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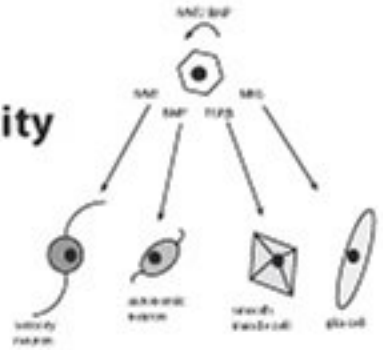
Neural Plasticity and Repair

Editor
Paul Herrling, Basel



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Preface

The National Centres of Competence in Research (NCCRs) were set up as a research promotion tool of the Swiss government and realized by the Swiss National Science Foundation. By promoting the NCCRs, Switzerland intends to strengthen research in strategically important areas, such as innovative technologies or diseases of major societal impact. Federal support of an NCCR lasts around 10 years. The currently ongoing NCCRs were rigorously selected among hundreds of applications and were considered as being of nationwide importance, high quality with a particular emphasis on interdisciplinary and innovative approaches within the disciplines.

Each NCCR consists of a 'leading house' and a network of partners from the university or extra-university spheres. Zurich took such a lead successfully for the NCCR 'Neural Plasticity and Repair' (NCCR Neuro). Diseases of the central nervous system (CNS) are among the most debilitating illnesses, putting an enormous strain on both social and health care budgets in Switzerland. To date, only limited help is available to patients suffering from disorders such as Alzheimer's disease, stroke, epilepsy, multiple sclerosis or traumatic CNS injury, which affect more than 200,000 patients in Switzerland. Altogether, the underlying aetiologies are still poorly understood. There are currently no treatments available that can halt or prevent, let alone reverse nerve cell degeneration. The NCCR Neuro was designed to improve our molecular understanding of the aforementioned devastating diseases to develop better treatments or even cures. In particular, the NCCR has two principal tasks: to develop innovative treatments and new forms of rehabilitation in order to significantly improve the quality of life of the patients affected, and to train recruits for the research professions to an internationally recognized level. Scientists from the domains of basic and clinical research work together towards this goal in a total of 53 research groups. At present, the NCCR Neuro consists of eight projects: (1) Neural Stem Cells: An Integrated Approach to Basic Knowledge and Therapeutic Applications; (2) Alzheimer's Disease; (3) Functional Recovery after Stroke; (4) Epilepsy: New Models and Therapeutic Strategies; (5) Cortical Plasticity; (6) Infection and Immunity of the Central Nervous System; (7) Spinal Cord Repair; (8) Rehabilitation Technology Matrix. Additional support is provided from four 'Centers of Technical Expertise' with the topics 'Novel Transgenic Systems', 'Advanced Assessment of Animal Behaviour', 'Proteomics' and 'Animal Imaging'.

This special issue has selected a few highlights from this programme demonstrating the progress made in the fields of Alzheimer's disease, stroke, epilepsy, spinal cord injury and brain inflammation. Particular emphasis is placed on innovative therapeutic strategies including the use of stem cells, rehabilitation technologies and mechanisms of neuronal plasticity. It is hoped that such integrated endeavours will help to identify novel avenues for treatment and prevention of CNS diseases in the future.

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The Neural Crest: Understanding Stem Cell Function in Development and Disease

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Key Words
Neural crest · Stem cells · Signaling · Cancer stem cells

Abstract
Complex organs like the nervous system are composed of different cell types which are all derived from multipotent stem cells. In vertebrates, a transient population of stem cells, the neural crest, generates the entire peripheral nervous system as well as non-neural progeny. The developmental processes of cellular differentiation and proliferation require precise coordination and control. Errors in the programs that regulate stem cell function can lead to defects that manifest in developmental disorders, in some cases they might even induce cancer. It is therefore of fundamental interest to understand the mechanisms of stem cell maintenance and differentiation. Using the neural crest as a model system helps us not only to understand the role of stem cells in development but might also lead to new aspects for the cure of stem cell-related diseases.

Introduction

Stem cells are the source of all cell types that exist in our bodies. Stem cells are usually defined in terms of two characteristics: first, the ability to produce several types of differentiated progeny (multipotency) and second, the property of self-renewal, meaning that cell division of a stem cell leads again to at least one new stem cell. Not surprisingly, as more has become known about how stem cells function, their therapeutic potential has been envisaged and stem cells have become a research field of major interest. Still, in order to use stem cells as a source for tissue repair and cell replacement therapies we first need to understand in detail how they fulfill their extraordinary functions. Clearly, the processes of differentiation and self-renewal require tight coordination and regulation. Hence, the elucidation of the genetic programs that are at the base of stem cell regulation are of fundamental interest. Furthermore, the question arises of what might happen if these regulatory programs undergo fatal errors. Indeed, malformations may occur when stem cells differentiate at the wrong time, in the wrong place or when their ability to differentiate or self-renew is unexpectedly altered in some way. Especially during development this may prove fatal and lead to developmental disorders manifesting in a disease. Moreover, there is increasing evidence that deregulation of stem cells may lead to malignancy and produce aggressive cancers [1].

In order to understand the abilities of stem cells we need appropriate model systems that allow us to tackle our unresolved questions. One of these suitable systems is provided by neural crest stem cells (NCSCs), a migratory population of cells that arises during development from the dorsal neural tube at the time of neural tube closure [2]. Neural crest cells are of ectodermal origin

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and only exist in vertebrates. Their appearance in evolution is not fully understood, but because of their significant contribution to the cellular diversity in vertebrates and their unique features some might consider the neural crest as an addition to the three germ layers, ectoderm, mesoderm and endoderm [3]. NCSCs can give rise to both, neural and non-neural structures. For example, most cells of the peripheral nervous system, e.g. sensory neurons in the dorsal root ganglia, sympathetic and parasympathetic neurons of the autonomic nervous system, chromaffin cells of the adrenal gland and the enteric nervous system of the gut are all of neural crest origin. Non-neural cells comprise melanocytes (the pigment cells of the skin), smooth muscle cells of the heart outflow tract and cranial blood vessels and craniofacial bone and cartilage. This large variety of cells that arise from NCSCs makes them a highly interesting source to investigate how cellular diversity is achieved. Several model organisms are commonly used in neural crest research. The most common are zebra fish, chicken, rat and mouse, while quail-chick chimeras have been proven useful for cell grafting experiments [4–6]. The development of in vitro systems, such as for example neural tube explant cultures, that allow the cultivation of pure NCSC populations, significantly contributed to the field of stem cell research [7]. Altogether, the neural crest serves as a valuable model system for a range of developmental processes, including induction, specification and differentiation, as well as guided cell migration and epithelial-to-mesenchymal transition [8, 9].

In the following we will summarize current knowledge regarding some of the factors that regulate NCSCs, findings that may be fundamental on the way to therapeutic applications of stem cells. We will approach the link between the neural crest and certain diseases, showing the molecular and cellular events that may lead to a specific disease. Finally we will evaluate current knowledge concerning neural crest-derived tumors, whether NCSCs might be at the origin of certain cancers, and how this might suggest new ideas for cancer therapies.

Factors Regulating NCSCs

Neural crest cells arise at the border of neural plate and epidermis at the time of neural tube closure [8]. Following the folding process and neural tube closure neural crest precursors are localized in the dorsal part of the neural tube. There they undergo an epithelial-to-mesenchymal transition and detach from the neural tube in a process called delamination. They acquire unique features that distinguish them from their tissue of origin and begin to migrate extensively throughout the embryo. Significant progress has been made in identifying the mechanisms leading to the induction and specification of the neural crest. We will only outline the factors involved in these steps and refer the interested reader to already existing reviews that give more detailed information [8, 10].

One of the early inductive signals, BMP4, is expressed in the border region of the epidermis and the neural plate. It was shown that BMP has a dose-dependent effect during neural crest induction. Indeed, high levels of BMP give rise to epidermis, intermediate levels to neural crest and low levels to neural tissue [8]. In addition to BMP, the WNT signaling pathway plays a major role in neural crest induction. Gain and loss of function experiments with members of the WNT signaling pathway have established a requirement for WNT signaling in the induction of the neural crest [11–13]. The actions of early inducing signals trigger the activation of transcription factors in the prospective neural crest leading to further specification. Among these are AP-2, FOXD3, SLUG and SNAIL, c-MYC, RHOB, ZIC-family and SOXE transcription factors (SOX8, SOX9 and SOX10) [9, 10].

Interestingly some of the factors that act during early neural crest induction and specification will later on play a role in cell fate decisions of the neural crest. SOX9 seems to be required during early neural crest formation, is down-regulated in migratory NCSCs, but is again expressed in neural crest progenitors that form craniofacial structures [14, 15]. The same is true for canonical WNT signaling and the BMP pathway. While both have an 'early' role in neural crest induction, they will at a later timepoint have instructive roles in cell fate specification of multipotent neural crest cells, as we will see in the following paragraph.

Following delamination neural crest cells migrate via defined pathways to various locations to contribute to diverse tissues. During and after migration they undergo specific differentiation steps which are influenced by environmental signals (fig. 1). Again BMP signaling plays an important role. More precisely, BMP2 acts as a growth factor and instructs NCSCs to become autonomic neurons of the peripheral nervous system [16]. In addition, canonical WNT signaling acts instructively to promote sensory neurogenesis from NCSCs [17, 18]. The role of canonical WNT signaling in cell fate specification of neural crest cells contrasts with its known function in stem cells from other tissues: in embryonic, intestinal,

skin and hematopoietic stem cells, WNTs mainly regulate proliferation of the respective stem cell pools and do not influence fate specification [19]. This suggests that responsiveness to WNTs depends on cell-intrinsic features and the environmental context. It also clearly separates the behavior of NCSCs in the presence of WNT signaling from that of other types of stem cells. Interestingly, the simultaneous action of WNT and BMP signaling on NCSCs does not induce one particular cell fate, but rather keeps these cells in a nondifferentiated multipotent state [20]. Other instructive factors that are involved in neural crest differentiation are glial growth factor neuregulin (NRG), which is required for peripheral gliogenesis and transforming growth factor-β (TGF-β) signaling, which induces non-neuronal fates, for example smooth muscle cells [2].

The transcription factor SOX10, a member of the SRY-related high mobility group (HMG) of proteins, fulfills a dual role in the neural crest. During the early phase of migration SOX10 is expressed in all NCSCs, whereas later its expression is mainly restricted to cells of the glial lineage [21]. In addition to its role in gliogenesis, studies have demonstrated a requirement for SOX10 to maintain multipotency of migratory and postmigratory NCSCs. SOX10 keeps NCSCs responsive to instructive growth factors that induce different cell lineages [22, 23]. Furthermore, lack of SOX10 leads to increased apoptosis in undifferentiated postmigratory neural crest cells [22].

It may appear surprising that a relatively small number of signaling molecules are sufficient to control the diverse phenomena of stem cell maintenance and their fate decisions. Possibly combinations of these signals have synergistic or antagonistic effects on one another, creating a network of possible inputs on stem cells. Cellular response to these signals may further depend on the local environment of the cell, where the extracellular matrix and neighboring cells influence the cellular interpretation of instructive signals.

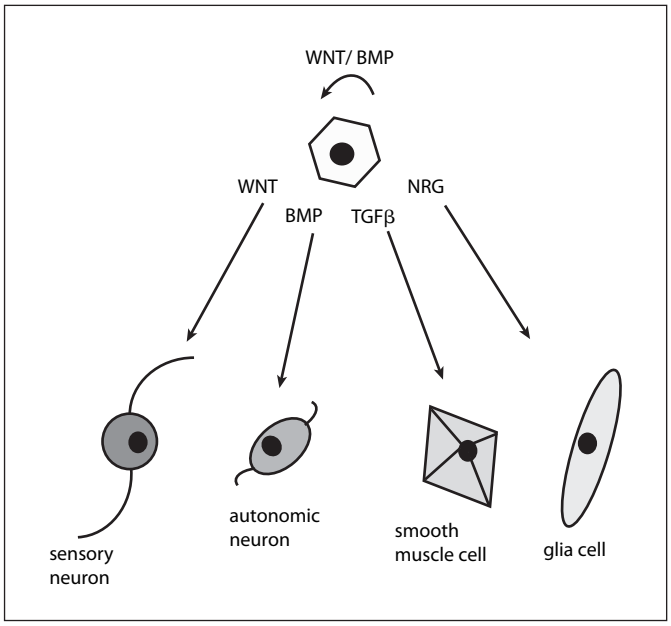

Fig. 1. NCSC response to instructive signals. The combination of WNT and BMP signaling maintains NCSCs in a multipotent state. WNT signaling alone instructs NCSCs to induce sensory neurogenesis and BMP signaling alone induces autonomic neurogenesis. Glial differentiation is induced by NRG, while non-neural differentiation, i.e. into smooth muscle cells, requires TGF-β signaling.

