, SEMA, slits, ephrins and vasoactive intestinal peptide [21] provide the cues required for accurate patterning of axonal projections in the nervous system. However, by summarizing the literature, multiple paradigms by which these molecules interact with integrin adhesion receptors in and outside the neuronal tissues [22], and, above all, in tumor tissues, are described, therefore, providing the archetype of molecular transmitters of a neuro-neoplastic synapse.

Perineural Invasion of Carcinoma Cells

Prostate carcinoma is often associated with perineural (PN) invasion, as Muller has mentioned above. PN invasion by prostate carcinoma cells may not only be a volume effect of growing carcinomas; the neural components may favor the growth of carcinoma cells by inhibiting apoptosis, presumably through a paracrine mechanism, and thereby facilitate the spread of carcinomas along nerves. The heterogeneity in growth potential of prostate carcinoma cells may be determined by their local microenvironments, such as an association with neural components [23]. Indeed, perineurium production of caveolin-1 is involved in a paracrine anti-apoptotic loop in PN invasion. Transforming growth factor-β1 is up-regulated in the cancer cells as they approach the nerve and is thought to up-regulate caveolin-1 in the perineurium of nerves within prostate cancer. Caveolin-1 is then secreted into the microenvironment and used by prostate cancer cells to inhibit apoptosis [24]. The quantification of cancer invasion into the PN space influences the prognosis of patients treated with radical prostatectomy; PN invasion is an independent predictor of prognosis [25].

To understand the relationship between disease progression and pain in pancreatic cancer a transgenic mouse model was developed [26]. In these mice precancerous cellular changes were evident at 6 weeks and these included an increase in microvascular density, macrophages that express nerve growth factor and the density of sensory and sympathic fibers that innervate the pancreas, with all of these changes increasing with tumor growth. Samples of tissue from patients undergoing resection of pancreatic carcinoma were studied by electron microscopy and light microscopy by Bockmann et al. [27]. The adenocarcinoma is not confined to the periphery of nerves. It penetrates the perinuerium and becomes intimately associated with Schwann cells and axons in the endoneurium. Transforming growth factor-α is abundant in nerves in the pancreas. Interaction of transforming growth factor-α in nerves with EGFR on cancer cells constitutes a possible paracrine mechanism that provides a growth advantage for pancreatic adenocarcinoma and serves as an example of potential

cross-talks that might be active in biological interaction of cancer with nerves, called neuro-neoplastic synapse.

Human Tumor Dormancy: A Neurogenic Switch from the Non-Neurogenic Phenotype

Microscopic human cancers can remain dormant for life. Recently, a model of tumor progression was introduced, depending on sequential events, including a switch to the angiogenic phenotype, e.g. initial recruitment of new vessels [28]. For some tumor entities, e.g. breast, prostate, colon, pancreas and lung tumors, this model can be extended by including the formation of a neuro-neoplastic synapse as a cue for tumor cell progression, induction of migration and acquisition of a metastasizing phenotype. Human tumors contain cell populations that are heterogeneous in angiogenic activity, proliferation capacity, tumor cell stromal cell cross-talk signaling, and, if ever, in apoptosis induction, or inhibition. With the described molecular links to angiogenesis, we introduce a conceptual framework by including the neuro-neoplastic synapse that dormant tumor cells in a tiny tumor cell conglomerate are stimulated by the different neuronal-derived cues and, thus, are converted into a proliferative and invasive phenotype by induction of: (i) proliferation, (ii) migration, (iii) expression of metalloproteases, (iv) escaping from immunosurveillance [29] and (v) gaining the capability to settle in distant organs, if neo-angiogenesis and neo-lymphangiogenesis [30] provide the appropriate intra-tumoral vasculare architecture to facilitate the evasion of these cells – already from a small and solid but growing tumor mass.

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