Neural Crest in Human Congenital Diseases

With the neural crest contributing to a variety of tissues and organs it is not surprising that it is also of clinical relevance. In fact, developmental abnormalities of the neural crest are implicated in several congenital diseases, called neurocristopathies. These may be caused by mutations that affect NCSC function. Such links between molecular events of stem cell regulation and disease are of particular interest as they may serve to better understand both developmental regulation and disease mechanism. One good example is Hirschsprung's disease (HSCR), also called aganglionic megacolon, a relatively common neurocristopathy affecting the enteric nervous system. Patients with HSCR show absence of enteric ganglia (agangliosis) along a variable length of the intestine, with the distal part of the gut being most often affected. HSCR manifests itself in chronic constipation, as a lack of innervation impairs gut motility and enzyme secretion into the intestinal lumen. In HSCR, enteric progenitor cells (EPCs) fail to completely colonize the gut. Normal EPCs are self-renewing and multipotent, giving rise to diverse neurons and glia of the enteric nervous system, and persist in the adult organism [24–26]. Known causes of incomplete colonization are either premature differentiation or impaired migration of EPCs [24, 27]. The primary genes affected in HSCR are indeed required for proper EPC development and function. Among these are the receptor-tyrosine kinase (RET), endothelin receptor type B (EDNRB), endothelin 3 (EDN3), glial cell line-derived neurotrophic factor (GDNF), SOX10 and others such as endothelin converting enzyme 1 (ECE1) or smad inter-

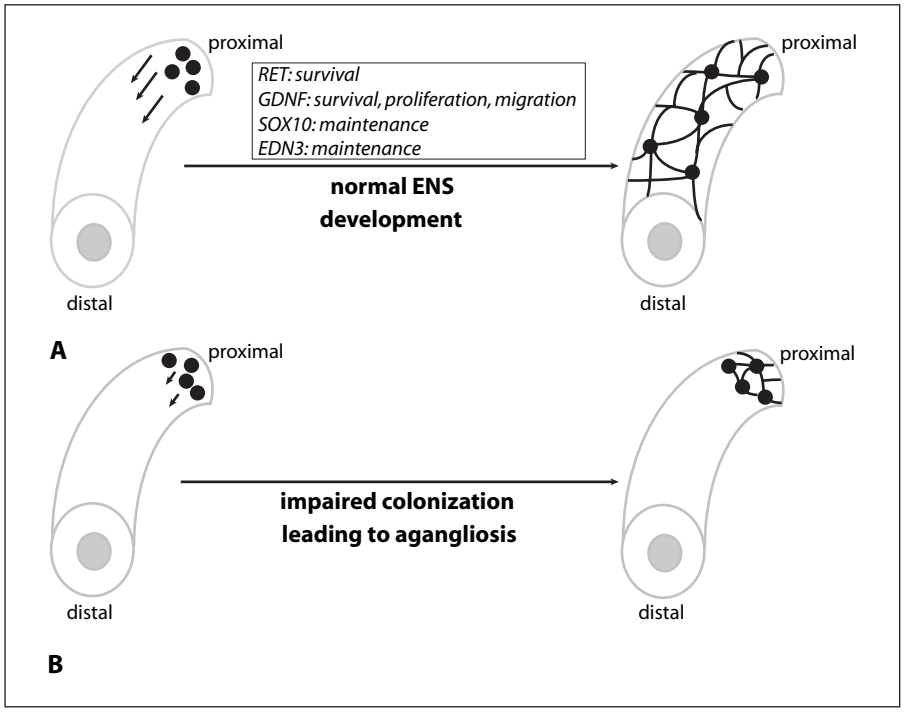

Fig. 2. A simplified model of gut colonization by enteric nervous system precursor cells. **A** EPCs migrate along the gut in proximal to distal direction. EPCs colonize the entire intestine and differentiate into the cells of the enteric nervous system. **B** Mutations in important regulatory genes lead to impaired EPC colonization of the gut. As a result, innervation of the gut is incomplete.

acting protein 1 (SIP1). GDNF acts as a chemoattractant and promotes proliferation and survival of enteric neural crest cells in vitro [28]. The GDNF receptor RET, however, is specifically expressed in EPCs and is necessary for their migration and survival in the gut [27, 29]. Mutations in RET are among the most common in HSCR patients. EPCs that carry a heterozygous mutation in the transcription factor SOX10 initially show normal migration into the proximal intestine. While their survival capacity remains unaffected, these cells are unable to maintain their progenitor state, leading to premature differentiation and hence to a reduction of the progenitor cell pool size [24]. This depletion then causes incomplete colonization of the more distal gut (fig. 2). Further confirmation comes from a recent study showing that SOX10 overexpression inhibits neuronal and glial differentiation of ENS progenitors without affecting their multipotency [30]. The authors also addressed the function of EDN3 in enteric nervous system development. The data indicates that EDN3 is needed for the maintenance of EPCs. EDN3 is a ligand for EDNRB, a receptor found to be mutated in HSCR patients [31, 32]. Whether the requirement for EDNRB in enteric nervous system development concerns migration or maintenance of enteric precursors has not been clarified so far.

Other neurocristopathies can also affect non-neural derivatives of the neural crest. Promotion of non-neural lineages in NCSCs largely depends on TGF-β signaling. Indeed, mice lacking the TGF-β type II receptor in neural crest cells show disorders of eye development and additionally display a phenotype resembling DiGeorge syndrome, characterized by anomalies in the development of craniofacial bone and cartilage, the outflow tract of the heart, as well as thymus and parathyroid glands [33, 34].

Taking the example of the enteric nervous system development, it appears that neural crest cells have to integrate a variety of intrinsic and extracellular signals. These processes conceivably involve interactions between distinct factors. Unraveling these interactions between already established and yet unknown regulators will be an important step for the further understanding of developmental and disease mechanisms.

Neural Crest in Cancer

Tumors are characterized by uncontrolled proliferation of tissue that does not serve a physiological function. They usually show a heterogeneous cellular composition; indeed not all cells within a tumor have the same malig-

nant potential. This may appear surprising as many tumors have a clonal origin. So what is the origin of this heterogeneity? Recent reports have established the concept of a cancer-initiating cell (or cancer stem cell) that has the potential to initiate and form tumors [35–38]. In analogy to a normal stem cell, such a cancer stem cell can self-renew and undergo multilineage differentiation, giving rise to more benign progeny with limited proliferative potential (fig. 3). While the benign progeny cells compose the major part of the tumor, a relatively small number of proliferative cancer stem cells are sufficient to cause aggressive tumor growth. These findings open up new perspectives for cancer therapies as it might be more efficient to target a specific cell population within a tumor instead of assuming the tumor to be functionally homogeneous. Data on molecular and cellular analysis of tumor biology might need to be reviewed in the light of these findings, as many studies have simply addressed tumors as a single entity. Identification of a specific subset of cells in a tumor requires availability of suitable markers. For example, in the case of medullo- and glioblastomas, aggressive cancers of the central nervous system, the marker CD133 was successfully used to isolate a population of malignant cells with tumor-initiating potential. Moreover these cells showed features reminiscent of neural stem cells [36]. In the case of breast cancer, an epithelial tumor, a fraction of cells expressing CD44 but little or no CD24 (CD44+CD24–/low) showed sustained tumor initiation and growth by serial passaging in a xenotransplantation assay. Furthermore, these cells also generated populations of nontumorigenic cells with different marker expression [35].

The question arises as to where these cancer stem cells originate. Several theories exist, assuming that mutations in somatic stem cells might cause these cells to exit from tight proliferation control. More restricted progenitor or differentiated cells might acquire malignant stem cell properties through mutations. Also, cell fusion events which normally occur in development, e.g. in muscle formation, might be a source of cancer, as fusion events might generate cells with malignant properties [39]. In any case, the influence of the cellular environment should be considered as an additional trigger for the appearance and dissemination of cancer stem cells.

With the extensive contribution of neural crest cells to the vertebrate body, several tumors can be assigned to a neural crest origin. Among these are melanoma, skin tumors which arise from melanocytes, neuroblastoma, a cancer of sympathoadrenal cells and pheochromocytoma, tumors of chromaffin cells of the adrenal medulla or extra-adrenal paraganglia. Might the concept of tumor appearance through cancer stem cells also apply to neural crest-derived tumors?

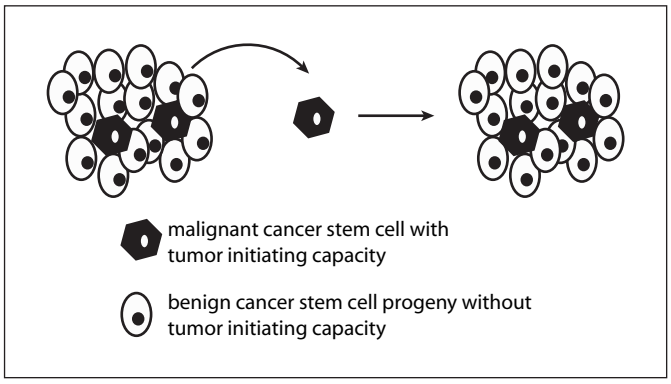

Fig. 3. Tumor initiation and growth can be driven by cancer stem cells. Besides their malignant proliferative potential, these cells are able to self-renew and to give rise to benign progeny cells that represent the major part of the tumor. Benign cancer cells show reduced proliferative potential and are not able to induce tumors de novo.

In a recent study a subpopulation of cells was found in metastatic melanomas, which could differentiate in vitro into cells reminiscent of some neural crest lineages. These cells might thus have similarities to neural crest stem or progenitor cells, although this has not yet been analyzed [40]. Moreover, upon transplantation of labeled adult human metastatic melanoma cells in ovo into the premigratory neural crest of chicken embryos, melanoma cells distributed along neural crest migratory pathways. They seemed to have lost their tumorigenic potential and partially acquired normal neural crest features. The authors conclude that reprogramming through the embryonic microenvironment occurred [41]. Although this is no proof of any involvement of NCSCs in melanoma formation it is interesting that melanoma cells are able to adopt characteristics of non-pigment neural crest derivatives.

Neuroblastomas are among the most common pediatric tumors. They arise from precursor cells of the neural crest-derived sympathoadrenal lineage. Neuroblastoma is often characterized by an enigmatic clinical behavior: these tumors may regress spontaneously, especially when occurring before 1 year of age. However, aggressive tumor growth and metastatic disease are often observed when neuroblastoma is diagnosed after 1 year of age. It is still unclear whether favorable and unfavorable neuroblastomas arise from a common precursor or if they rather have distinct origins. The occurrence of neuroblasto-

mas at a rather early time in life suggests that their occurrence and behavior may be linked to developmental programs that are still ongoing after birth and that might involve rather undefined stem or precursor cells of the sympathoadrenal lineage. In agreement with this idea, certain neuroblastoma cell lines contain cells with morphological features and differentiation behavior characteristic of prospective stem cells [42]. Compared to neuroblastoma cell lines without stem cell features, candidate cancer stem cells showed the ability to differentiate, had a growth advantage in vitro and displayed higher tumorigenicity when transplanted into athymic mice [42]. The authors state that these prospective cancer stem cell lines showed expression of CD133 and c-KIT (CD117), markers that are more commonly assigned to hematopoietic (CD133 and c-KIT) and neural stem cells (CD133). In another study the occurrence of a 'side population' (SP) was reported in primary neuroblastomas and cell lines. SP cells are characterized by a high efflux capacity for antimitotic drugs. Interestingly, SP cells from neuroblastomas showed the capacity to generate both SP and non-SP cells in vitro, indicating the potential to self-renew and differentiate [43].

It is tempting to speculate that cancer stem cells might be a source for at least some cases of neural crest tumors. To clarify this point it is necessary to better define the cellular composition of neural crest-derived tumors, such as melanomas and neuroblastomas mentioned here. Detailed analysis of relevant marker expression and better functional characterization of tumor cells at a cellular and molecular level should help to understand the etiology of tumorigenesis in neural crest cells. Thus, detailed analysis of the mechanisms regulating NCSC fates during development and disease may be worthwhile goals on the way to establish novel anticancer treatments.

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Amyloid-β Aggregation

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Key Words
Amyloid-β · Alzheimer's disease · Protein misfolding · Neurodegeneration · Oligomers

Abstract
Alzheimer's disease (AD) is the most prevalent neurodegenerative disease in the growing population of elderly people. A hallmark of AD is the accumulation of plaques in the brain of AD patients. The plaques predominantly consist of aggregates of amyloid-β (Aβ), a peptide of 39–42 amino acids generated in vivo by specific, proteolytic cleavage of the amyloid precursor protein. There is a growing body of evidence that Aβ aggregates are ordered oligomers and the cause rather than a product of AD. The analysis of the assembly pathway of Aβ in vitro and biochemical characterization of Aβ deposits isolated from AD brains indicate that Aβ oligomerization occurs via distinct intermediates, including oligomers of 3–50 Aβ monomers, annular oligomers, protofibrils, fibrils and plaques. Of these, the most toxic species appear to be small Aβ oligomers. This article reviews the current knowledge of the mechanism of Aβ assembly in vivo and in vitro, as well as the influence of inherited amino acid replacements in Aβ and experimental conditions on Aβ aggregation. Challenges regarding the reproducible handling of the Aβ peptide for in vitro assembly studies are discussed.

Introduction

*The Amyloid Hypothesis – Which Role Does Amyloid-*β *Play in Alzheimer's Disease?*

Alois Alzheimer described the first case of fatal progressive dementia known as Alzheimer's disease (AD) 100 years ago [1]. AD is characterized by accumulation of extracellular amyloid plaques and intracellular neurofibrillar tangles in human brain tissue. The extracellular deposits are predominantly composed of aggregated amyloid-β (Aβ) peptide [2], whereas neurofibrillar tangles are aggregates of hyper-phosphorylated forms of the neurofilament-associated protein tau [reviewed in ref. 3]. Today, AD is considered the most frequent of more than 20 known amyloidoses with fibrillar protein deposits [reviewed in ref. 4, 5].

The sequence of the Aβ peptide was first determined for Aβ samples derived from meningeal blood vessels of AD and trisomy 21 patients [6]; it is a normal product of cellular protein degradation [7]. The 39–42 amino acid peptide (Aβ1–39 and Aβ1–42) is cleaved from the C-terminal segment of the amyloid precursor protein (APP) [8], an integral membrane protein involved in signal transduction, with different isoforms caused by alternative splicing [9, 10]. The N-terminus of Aβ is generated by β-secretase cleavage followed by C-terminal cleavage by the γ-secretase complex, containing presenilin (PS) 1 and 2 [reviewed in ref. 11].

Numerous investigations led to the formulation of the 'amyloid hypothesis', stating that Aβ aggregation is the

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cause rather than an effect of AD [reviewed in ref. 12]. The most significant facts favoring the amyloid hypothesis are the colocalization of Aβ aggregates with dying neurons, the association of amino acid replacements in Aβ with early-onset familial AD (FAD), the increased aggregation tendency of FAD-linked Aβ variants in vitro [13], and the generation of an AD phenotype in transgenic mice overexpressing APP or overproducing Aβ by enhanced cleavage of APP [14–16]. As AD is also associated with an increase in the ratio between Aβ1–42 and Aβ1–40, it appears that Aβ1–42 is the predominantly toxic and/or amyloidogenic species [16, 17], although there is generally a tenfold excess of Aβ1–40 over Aβ1–42 [18]. Arguments against a causative role of the protein tau, which forms the neurofibrillar tangles in neurons of AD patients [3], are that Aβ aggregation precedes formation of neurofibrillar tangles [19] and that, in contrast to Aβ deposits, tangles are not necessarily present in human AD brain tissues [20]. In addition, inherited mutations in tau lead to frontotemporal dementia with parkinsonism, but do not induce AD [21, 22].

*How Can A*β *Exhibit Neurotoxic Characteristics?*

The question of how assemblies of misfolded protein cause toxicity is perhaps more complex than the mechanism of the aggregation process itself. The proposed neurotoxic effects of Aβ are oxidative stress, the formation of ion channels and membrane disruption, recruitment of cellular factors or activation of cellular processes such as apoptosis and inflammation [12, 23–25]. Initially, the attention focused on Aβ fibrils [26], but evidence has accumulated during the last years that lower-order Aβ assemblies are the neurotoxic species. AD symptoms such as memory and cognitive deficits as well as synaptic loss correlate better with the amounts of soluble Aβ oligomers than with the appearance of insoluble plaques in the brains of AD patients as well as in mouse models [27–31], and initial AD symptoms even occur before plaques accumulate [32, 33]. In addition, the concentration of soluble, lower-order Aβ oligomers is elevated in human AD brain tissue [34, 35] and the formation of soluble oligomers precedes the development of AD symptoms [36, 37]. Recently, defined oligomeric species isolated from murine AD brains and cell cultures have been shown to exhibit toxic characteristics [38, 39].

The extracellular location of AD plaques initially led to the assumption that toxicity results from extracellular attack of neurons by Aβ [40], but Aβ has also been shown to exist intracellularly in cell cultures and in rat brain tissue [41], and there is evidence that intracellular, nonfibrillar Aβ oligomers cause cytotoxicity in human neurons that strongly exceeds that of extracellular Aβ species [42].

Identified and Proposed Key Players – from Aβ Monomers to Plaques

*Isolation and Production of Intermediates in the Pathway of A*β *Aggregation*

Enormous efforts have been made to identify and characterize intermediates in the pathway of Aβ aggregation. Several approaches focused on the isolation of aggregated Aβ species from postmortem tissue and body liquids such as cerebrospinal fluid (CSF), which were mostly purified by size exclusion chromatography or ultracentrifugation, and probably represent the in vivo situation in the most reliable manner [35, 38, 39, 43]. On the other hand, numerous in vitro investigations such as kinetic and structural studies with synthetic or recombinantly expressed Aβ peptides were carried out [44] [see refs. 18, 45 for synthesis and purification methods]. Stabilization of transient intermediates during in vitro assembly of Aβ has turned out to be very challenging [46]. The following section reviews the so far identified Aβ species in the Aβ aggregation zoo (table 1).

Monomer Structure

The Aβ sequence has an amphipathic character, as the N-terminal segment is hydrophilic, whereas the 12–14 C-terminal amino acids are very hydrophobic (fig. 1A). This is consistent with the predicted localization of Aβ residues 1–28 in a soluble, extracellular APP domain, while residues 29–42 are contained in a predicted transmembrane helix of APP [11]. In water-alcohol mixtures or micelle solutions, Aβ adopts α-helical conformation with two helical segments interrupted by a so-called kink region, which adopts disordered or β-turn conformation [47, 48]. In aqueous solution, a random coil structure of Aβ1–40 has been observed [49]. Aβ1–42 adopts β-sheet structure rapidly at physiological pH, in contrast to Aβ1–39, which has the tendency to preserve the random-coil conformation over longer times prior to self-assembly into β-sheet-rich oligomers [50]. Some studies suggest that β-sheet conformation in Aβ is initiated by a β-turn formed by residues 24–28 prior to oligomerization [51, 52].

Dimers

Stable Aβ dimers have been detected in in vitro experiments with synthetic Aβ1–40 and Aβ1–42 [53, 54],

Table 1. Intermediates in the pathway of Aβ fibrillization

Aβ species	Characteristics	References
Monomers	soluble amphipathic molecule; generated from APP; potential α-helical, random coil or β-sheet conformation	11, 49–52
Dimers	intracellular localization in vivo, in human brain extracts and in vitro; hydrophobic core; diameter of about 35 nm	53–57, 199
Trimers	observed in vivo in mouse models; potential key role as subunit of toxic oligomers	39, 62
Small oligomers	observed in vivo in AD patients as well as in mouse models and in vitro; heteromorphous; comprising of 3–50 monomers; mostly transient and unstable, but toxic	28, 30, 31, 34, 35, 38, 39, 46
Annular oligomers	observed in cell culture and in vitro experiments; potential role as membrane-disrupting pores or ion channels	68–71, 200
ADDLs	observed in murine and human brain extracts as well as in vitro; nonfibrillar; neurotoxic; 17–42 kDa; trimers to 24mers	63–67, 201
Protofibrils	observed in vitro; short, flexible, rod-like structure; maximum size 8 × 200 nm; binding Congo red and thioflavin T; precursor of mature fibrils; toxic	66, 72–80, 202
Fibrils	observed in AD patients as well as in mouse models and in vitro; bind Congo red and thioflavin T; stable, filamentous Aβ aggregates composed of repeating Aβ units perpendicular to the fiber axis	3, 81, 82, 84, 92, 203
Plaques	observed in vivo in AD patients as well as in mouse models; large extracellular Aβ deposits; predominantly composed of fibrils; not toxic; surrounded by dystrophic dendrites, axons, activated microglia and reactive astrocytes	11, 36, 96, 97

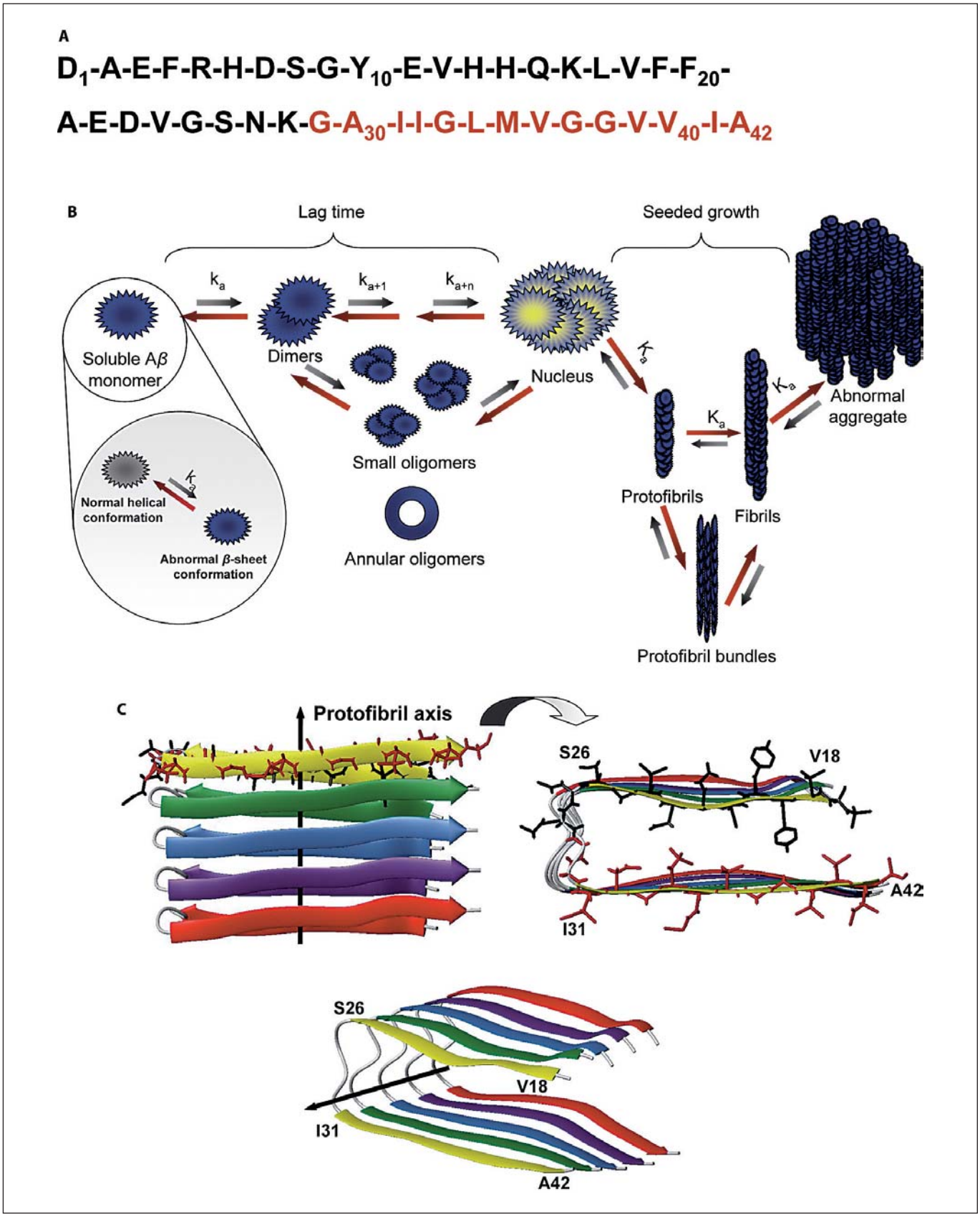
human brain homogenates [55] and cell culture media [56], and Aβ dimers detected in vivo are supposed to form intracellularly [57]. Aβ dimers isolated from the human brain seem to have a hydrophobic core and a diameter of about 3–4 nm [55].

Small (Globular) Oligomers

Small Aβ oligomers are probably the most intensively studied group of intermediates in Aβ aggregation, and comprise oligomers of 3–50 Aβ subunits. The smallest low-molecular-weight oligomers have been found in in vitro experiments, in the growth medium of Aβ-secreting cells, the CSF and human brain homogenate [30, 31, 34, 46]. The small Aβ oligomers are in the center of interest because they generally exhibit much higher cytotoxicity than insoluble Aβ fibrils [28, 35, 38, 39]. It has been postulated that the initial phase of oligomerization of Aβ1–42 in vitro, in contrast to that of Aβ1–40, involves the formation of mostly unstructured pentamers/hexamers termed paranuclei. Notably, formation of paranuclei is prevented when methionine 35 (M35) of Aβ is oxidized to methionine sulfoxide [58, 59]. Further small oligomeric species that have been identified include a stable 60-kDa oligomer of Aβ that was found in vivo in murine and in human brain as well as in vitro and has been termed Aβ1–42 globulomer [60], and an Aβ 48mer only obtained in in vitro experiments [61]. Very recently, the 56-kDa assembly Aβ*56, corresponding to an Aβ dodecamer, was isolated from mouse brain and shown to exhibit cytotoxicity in vivo [39]. Another recent study revealed a potential role of trimers as building blocks of Aβ oligomers in mice [62].

*A*β*-Derived Diffusible Ligands*

Aβ-derived diffusible ligands (ADDLs) are nonfibrillar and neurotoxic *A*β oligomers ranging in size from 17 to 42 kDa. They were first described for in vitro fibrillization experiments, but have also been found in soluble extracts of human and murine brain tissue [63–65]. They could also be allocated to the group of small Aβ oligomers [64]. ADDLs contain primarily trimers to hexamers, but also larger species up to 24mers and morpho-


A
D1-A-E-F-R-H-D-S-G-Y10-E-V-H-H-Q-K-L-V-F-F20-
A-E-D-V-G-S-N-K-G-A30-I-I-G-L-M-V-G-G-V-V40-I-A42
B
Lag time
Seeded growth
ka
ka+1
ka+n
Soluble Aβ monomer
Dimers
Nucleus
Small oligomers
Annular oligomers
Normal helical conformation
Abnormal β-sheet conformation
Protofibrils
Fibrils
Abnormal aggregate
Protofibril bundles
C
Protofibril axis
S26
V18
A42
I31
S26
V18
I31
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logically resemble spherical protofibrils [66]. Interestingly, the ADDL levels in brain tissue and CSF correlate with the cognitive defects of AD patients [67]. Moreover, ADDLs are also neurotoxic in vitro [64, 65].

Annular, Pore-Like Oligomers and the Channel Hypothesis

The observation and characterization of annular Aβ assemblies in in vitro experiments and in cell culture led to the channel hypothesis, according to which annular Aβ oligomers are pathogenic, membrane-disrupting, unspecific and unregulated pores [68–70]. Some data suggest that annular Aβ species can induce cellular Ca^{2+} influx by forming channels or by activating cell surface receptors coupled to calcium influx [71]. Identified annular species exhibit various sizes, possess channel selectivity and regulatory properties [reviewed in ref. 68]. Concerning the mechanism of Aβ amyloid formation, it remains to be shown whether annular Aβ oligomers are on- or off-pathway intermediates.

Protofibrils

The characteristic feature of Aβ protofibrils, the precursors of Aβ fibrils, is a relatively flexible, rod-like structure with a size of <8 × <200 nm. They exhibit a core that is resistant to hydrogen exchange and bind the dyes Congo red and thioflavin T, suggesting a high β-sheet content [66, 72–77]. They have primarily been observed in vitro, are considered as direct, soluble precursors of mature Aβ fibrils and are likely to consist of a regular β-sheet array of Aβ monomers (fig. 1C). Protofibrils can be rapidly converted to Aβ fibrils in vitro when seeded by a small amount of preformed fibrils [73]. Furthermore, protofibrils appear to be in a dynamic equilibrium with lower-order oligomers [78] and elongate by association of smaller protofibrils with a rate depending on Aβ concentration, pH, ionic strength and temperature [72]. In vitro, protofibrils are potentially toxic [79], and several studies indicate that protofibrils are also neurotoxic species in AD [73, 80].

Fibrils

Aβ fibrils are amyloid fibrils that have been defined as 'thermodynamically stable, structurally organized, highly insoluble, filamentous protein aggregates being composed of repeating units of β-sheets aligned perpendicular to the fibre axis, with a distinctive X-ray diffraction pattern (cross-β) that is similar to crystalline silk and consistent with high β-sheet content' [3]. Like Aβ protofibrils, Aβ fibrils bind Congo red and thioflavin T [81, 82]. Importantly, Aβ fibrils formed in vitro share many similarities with those extracted from human AD plaques [54, 83]. Strong effort has been put into the elucidation of the structure of Aβ fibrils. It was proposed that they are composed of multiple protofibrils twisted around each other, which results in filamentous structures with a diameter of 6–10 nm [84]. The elucidation of the repetitive tertiary structure of Aβ in fibrils has been challenging for a long time. Aβ fibrils contain a core that is highly resistant to hydrogen exchange. Limited proteolysis suggested that the N-terminal segment of Aβ is not involved in the β-sheet fraction [85]. Further studies gave hints to both a potential parallel and an antiparallel arrangement of the β-sheets [86–90; reviewed in ref. 18, 91]. This controversy was essentially resolved through an NMR study of Luhrs et al. [92], who showed that the Aβ1–42 monomer in the Aβ fibril forms a β-turn-β motif and assembles to two parallel in-register β-sheets in which the Aβ residues 18–26 and 31–42 provide the respective β-strands (fig. 1C). Recently, Sachse et al. [93] investigated the quaternary structure of the Aβ(1–40) fibril in a morphologically homogeneous pool of fibrils and observed an S-shaped fibril cross section formed by two or three protofibrils arranged to a left-handed superhelix. Aβ, however, has the ability to form multiple fibrillar structures, as different fibril morphologies with different molecular structures can be obtained in vitro by varying the aggregation conditions [94]. This most interesting observation pro-

Fig. 1. A Amino acid sequence of the human Aβ1–42 peptide. The hydrophobic transmembrane segment of Aβ is indicated in red. D = Aspartate; A = alanine; E = glutamate; F = phenylalanine; R = arginine; H = histidine; S = serine; G = glycine; Y = tyrosine; V = valine; Q = glutamine; K = lysine; L = leucine; N = asparagine; I = isoleucine; M = methionine. **B** Schematic representation of Aβ fibrillization. A fraction of Aβ monomers might adopt an aggregation-competent β-sheet conformation that enters the aggregation pathway, which includes the formation of small oligomers, protofibrils, fibrils and larger protein deposits. This reaction is supposed to be a nucleated polymerization, characterized by a lag-phase that is followed by rapid growth. Small globular and annular oligomers are considered to be potential on-pathway or off-pathway intermediates; they are proposed to exhibit the highest neurotoxicity among all Aβ species. **C** Ribbon representation of the structure of Aβ protofibrils in the context of mature fibrils produced in vitro, described by Luhrs et al. [92] (PDB: 2PEG). The two proposed, parallel β-sheets formed by the Aβ segments 18–26 and 31–42 are indicated by arrows. The turn between the two strands (residues 27–30) in each monomer is depicted as a gray spline curve. The residues of Aβ in the upper molecule are shown in the same color as in **A**.

vides evidence for the existence of discriminative strains of Aβ deposits, analogous to the existence of prion strains in transmissible spongiform encephalopathies [see ref. 95 for review]. It remains to be shown whether Aβ deposits with slightly different quaternary structures exist in AD, and whether they can be correlated with disease symptoms.

Plaques

Amyloid plaques found in AD patients' and in murine brain represent the final state of the Aβ aggregation process in vivo. They are large extracellular Aβ deposits composed of insoluble Aβ amyloid fibrils [96], and intimately surrounded by dystrophic dendrites, axons, activated microglia and reactive astrocytes [11]. The presence of plaques does not correlate with neurotoxicity, as had been proposed initially. In contrast, plaques could contribute to the removal and inactivation of smaller, neurotoxic Aβ species [36, 97].

The Pathway of Aβ Aggregation

Proposed Models and Mechanisms

The elucidation of the pathway of Aβ aggregation is a very complex task [57, 98] in which the following questions need to be addressed: Is Aβ aggregation a seeded or a linear process and are there multiple assembly pathways with alternative intermediates [99–101]? Which conformational changes occur at the level of secondary, tertiary and quaternary structure? Are there even different forms (strains) of Aβ fibrils? What is the in vivo localization of the different assembly intermediates and their neurotoxicity? Is Aβ1–42 the main amyloidogenic species?

The model of Aβ fibrillization favored by most scientists is a nucleation-dependent polymerization mechanism that requires seeding by an ordered nucleus, followed by the growth of oligomers through incorporation of further Aβ molecules. In this model, the formation of nuclei with seeding activity is the rate-limiting step, which agrees with the observed lag phase in formation of Aβ fibrils that can be eliminated by addition of preformed seeds [13, 72, 73, 94, 102, 103]. Seeding of Aβ has been found to be specific; seeds from other amyloidogenic proteins are not able to trigger Aβ fibrillization efficiently [104]. In addition, formation of Aβ fibrils requires a critical, minimal Aβ concentration, which is 10–40 μM for Aβ1–40 fibrillization and about fivefold lower for Aβ1–42. This compares with physiological Aβ concentration in the low nanomolar range in the CSF (<10 nM) of healthy individuals, which is not significantly higher in AD patients' brains [105 and references therein]. In in vitro aggregation experiments the initial Aβ concentration is generally inversely proportional to the time the peptide remains soluble in vitro [73]. At 50-fold supersaturated Aβ concentrations, which exceed the physiological Aβ concentration about 10,000-fold, unspecific Aβ aggregation becomes dominant, and seeding no longer triggers Aβ fibril formation. At about ten times lower concentrations, Aβ1–39 and Aβ1–40 are soluble for several days, whereas Aβ1–42 aggregates rapidly into fibrils [13, 106, 107]. These results show that, on the one hand, a minimal Aβ concentration is required for ordered aggregation, and that, on the other hand, a maximal concentration exists above which unspecific aggregation prevents specific polymerization of Aβ. Notably, the aggregation pathways for the same Aβ peptide at different initial Aβ concentrations might differ [108]. There is also evidence that Aβ1–40 and Aβ1–42 aggregate through distinct pathways [46, 51]. Overall, the common features of the deduced assembly models include the formation of an Aβ oligomer as a nucleus that is significantly smaller than Aβ fibrils, the growth of the nuclei via protofibrils and fibrils that, in vivo, eventually associate to plaques, as well as the formation of off-pathway intermediates [72, 103, 109–111] (fig. 1B).

Experiments with full-length and N- and C-terminally truncated Aβ peptides indicate that Aβ residues 17–21 and conformational transitions in this Aβ segment are critical for the early assembly steps [54, 112, 113]. Models exist for the monomer-to-oligomer transition. According to the first model, Aβ monomers exist in equilibrium between α-helical and β-sheet conformation, where only the β-sheet fraction is assembly-competent and pulled from the equilibrium. Recently, it has been reported that β-turn formation in the Aβ segment 24–28 induces the monomer conformation required for fibrillization [51]. High-resolution atomic force microscopy studies suggest the formation of dimers, tetramers and octamers of Aβ with β-sheet structure as early assembly intermediates [100]. A second model is based on evidence that an α-helical, oligomeric intermediate accumulates in the fibrillization process [114]. The transition to a β-sheet conformation might thus also occur at the level of oligomers, as soluble, low-molecular-weight oligomers with α-helical structure have been described, whereas higher-order and insoluble oligomers only show β-sheet structure [78]. Formation of fibrils from protofibrils may be entropically driven through hydrophobic contacts between 3 and 6 protofibrils that are supposed

to form a fibril [115]. Real-time monitoring of fibril growth indicates that the reaction is a cooperative process with a constant elongation rate [116]. In addition, it has been observed that 'molecular recycling' takes place in fibrils, where Aβ can dissociate from the end of a fibril and reassociate with another fibril [117]. This suggests that fibril formation is in principle reversible, although real equilibria between fibrils and soluble Aβ monomers may only exist at the fibril end. In the case of Aβ1–40, an equilibrium concentration of 0.7–1.0 μM has been measured for the soluble Aβ1–40 monomer, which is independent of the total concentration of Aβ1–40 in fibrillization experiments [110].

Aβ is produced intracellularly and subsequently released into the extracellular space in healthy individuals. It is generated in endocytic vesicles, has been detected in the endoplasmic reticulum and Golgi apparatus as well as in recycling endosomes and at the plasma membrane [118–123]. There is evidence for in vivo seeding in marmosets after inoculation with brain tissue from a FAD patient [124] and for the intracellular existence of Aβ aggregates in cultured neurons and in human brain tissue [57, 125, 126]. In late AD stages, intracellular Aβ aggregates could not be detected [127].

Effects of Mutations Linked with Inherited AD

About 90–95% of the AD cases are sporadic, whereas 5–10% represent FAD cases, which have an average age of onset below 65 years. Hitherto, three genes have been identified wherein mutations cause FAD, one of them being the gene encoding APP. However, only about 1% of the FAD cases are linked to mutations in the APP gene [reviewed in ref. 12]. The vast majority of FAD patients bear mutations in the genes encoding PS1 and PS2 [128, 129], which are the proteolytic subunits of the γ-secretase complex generating the C-termini of Aβ.

There are FAD mutations in APP outside and within the Aβ sequence. Mutations outside Aβ promote proteolytic cleavage of APP and either increase the total Aβ level (Swedish mutation) or the ratio between Aβ1–42 and Aβ1–40 (French mutation) [17, 130–132]. In total, more than 150 mutations in APP, PS1 and PS2 have been identified to date that affect APP processing either by increasing the cleavage efficiency or by favoring Aβ1–42 production [133]. The most prominent example of elevated Aβ concentration is the gene dosage effect in trisomy 21 patients [37]. Recent data show that FAD can also result from duplication of the APP gene locus [134]. In summary, all FAD mutations that do not change the amino acid sequence of Aβ itself nevertheless favor assembly of Aβ amyloids through increased intracellular Aβ concentrations or an increased fraction of the most amyloidogenic Aβ form, Aβ1–42. Thus, fibrillization of Aβ1–40 in vivo might be triggered by seeds of Aβ1–42, which agrees with the hypothesis that an increased Aβ1–42/Aβ1–40 ratio might be associated with most sporadic AD cases [135].

In the Aβ sequence itself, various FAD-linked amino acid replacements have been identified. These predominantly affect residues 22 and 23, for instance the Dutch (E22Q), Italian (E22K), Iowa (D23N), Flemish (A21G) and arctic (E22G) mutations. It has been found that arctic, Dutch, Italian and Iowa variants of Aβ aggregate faster than Aβ wild type [114, 136–139]. The Flemish mutation slows fibrillization, but assembly intermediates of the Aβ variant carrying this mutation have been shown to be more soluble than and as toxic as wild-type Aβ in cell culture [44, 140]. The arctic mutation as a prominent example leads to enhanced Aβ1–42 levels with enhanced tendency to aggregate in vitro, and an increased fraction of protofibrils [138, 141].

All FAD patients with amino acid replacements in Aβ are heterozygous and thus produce mixtures of wild-type and variant Aβ. The fibrillization pathways of the two Aβ peptide variants seem to differ. Wild-type Aβ has been reported to be excluded from protofibrils and fibrils in in vitro aggregation reactions with equimolar amounts of wild-type and mutant Aβ; furthermore, the morphology and size distribution of aggregation intermediates seem to differ for both Aβ forms [75, 102, 141], whereas e.g. human and mouse Aβ coaggregate and form heteropolymers [142].

The difference in neurotoxicity between Aβ1–40 and Aβ1–42 oligomers seems to be stronger than that between the known Aβ variants with mutations at position 22 and 23 and wild-type Aβ. In cell cultures, the difference in toxicity of wild-type Aβ1–42 compared to that of wild-type Aβ1–40 exceeds the difference between Aβ variants and the wild type with equal length in cell culture [reviewed in ref. 18]. Variation of residues 41 and 42 showed that the aggregation propensity of Aβ correlates with the hydrophobicity and β-sheet propensity of these residues [143].

Reactions and Covalent Modifications of Aβ

Oxidative stress is associated with AD [reviewed in ref. 144], as the concentrations of reporter molecules indicative of oxidative stress are increased in AD brains, as well as the concentration of enzymes that reduce oxidative stress. There are several hints that Aβ paradoxi-

cally plays a beneficial physiological role as antioxidant and in metal homeostasis [reviewed in ref. 145, 146]. APP translation is also up-regulated by iron ions [147]. In the normal brain most of the Aβ is bound to membranes, where it is believed to form a metal-chelating hexamer [148].

There are numerous studies on the role of metal ions in AD. Of these, copper and potentially zinc ions seem to play a dominant role for the development of AD and AD pathogenesis [see ref. 149 for a review on metals in AD]. High concentrations of metal ions (e.g. copper, zinc, iron) have been found in amyloid deposits in the human brain [150]. Micromolar concentrations of Zn^{2+} induce aggregation of Aβ in vitro [151], Fe^{3+} and Cu^{2+} have the same effect under appropriate conditions [152]. This argues in favor of a metal-induced initiation of Aβ aggregation in synaptic regions where the metal ion concentration is increased during neuronal activity [153]. As Cu^{2+} coordinated by the three histidine residues 6, 13 and 14 of Aβ produces H_2O_2 from O_2 in vitro, Aβ-dependent formation of H_2O_2 may contribute to neuronal death [154, 155]. In vitro, Cu^{2+} can be reduced by Aβ to Cu^{1+} while H_2O_2 is formed and Tyr10 of Aβ is converted to the tyrosyl radical [144, 156]. Interestingly, in contrast to human Aβ, rodent Aβ (with the replacements R5G, Y10F, and H13R relative to human Aβ) does not produce H_2O_2 in conjunction with metal ions [reviewed in ref. 18].

Residue M35 of Aβ probably plays an important role in Aβ neurotoxicity [157]. It can be oxidized to methionine sulfoxide by reactive oxygen species, and this reaction is catalyzed by metal ions. Oxidation of M35 might also cause different conformations in Aβ oligomers [see ref. 158 for review]. In vivo, methionine sulfoxide reductases catalyze reduction of Aβ with oxidized M35. Aβ with oxidized M35 is a natural Aβ product in healthy individuals, seems to be less neurotoxic compared to the reduced form and exhibits a drastically reduced tendency to aggregate and associate with membranes [64, 159, 160].

There is evidence that individuals bearing the epsilon 4 allele of apolipoprotein E (ApoE4) have an enhanced AD risk compared to those bearing other ApoE alleles. ApoE4 is linked with a lower average age of AD onset, increased Aβ fibril and plaque formation, and elevated Aβ immunoreactivity [161–163]. It is the only known gene that represents a genetic AD risk factor except for inherited mutations in APP, PS1 and PS2, albeit there is evidence for the involvement of other proteins, for instance α_2-macroglobulin [164]. Early-onset AD cases with no mutations in any of the above genes will certainly lead to the discovery of more genetic AD risk factors [165].

Aggregation Conditions in vitro and in vivo

It is difficult to deduce general 'traffic rules' for Aβ aggregation in vitro, because the aggregation process is strongly dependent on the reaction conditions. For example, the aggregation tendency of Aβ in vitro increases with solvent hydrophobicity and depends on the storage condition for Aβ prior to initiation of assembly. Aβ aggregation in phosphate-buffered saline is faster when Aβ is predissolved in 35% acetonitrile/0.1% trifluoroacetic acid (TFA) compared to 0.1% TFA or dimethylsulfoxide, possibly due to different initial conformations prior to dilution in phosphate-buffered saline [166]. The presence of detergents also favors Aβ aggregation [167]. In addition, high ionic strength favors the aggregation process, and addition of salts to Aβ solutions can be used to initiate aggregation [168]. Another critical parameter is the pH value. The aggregation rate seems to be maximal at pH 5–6, whereas more acidic or alkaline conditions slow down or inhibit fibrillization. It has been reported that the α-helical conformation in the Aβ monomer is favored at pH 1–4 and 7–10, whereas the β-sheet conformation is the predominant conformation at pH 5–6. These observations agree with the hypothesis that the fraction of Aβ monomers with β-sheet conformation is the aggregation-competent Aβ form. Moreover, oligomeric intermediates formed at different pH values exhibit different morphologies and neurotoxicity [169–172]. As described above, Aβ fibrillization also depends on Aβ peptide concentration and the presence of preaggregated forms of the peptide [73, 112, 173]. The temperature dependence of Aβ aggregation exhibits Arrhenius-like behavior. Conformational transitions in Aβ depending on temperature have also been observed [174, 175]. Metal ions and impurities in Aβ preparations may favor aggregation or lead to the formation of distinct oligomer morphologies [reviewed in ref. 176]. This, together with varying experimental conditions for Aβ aggregation used in different laboratories represents the main difficulty in extracting a general mechanism for Aβ aggregation in vitro. Besides the strong influence of experimental conditions on Aβ aggregation, the field also suffers from the fact that many in vitro studies performed so far focused exclusively on Aβ 1–40, although Aβ1–42 represents the main amyloidogenic species and appears to be more neurotoxic [177, 178, reviewed in ref. 18].

In vivo, the Aβ peptide can bear several posttranslational modifications besides the oxidation of M35, including the isomerization of Asp1 and 7 to isoaspartyl residues, the racemization of Ser8, Asp1, 7 and 23, and the cyclization of Glu3 and 11 [179–183]. Their role in Aβ aggregation still has to be clarified. Overall, the current knowledge on Aβ fibrillization is still rather limited, and there may well be distinct, parallel fibrillization pathways depending on the respective in vivo and in vitro conditions.

In vitro Experiments versus Transgenic Animals versus Human Brain

Aβ aggregation has been investigated both in vitro (cell culture, test tube) and in vivo (mouse models, clinical trials). The majority of in vitro experiments involve initial Aβ concentrations in the micromolar range and time scales of minutes to days for the aggregation process. In vivo, however, the Aβ concentrations are in the nanomolar range and the estimated time scale of amyloid plaque formation is 40–60 years in humans [184]. Nevertheless, Aβ fibrils produced in vitro have similar morphologies and share their characteristic biochemical properties with those found in vivo [83]. Further evidence for the reproducibility of Aβ fibrils derived from human brain by in vitro aggregation of synthetic Aβ comes from passive vaccination trials in which synthetic Aβ was used for the generation of antibodies [185, 186]. Aβ fibrils produced in vitro were also shown to be toxic in cell culture, in contrast to amorphous aggregates [26, 173]. In addition, oligomers of Aβ isolated from cell culture are toxic in rats [38], and active vaccination trials in humans as well as in mice with aggregates of synthetic Aβ resulted in beneficial effects in antibody responders [187–189]. These results support the view that the in vivo pathway of Aβ aggregation can be elucidated in vitro.

In vivo, Aβ occurs in various cellular compartments, and intracellular as well as extracellular aggregation has been observed. Thus, it may well be that the pathway of Aβ aggregation in vivo depends on the respective molecular environments. Current models with transgenic mice producing human APP and tau represent very valuable tools for studying AD in vivo, as these mice show Aβ pathology, cognitive decline and behavioral alterations similar to human AD symptoms. However, they do not develop neurofibrillar tangles and do not show neuronal loss [14–16, 190–194]. Thus, the rodent brain appears not as susceptible to Aβ toxicity as the human brain [195]. In addition, Aβ aggregation does not seem to be a prerequisite for neurotoxicity in these animals [196, 197].

Future Prospects and Challenges for Characterization of Aβ Fibrillization in vitro

How to Ensure Reproducibility – Handling 'the Peptide from Hell'

Aβ, and in particular Aβ1–42, has been termed 'the peptide from hell' due to its strong aggregation tendency and the difficulties in obtaining reproducible results in in vitro studies on Aβ fibril formation [176, 198]. In addition, there seem to be differences between synthetic Aβ batches from commercial sources with respect to fibrillogenesis [112]. To us, the most likely explanation for these difficulties, besides impurities in synthetic Aβ, appears to be different concentrations of fibrillar seeds in different Aβ preparations and stock solutions of the Aβ monomer generated under different storage conditions. Consequently, establishment of solvent conditions that guarantee the monomeric state of Aβ (with a defined redox state of M35) in concentrated stock solutions prior to initiation of Aβ fibrillization seems to be a prerequisite of the reproducibility of in vitro aggregation studies.

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Adenosine-Based Cell Therapy Approaches for Pharmacoresistant Epilepsies

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Abstract
Despite recent medical advances pharmacoresistant epilepsy continues to be a major health problem. The knowledge of endogenous protective mechanisms of the brain may lead to the development of rational therapies tailored to a patient's needs. Adenosine has been identified as an endogenous neuromodulator with antiepileptic and neuroprotective properties. However, the therapeutic use of adenosine or its receptor agonists is largely precluded by strong peripheral and central side effects. Thus, local delivery of adenosine to a critical site of the brain may provide a solution for the therapeutic use of adenosine. The following rationale for the local augmentation of the adenosine system as a novel therapeutic principle in the treatment of epilepsy has been established: (1) Deficits in the adenosinergic system are associated with epileptogenesis and these deficits promote seizures. Thus, reconstitution of an inhibitory adenosinergic tone is a rational therapeutic approach. (2) The focal paracrine delivery of adenosine from encapsulated cells suppresses seizures in kindled rats without overt side effects. (3) The anticonvulsant activity of locally released adenosine is maintained in models of epilepsy which are resistant to major antiepileptic drugs. This review summarizes the rationale and recent approaches for adenosine-based cell therapies for pharmacoresistant epilepsies.

Epilepsy affects around 60 million people worldwide. According to conventional theories, epilepsy is thought to be due to an imbalance between glutamate-mediated excitatory and GABAergic inhibitory neurotransmission. Consequently, pharmacotherapy of epilepsy has largely been postsynaptic by focusing on these two transmitter systems and on the ion channels involved in neurotransmission. However, despite optimal treatment with currently available antiepileptic drugs, seizures persist in about 35% of patients with complex partial epilepsy and pronounced side effects limit the most favorable use of the antiepileptic drugs [1].

Recently, new insights into the role of presynaptic modulation in controlling neuronal excitability and epileptogenesis [2] have opened new prospects for the development not only of novel anticonvulsant but also of antiepileptogenic therapies. Thus, neuromodulators, such as neuropeptide Y, galanin, or adenosine are thought to have potent anticonvulsive and antiepileptogenic effects. This review

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focuses on the role of the endogenous adenosine system in regulating neuronal excitability and on the translation of recent findings into novel therapeutic approaches.

Adenosine – an Endogenous Anticonvulsant of the Brain

The effects of adenosine are mediated by activation of seven-membrane-spanning domain adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) that couple to heterotrimeric G proteins to access diverse intracellular signaling pathways. Thus, by specific interaction with different G proteins, by specific affinities for adenosine, and by specific spatial distribution within the brain, these receptors allow a high degree of complexity in the effects of adenosine [3]. Adenosine exerts its potent inhibitory effects mainly by activation of the high-affinity A_1 receptors that are linked to inhibitory G proteins, which (i) inhibit adenylyl cyclase, (ii) activate G-protein-dependent inwardly rectifying K^+ channels, (iii) inhibit Ca^{2+} channels, and (iv) activate phospholipase C. As a consequence, the release of various neurotransmitters, in particular of glutamate, is inhibited via presynaptic A_1 receptors [3], and in hippocampus the tonic activation of A_1 receptors by ambient adenosine profoundly affects paired-pulse facilitation in mossy fiber-CA3 synapses [4]. Pharmacologically, adenosine and its A_1-selective analogues are effective in seizure suppression [5] and neuroprotection [6]. Furthermore, A_1 receptor activation is efficient in the suppression of pharmacoresistant seizures [7, 8] and a prerequisite to keep an epileptic focus localized [9]. Thus, adenosine is a potent anticonvulsant with efficacy in pharmacoresistant seizures.

Due to the manifold actions of adenosine via its receptors, a tight regulation of adenosine levels is of crucial importance. Since intra- and extracellular adenosine is rapidly equilibrated by bidirectional, equilibrative nucleoside transporters [10], the extracellular concentrations of adenosine are regulated by the interplay of intra- and extracellular enzymes of adenosine metabolism (fig. 1A). Physiologically, intracellular adenosine is formed by dephosphorylation of AMP by 5′-nucleotidase or by hydrolysis of S-adenosyl-homocysteine, while extracellular adenosine is formed by a cascade of ectonucleotidases with at least eight distinct members [11] including ecto-ATPase and ecto-5′-nucleotidase. Adenosine deaminase and adenosine kinase (ADK) reduce adenosine concentrations by forming inosine and AMP, respectively. While catalyzing a salvage pathway for the recycling of adenosine into adenine nucleotides, ADK is not involved in the de novo synthesis of purines, which, like the classical purine salvage pathway via hypoxanthine phosphoribosyltransferase, results in the formation of IMP. Based on its low K_m for adenosine, ADK is the primary route of adenosine metabolism [12]. Thus, pharmacological inhibition of ADK provides seizure suppression in various models of epilepsy [13]. In contrast, inhibition of adenosine deaminase has little influence on the concentration of extracellular adenosine [14]. A high flux rate in a substrate cycle between adenosine and AMP, involving 5′-nucleotidase and ADK, allows minor changes in the activity of ADK to be rapidly translated into major changes in adenosine [12]. ADK can rapidly be inactivated by ADP-mediated aggregation [15]. Consequently, under conditions of stress, e.g. during seizure activity or ischemia, in which ATP consumption exceeds its formation, ADK could rapidly be inhibited by an increase in ADP and subsequent aggregation. Thus, ADK is rapidly inactivated during status epilepticus in mice [8] or after oxygen glucose deprivation in cultured neurons [16] (fig. 1B). Extracellular levels of adenosine can also be regulated by changes in pH, which in turn depend on carbon dioxide (CO_2) partial pressure [17]. Thus, decreased CO_2 partial pressure (hypocapnia) and alkalized pH lowered extracellular adenosine and induced epileptiform activity in hippocampal slices, whereas increased CO_2 partial pressure (hypercapnia) and acidified pH increased extracellular adenosine and reduced excitatory postsynaptic potentials in field recordings [17]. Interestingly, ecto-ATPase is inhibited under alkalized conditions, thus reducing adenosine formation under hypocapnia [17], while ADK is inhibited under slightly acidified conditions [12] and thus may contribute to increases in extracellular adenosine under hypercapnia.

Deficits in Adenosinergic Neuromodulation in Epilepsy

In healthy brain, endogenous adenosine prevents the development and spread of seizures via a tonic anticonvulsant effect. Normally, the adult brain is kept under an inhibitory adenosinergic tone by a restriction of ADK expression mainly to astrocytes [8]. However, a deficit of this beneficial adenosine response was recently described in the hippocampus of epileptic rats and found to be due to a combined decrease in the density of A_1 receptors and to metabolic changes that led to lower basal levels of adenosine [18]. Indeed, overexpression of ADK in epilep-

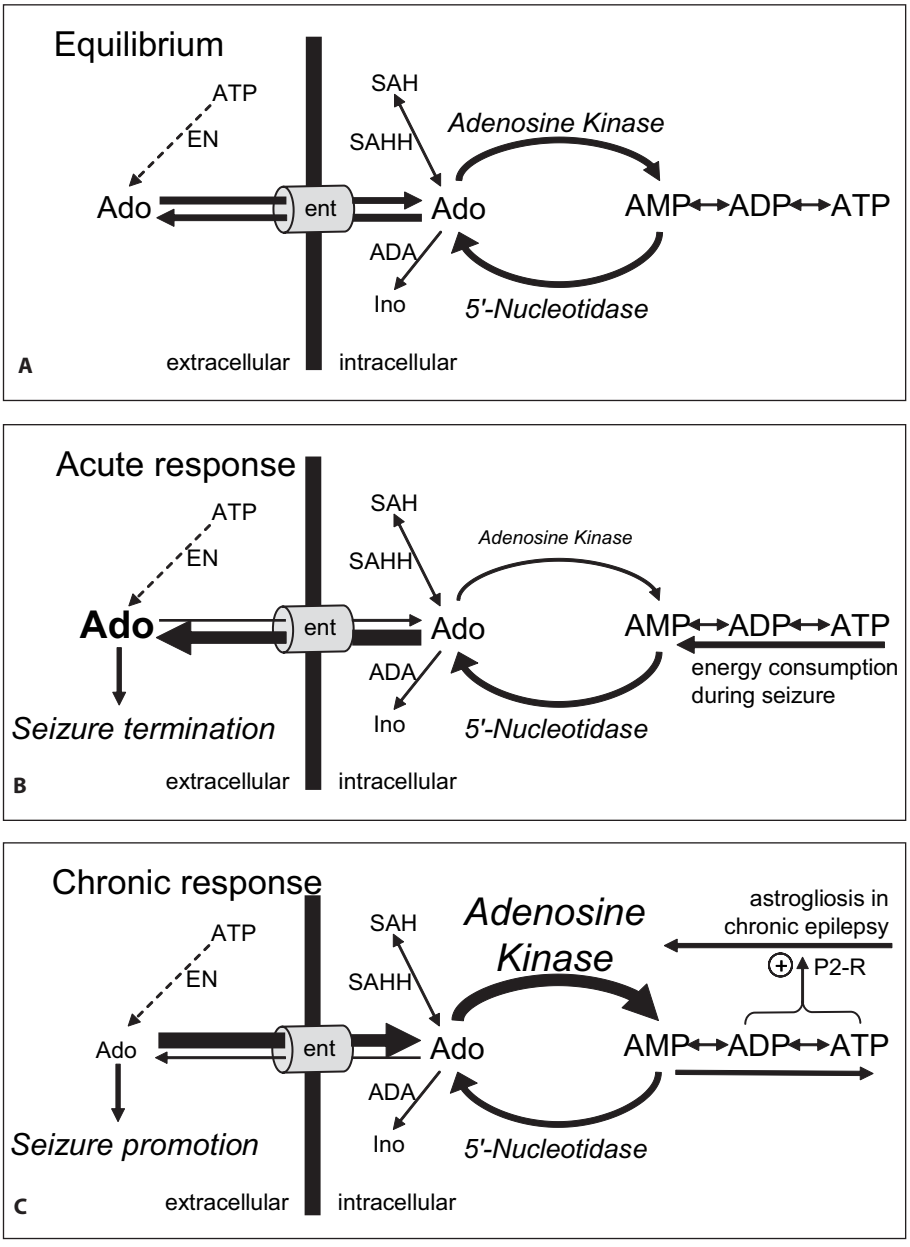

Fig. 1. Regulation of adenosine levels in astrocytes by ADK. **A** Under normal conditions adenosine (Ado) levels in adult brain are mainly dependent on the activity of ADK, which together with 5′-nucleotidase is part of a substrate cycle between AMP and adenosine. This substrate cycle is directly linked to the energy pool (ATP/ADP) of the cell. In brain, alternative metabolic routes of adenosine involving the reversible hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase (SAHH), or the deamination of adenosine to inosine (Ino) by adenosine deaminase (ADA) constitute only minor pathways for the regulation of adenosine. Intra- and extracellular concentrations of adenosine are rapidly equilibrated by equilibrative nucleoside transporters (ent). Thus, extracellular concentrations of adenosine depend largely on its intracellular metabolism. In addition, the extracellular cleavage of ATP (apyrase) can contribute to the formation of adenosine. **B** As an acute response to seizure activity ADK is rapidly down-regulated. In addition, excessive energy consumption leads to the degradation of ATP. Thus, more AMP is fed into the substrate cycle. As a consequence, intracellular adenosine levels rise and lead to the efflux of adenosine. The rise in extracellular adenosine during acute seizure activity is thought to be responsible for the termination of seizures. **C** In chronic epilepsy ADK is up-regulated as a consequence of astrogliosis. Thus, the direction of adenosine flux is reversed and the formation of adenine nucleotides is favored. This leads to a reduction of extracellular adenosine and the promotion of seizures. In addition, increased levels of adenine nucleotides may stimulate the process of astrogliosis via activation of P2 receptors, thus forming a vicious circle.

tic hippocampus has been associated with astrogliosis and seizure activity in a mouse model of intrahippocampal kainic acid induced status epilepticus (KASE) [8] (fig. 1C). Overexpression of ADK in astrocytes, and, consequently, a reduction of ambient adenosine and an increase in adenine nucleotides, may actually promote further astrogliosis, since adenosine inhibits astrocyte proliferation via A_1 receptors and adenosine nucleotides stimulate astrocyte proliferation via P2 receptors [19]. To clarify the causal relationship between astrogliosis, up-regulation of ADK and recurrent seizures in temporal lobe epilepsy a new mouse model was created in which the endogenous astrocytic ADK was replaced by a transgenic ADK providing a novel neuronal expression of ADK [20]. These mice develop KASE-induced astrogliosis in the absence of endogenous ADK. Therefore, overexpression of ADK in wild-type mice is a consequence of KASE. In addition, the mutants display spontaneous seizure activity. Therefore, overexpression of ADK can be a cause for seizures. Consequently, therapeutic strategies, which augment the adenosine system after astrogliosis-induced up-regulation of ADK, constitute a rational treatment approach.

Rationale for Local Cell-Based Therapies

Although adenosine, A_1 receptor agonists, and ADK inhibitors are effective in seizure suppression [5], their systemic application is precluded by peripheral side effects. Therefore, focal adenosine delivery becomes a necessity. In recent years focal drug delivery in refractory epilepsy, which can be achieved with slow-release polymers or pump systems, was demonstrated to be well tolerated and devoid of major side effects [21]. Thus, intracerebral implants of GABA- and adenosine-releasing synthetic polymers provided seizure suppression in kindled rats [22, 23]. However, during the lifetime of an epilepsy patient, even the most advanced drug delivery systems require repeated implantation or refill procedures with the attendant risks of complications. In contrast, local cell therapies offer the potential to achieve regeneration and repair combined with the sustained delivery of therapeutic compounds. Thus, many cell therapies are currently under development for the treatment of neurological disorders [24]. However, the development of cell therapies for epilepsy has so far been delayed by the lack of an identifiable uniform defect to be restored. Grafting of fetal hippocampal tissue to repair pathological changes of hippocampal sclerosis led to a partial reversal of these structural alterations in a kainate rat model of temporal lobe epilepsy, however the effect of the graft on the occurrence of epileptic seizures was not investigated [25]. Attempts to graft therapeutic cells, which release anticonvulsant substances, have been limited so far to the use of GABA- [26, 27] and adenosine-releasing cells [5]. Thus, grafts of fetal GABAergic neurons provided a transient reduction of the severity of kindled seizures [27]. More recently, immortalized neurons, engineered to release GABA under the control of doxycycline were transplanted into the dentate gyrus of rats and demonstrated to affect afterdischarge thresholds and kindling development, but seizure suppression was not demonstrated [26].

Seizure Suppression by Encapsulated Adenosine-Releasing Cells

Adenosine-based cell therapy approaches for partial epilepsy have recently been developed by engineering BHK fibroblasts and C_2C_{12} myoblasts to lack ADK. These adenosine-releasing cells were encapsulated into semipermeable polymer membranes to selectively study paracrine effects of adenosine and to avoid immune rejection. Adenosine-releasing intraventricular brain implants provided protection from convulsive seizures and from epileptiform electric afterdischarges in rats kindled in the hippocampus, a model of temporal lobe epilepsy [14, 28]. This focal delivery of adenosine, as opposed to the systemic application of an adenosine A_1 receptor agonist, did not cause sedation [28]. The anticonvulsant effect lasted up to 8 weeks and corresponded to the life expectancy of the encapsulated cells. The effect was adenosine-mediated since seizure suppression was transiently reversed after application of an A_1 receptor-selective antagonist. Thus, the paracrine focal adenosine delivery by cell grafts is a promising strategy to control seizures without accompanying sedative side effects.

Stem Cells for Intracerebral Drug Delivery

Based on the anticonvulsant properties of adenosine and on the long-term survival potential of stem cell-derived brain implants, adenosine-releasing stem cells may constitute a novel tool for the treatment of epilepsy. In contrast to encapsulated cell grafts, which provide therapeutic effects exclusively by paracrine action, direct stem cell-derived brain implants may combine paracrine effects with network interactions [29]. Pluripotency and self-renewal make embryonic stem (ES) cells a particular versatile donor source for transplantation. The directed differentiation of ES cells provides new perspectives for the generation of clinically relevant cell types, including neurons [30] and glia [31]. Following transplantation into the rodent CNS, ES cell-derived neural precursors integrated into the host tissue and yielded functional improvement [32, 33]. Since ES cells are amenable to a wide spectrum of genetic modifications, the ES cell technology holds great promise not only for cell replacement therapies, but also for cell-based drug delivery.

Seizure Suppression by ADK-Deficient ES Cells

With the aim to develop a stem cell-based delivery system for adenosine, both alleles of ADK were disrupted by homologous recombination in ES cells [34]. To avoid teratoma formation after transplantation, a protocol has been developed to differentiate ES cells into pure populations of glial precursor cells [31]. Accordingly, $Adk^{-/-}$ ES cells were recently subjected to a glial differentiation protocol and, as a result, gave rise to proliferating glial pre-

cursors, which were further differentiated into mature adenosine-releasing glia cells. Thus, a lack of ADK does not compromise the glial differentiation potential of ES cells [34].

An attempt was made to investigate the potential of differentiated *Adk*$^{-/-}$ ES cell progeny for seizure suppression by paracrine adenosine release. To isolate paracrine effects of stem cell-derived implants from effects caused by network integration, ES cell-derived embryoid bodies and glial precursor cells were encapsulated and grafted into the lateral brain ventricle of kindled rats. While seizure activity in kindled rats with wild-type implants remained unaltered, rats with adenosine-releasing *Adk*$^{-/-}$ ES cell-derived implants displayed transient protection from convulsive seizures and a profound reduction of after discharge activity in EEG recordings [35]. Long-term seizure suppression was precluded by limited viability of the encapsulated cells. Thus, proof has been established that *Adk*$^{-/-}$ ES cell-derived brain implants can suppress seizure activity by a paracrine mode of action.

Conclusions

Adenosine-releasing stem cells can be generated and used to augment the adenosine-based neuromodulatory system as a rational approach to treat pharmacoresistant focal epilepsies. This therapeutic strategy is based on three main findings: (i) Dysfunction of the adenosine system is associated with seizure activity, thereby providing a rationale for therapeutic intervention [8, 18, 20]. (ii) Augmentation of the adenosine system is sufficient to suppress pharmacoresistant seizures [7, 8]. (iii) Focal paracrine delivery of adenosine by encapsulated cells is sufficient to suppress seizures in kindled rats [14, 28]. The generation and characterization of human adenosine-releasing stem cell-derived brain implants thus might evolve into an exciting direction for future research.

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Cortical Plasticity: A View from Nonhuman Primates

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Key Words
Neocortex · Feedforward · Feedback · Perceptual learning · Adaptation · Decoding · Hand grasping

Abstract
The primate's large brain-to-body weight ratio and high complexity are unusual in the animal kingdom. There is compelling evidence that it is an evolutionary adaptation that allows its owner to live a long life because of its competence in solving a wide range of problems. How primates use their brain to achieve such competence is of course of central interest to us. Here we review some key aspects of the neocortex that can be explored in nonhuman primates. Studies of the cortical circuits in the visual cortex reveal that the two major types of pathways, called feedforward and feedback, involve a very small fraction of the total synapses that any area contains. Nevertheless these pathways may be critical for some important forms of cortical plasticity, like perceptual learning and tasks involving perception and action.

Introduction

Why should an animal need a modifiable brain at all? Why does the nervous system need to be plastic? Brains can be hardwired so that they elaborate a complex sequence of actions, like foraging, eating, drinking, and reproduction, without the need for learning. Such pre-programmed brains do, however, require that the environment in which its owner lives is predictable. Thus, animals such as insects, which live brief lives in a constant environment, can be preprogrammed to perform a wide range of essential behaviors that do not require learning. All animals use their brains to make predictions about the future, but the longer an animal lives, the more likely it is that conditions will change. If the future becomes less predictable, animals with brains that are able to generate well-adapted behaviors will have the best chances for survival. These animals possess a quality we call intelligence. Adaptation means that the brain has the capacity to change the neural representation of a particular brain area either qualitatively or quantitatively. This capacity is called 'plasticity' and it may be the main reason why large brains and long lives go together.

Primates have large brains for their size [1], are also long-lived and slow to reach sexual maturity. They are intelligent and well-equipped to respond adaptively to possible changes in future conditions. This flexibility in behavior extends not just over time but also over space. Because they tend to forage over wide areas, they also encounter a wide variety of conditions to which they also have to respond appropriately. Since there is a positive correlation between the relative size of the brain and the range of problems animals can solve [2], it is not surprising that there is also a positive correlation between the relative size of a primate's brain and the size of the territory over which it ranges [3].

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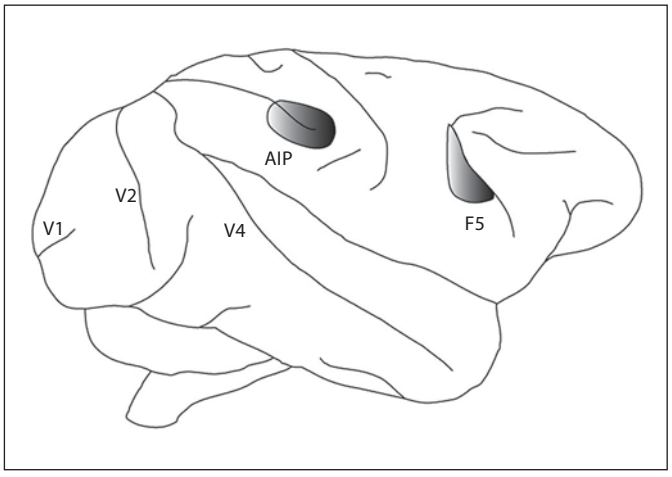

Fig. 1. Lateral view of a macaque brain. Labels indicate the visual areas: V1, V2, and V4. Shaded areas indicate AIP and premotor area F5.

In the following, we review key aspects of cortical connectivity and then give two examples of primate cortical research that raise particularly interesting questions in relation to cortical plasticity: perceptual learning and tasks involving perception and action. As we will see, cortical plasticity plays a central role in both of these topics while its mechanisms and neural implementation still await to be discovered.

Primate Neocortex

Although mammalian brains do have a similar 'bauplan', the primate brain is not simply an expanded rat or cat brain, because the rule that relates the volume of the neocortex to the volume of the whole brain is different for rodents, carnivores, and primates. The primate brain has proportionately more neocortex than the carnivore and even more than the rodent [2]. If the ratio of brain to body weight of an average mammal is expressed as an 'encephalization quotient' [1], then it is evident that the insectivores and rodents have small brains for their weight, the ungulates and prosimians have brains of moderate size, and the monkeys and apes have large brains. The change in brain size is largely due to an increase in the size of the neocortex. In carnivores the neocortex forms about 40% of the brain, whereas in primates it can vary from 53% in a cebus monkey to over 80% in the human brain. Other primates, like us, typically have brains that are large for their body weight compared to other animals. The significance of this for adaptive behavior is that brain size correlates with the animal's performance when faced with standardized problems. For example, in a set of 1,800 problems in visual discrimination learning, three species of primates (rhesus monkey, squirrel monkey and marmoset) outperformed cat, gerbil, rat and squirrel. The old world monkey outperformed by a considerable margin the new world primates [2]. This and other comparative evidence indicates that the old world primate in particular provides us with an important model for exploring the mechanisms of cortical plasticity in the human. The areas of neocortex that we will discuss here are shown in figure 1.

Cortical Circuits

Plasticity in brain circuits means that the effective connectivity between neurons is changed. These changes in effective connectivity may occur through many different mechanisms, from a change in the strength of a synapse to the growth of new connections. Thus, knowledge of the physical circuit is fundamental to understanding the changes in function that they produce during cortical plasticity. In the human, surprisingly, detailed maps of brain circuits do not exist. Our best knowledge of these circuits is indirect and comes mainly from studies of the brain circuits of macaques. Crick and Jones [4] have referred to this state of affairs as 'shameful', but unfortunately, it is not easy to remedy. Although new techniques of studying fiber tracts in humans are being developed (e.g., diffusion tensor imaging [5]), it seems likely that in vivo studies will always bump against the limits of spatial resolution. This means that for the foreseeable future, much of our understanding of the circuits of the human brain will come from studying brains of nonhuman primates and drawing analogies. However, even here there are caveats. Most of the detailed circuits from the macaque monkey are based on qualitative tract tracing analyzed at the light microscope level. Such studies show the existence of a connection between two structures, identity of the neurons of origin and, in association with other techniques, can identify target neurons as well. There are remarkably few quantitative studies of the brain circuits, particularly studies of the connections of the neocortex. Only through painstaking quantitative studies can we hope to go beyond the simple binary picture (i.e. connected, not connected) of cortical hierarchy, which has been the standard view for the past two decades.

Synaptic Connections of the Macaque Visual Cortex

Our own interest in cortical circuits has been at the level of a major site of plasticity: the synaptic connections between nerve cells. We made quantitative studies of the synaptic connections to the cortical visual areas in the macaque monkey. The visual system is organized in a bidirectional hierarchy: projections from the retina to the thalamus and onto the ascending levels of the visual cortex are defined as 'feedforward', whereas the descending projections are defined as 'feedback'. In short, V1 receives direct input from, and sends signals back to the thalamus. V1 is connected to area V2, which exchanges signals with a multitude of other extrastriate visual cortical areas, many of them also sending signals back to the thalamus. Most extrastriate cortical areas are thought to have a more specialized function than V1 or V2. Areas V3A and MT, for example, are thought to play an important role in depth and motion perception, while V4 is known to be important for form and color vision. We examined a number of feedforward projections in the macaque (summarized in fig. 2). These include the thalamic input to area 17 (V1) [6], the projections from V1 to areas V2 and MT [7; Anderson and Martin, in preparation], and from V2 to area V3A [8] and V2 to MT [9]. We also examined the feedback projections from V4 to V2 [10] and from V2 to V1 [Anderson and Martin, in preparation]. Remarkably, all these various projections show consistent patterns of synaptic connections. The ultrastructural appearance of their synapses is typical of excitatory glutamatergic synapses. Spiny (excitatory) neurons are the major targets (about 80% of targets) of these interareal projections, with about 20% of targets being smooth (inhibitory) neurons. All of these interareal projections and the projection from the thalamus to the primary visual cortex involve surprisingly few synapses. In all cases, only a few percent of the synapses formed with the dendritic tree of a single neuron actually come from a feedforward or a feedback. Neighboring excitatory neurons are the source of most of the remaining synapses [11].

The main differences between these two projection types, feedforward and feedback, are in the layers that they innervate. Feedforward projections between cortical areas resemble the thalamic input to the primary visual cortex in that the middle layers of the cortex (layers 3 and 4) are the major targets of innervation. In contrast, the feedback projections tend to avoid the middle layers of the cortex and target layers 1 and 5 (fig. 2). The feedback projection from V4 to V2, for example, forms most of its synapses on the distal dendrites of pyramidal cells in layer 1. The interplay between these ascending and descending cortical pathways is the subject of a great deal of speculation and some physiological data. The physiological data are extremely hard to obtain because isolating one or the other stream is virtually impossible, only one part of the cortical circuit can be silenced. Nevertheless, it seems clear that the interaction between these two streams is nonlinear. For example, when V1 is inactivated by cooling, then all visual responses are lost in V2, but the neurons in area MT remain active [12]. This means that the feedback projections from MT to V2 are not able to drive the V2 neurons. Nevertheless, when MT is cooled, the visual responses of neurons in V2 are substantially reduced [13]. The feedforward and feedback connections between areas thereby provide convenient means of modulating and altering the properties and functions of the visual system as a whole. Thus, it is readily conceivable that one mechanism of plasticity in these early visual areas involves rapid modifications of the interactions of the feedforward and feedback pathways, for example the rapid changes associated with a phenomenon called 'perceptual learning'.

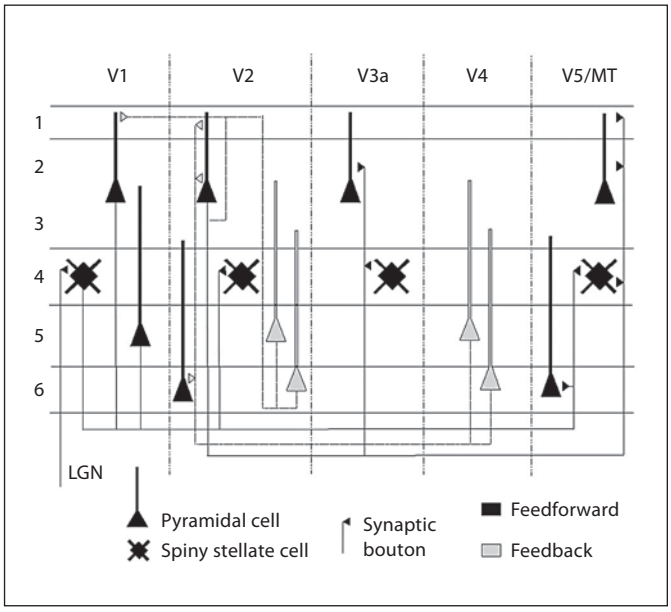

Fig. 2. Schematic representation of the connectivity between visual cortices in the macaque. The axonal projections were traced with light microscopy and the synaptic connections established by electron microscopy. Cortical laminae are numbered and the visual areas are indicated by V1, V2 etc. Feedforward connections: black, feedback connections: gray.

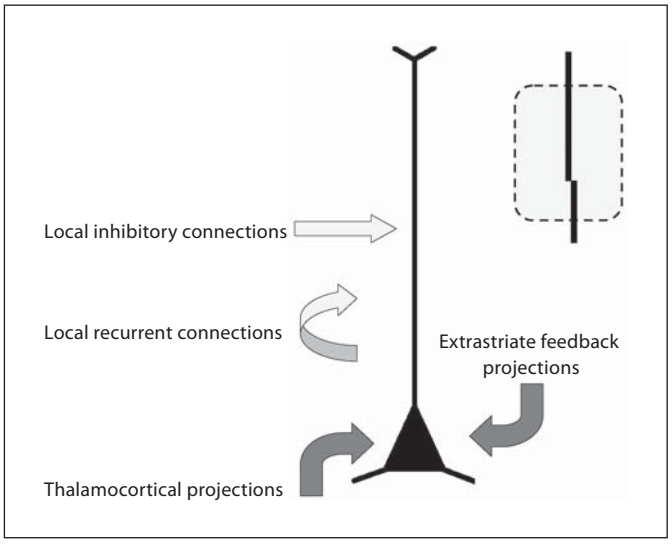

Fig. 3. A schematic primary visual cortex neuron with an elongated, oriented receptive field (dashed, light gray). Visual neurons with orientation-selective receptive fields can detect the presence or absence of an offset in a Vernier target (black lines over the receptive field). The receptive field properties can be modified either by plasticity of the synapses from external afferents (arrows) and/or internal connections.

The Model of Perceptual Learning

Among the many forms of plasticity shaping the properties of the primate cortex, those associated with practice in perceptual tasks provide researchers with a unique opportunity to probe the neural mechanisms of cortical plasticity. The improvement of performance due to practice is called perceptual learning (PL). To date, the mechanisms underlying PL are unknown. In most cases, it is not even clear which cortical areas are involved, let alone what type of synaptic or cellular processes are at play.

In the visual domain, PL is particularly strong in relative spatial position tasks, collectively known under the name of 'hyperacuities'. Although not the only type of task subject to PL [14, 15], hyperacuities improve significantly and rapidly with training. One form of hyperacuity, called Vernier acuity, has been used extensively to study the characteristics of PL and its neural basis. In a Vernier acuity task, a subject is asked to judge whether a line segment is offset to the left or to the right of an abutting reference segment (fig. 3). Vernier thresholds, which can be as low as a few seconds of arc in untrained subjects, can improve further by as much as a factor of 5 after a few hundred practice trials [16]. The training duration on the scale of hours necessary to observe significant PL is relatively long, and therefore consistent with many potential underlying mechanisms, such as synaptic potentiation or depression, or the establishment or deletion of synaptic contacts.

The high accuracy of Vernier judgments was shown to be consistent with the spatial properties of initial, oriented filters, which are known to exist in the primary visual cortex [17]. The nature and location of the neural events underlying improvement in performance are, however, unknown.

Thalamocortical Projections and PL

Assuming that the locus of PL is the primary visual cortex (V1; fig. 1, 2), PL could be implemented in at least three different elements of the neural circuitry. First, PL could reflect synaptic changes in the feedforward projections from the lateral geniculate nucleus (LGN) of the thalamus. Feedforward thalamocortical projections are known to play an important role in the generation of orientation selectivity in cortical neurons. Practice-induced changes in the synaptic weight of these connections could induce a sharpening of the neuron's selectivity for orientation, thereby yielding more acute performance in numerous hyperacuity tasks, including Vernier acuity. The fact that little or no interocular transfer is observed during learning in Vernier acuity is consistent with this hypothesis [16, 18]. Indeed, in primates most primary visual cortex neurons are binocular, although their LGN afferents are not. The lack of interocular transfer could be explained by modulation of the thalamocortical synapses, which are monocular (i.e. LGN afferents are from monocular neurons). Similarly, PL was shown to be specific to orientation: thresholds for Vernier targets at an orientation different from that trained do not benefit from the training [16]. This observation is also consistent with the hypothesis that PL is due to changes in the spatial characteristics of early, oriented, cortical filters. Another result consistent with the involvement of thalamocortical synapses is that PL is location-specific [16, 19]. Training one region of the visual field does not yield learning at other retinal locations. This suggests involvement of mechanisms with localized receptive fields. Thalamocortical projections are known to be highly location-specific, providing the basis for the small, localized, classical receptive fields of V1 neurons.

Local Cortical Circuits

The lack of interocular transfer, the orientation and location specificities of PL are all qualitatively consistent

with the hypothesis that PL results from modulation of the thalamocortical synapses. However, other mechanisms could be responsible as well. The orientation selectivity of primary visual cortex neurons is not only determined by feedforward projections, but is refined by local cortical circuitry. Local inhibitory feedback or recurrent excitatory connections have both been proposed as central mechanisms for the tuning of orientation selectivity [20]. In that view, local recurrent connections serve to amplify the signals coming from the thalamus, thereby enhancing a neuron's response to its preferred orientation. In addition, inhibitory signals from neighbor neurons tuned to other orientations can reduce a neuron's responses to nonpreferred orientations. Plasticity in these local synapses is thus a second potential mechanism that could induce sharpening of the cell's tuning, and support PL. This would be consistent with the orientation specificity of PL, as well as with its location specificity.

Distinguishing between the respective contributions of thalamocortical synapses and local cortical circuitry proves difficult, as exemplified by the controversy about their role in determining orientation selectivity [20]. The lack of interocular transfer favors involvement of the thalamocortical synapses, although it does not rule out alternatives [21].

Extrastriate Cortical Influences on PL

The data reviewed so far are qualitatively consistent with the notion that primary visual cortex neurons could be the site of PL. However, a number of observations are not consistent with this notion: quantitative measures of PL's orientation specificity, the role of perceptual feedback and attention during practice, PL's specificity to the complexity of the trained stimulus, and recent recordings of primary visual cortex neurons suggest involvement of extrastriate mechanisms in PL.

Several reports document the importance of feedback during practice [22, 23]. These studies demonstrate that PL is possible in the absence of behavioral feedback, but that it is considerably slower and less pronounced than when feedback is provided. These data indicate that signals pertaining to the correctness of behavioral responses influence the rate of learning. Because these signals are thought to originate in the extrastriate cortex, this result suggests the involvement of higher visual areas of the extrastriate cortex in PL. Similarly, an important role of attention in PL has been reported [24, 25]. Attentional signals are also known to be generated outside of V1. These results thus further support the notion that the extrastriate cortex has an important role to play in PL, although the precise mechanisms implementing that role are currently unknown.

Involvement of the extrastriate cortex in PL is also suggested by the fact that PL is not only orientation- and location-specific, but restricted to stimuli of the same complexity as that trained. Indeed, Poggio et al. [26] showed that PL does not transfer from a task trained with line segments to a similar task with dot stimuli. This is inconsistent with the implication of early oriented filters that should give similar responses to both types of stimuli. Similarly, Crist et al. [27] showed that PL is highly task-specific. Training in one task does not lead to improvement in other tasks thought to use the same underlying neural mechanisms. These high stimulus and task specificities are more reminiscent of extrastriate than primary visual cortex neurons.

Physiological Studies of PL

Numerous physiological studies attempted to find the mechanisms of cortical plasticity. Among those, a number of findings pertinent to PL have been described [for a review see ref. 28]. Because different tasks yield somewhat different results [15, 28], we focus on those using hyperacuity tasks, with particular emphasis on Vernier acuity. Although somewhat equivocal, the findings described above led most researchers to focus on the primary visual cortex. After training in a bisection task (in which monkeys indicated whether the central of three parallel line segments was closer to one or the other of the flanking lines), Crist et al. [27] failed to find any changes in V1 receptive field size, location, or orientation tuning. They did find, however, that contextual effects (measured through the effect of additional line segments placed outside the cells' classical receptive field) did increase with PL. Similarly, Ghose et al. [29] could not detect any change in orientation bandwidth, peak response, tuning amplitude, variance, preferred spatial frequency, spatial frequency bandwidth, or receptive field size in V1 and V2 cells of monkeys who had trained on an orientation discrimination task (a hyperacuity task thought to involve similar mechanisms as Vernier acuity). They did, however, report a slight decrease in the number of cells tuned to the trained orientation. To date, it is however not clear how a population decrease would yield better perceptual performance.

Few studies have reported classical receptive field changes following extensive training in a PL task [30]. Using an orientation task as well, the authors reported a decrease in the number of cells responding to the trained orientation, consistent with the findings of Ghose et al.

[29]. In addition, they reported changes in the neurons' orientation tuning, such that neurons tuned to the trained orientation showed a steeper slope in their orientation tuning function. Such a slope increase would be consistent with a higher ability to perform orientation discriminations. It is, however, not clear whether the changes reported by Schoups et al. [30] are sufficient to account for the observed reduction in behavioral thresholds.

Several years of research on the neural basis of PL have left many questions unanswered. Although there are strong indications that primary visual cortex neurons should exhibit PL, most studies aiming to reveal practice-induced changes in the classical receptive field of V1 neurons have failed. Because PL shows high intersubject variability, and because small changes in receptive field properties might be sufficient to account for the behavioral changes, it is possible that PL-related effects have gone unnoticed. Thus, it is necessary to perform additional, precise measures of the spatiotemporal properties of V1/V2 neurons during learning. If such changes indeed occur at later stages than V1/V2, the precise locus has to be determined. Moreover, quantitative analysis of the spatial and temporal characteristics of practice-induced changes will help us determine whether PL is induced by plasticity of the afferent synapses, the local cortical circuitry, or feedback from later stages of processing.

Cortical Circuits for Perception Action

The circuits of the occipital cortex are the best studied and form the basis of a wide range of concepts of cortical processing. The quantitative studies of synaptic connections referred to above have not been repeated for any other region of the neocortex. This lack of knowledge of the circuits is a major barrier to our understanding of the structural basis of plasticity in any of the other major divisions of the primate neocortex. This ignorance becomes particularly evident as we move from the occipital cortex to the temporal and parietal cortex. In these more rostral regions, the association of information from a variety of sources and modalities becomes more dominant. One of the most important perceptuomotor skills in primates is their dexterity of arm and hand movements, skills that are undoubtedly of considerable evolutionary significance. It is clear that the planning of purposeful hand movements from sensory information is a complex task that requires the coordinated action of many sensory and motor areas of the brain. This is immediately apparent from the fact that we normally use our hands in coordination with other actions like eye and arm movements and that all of these actions are based on a wealth of sensory, and in particular visual, information [31–34].

Hand movements are extremely versatile and span a range that extends from powerful grips to extremely delicate and precise manipulations of tools. In the premotor and parietal cortex, higher-level motor areas have been found that are involved in the formulation and generation of hand movement instructions. This is in contrast to neurons in the primary motor cortex that represent more precisely the hand movement details like trajectories and muscle forces. In the parietal cortex, the group of Sakata described neurons in the anterior intraparietal area (AIP, fig. 1) that encode the visual appearance of the object to be grasped [35, 36], or the grasping movement itself [37, 38]. These investigations emphasized the major role of this area for the transformation of visual information into high-level grasping plans. In the premotor cortex, the group of Rizzolatti found grasping neurons in the rostroventral aspect of the premotor area that are specifically active for a particular type of grasping or for hand orientation [39–41]. This area was termed 'frontal area 5' (F5, fig. 1) after careful histological examination [41, 42]. Taken together, the premotor and parietal grasping regions F5 and AIP play a prominent role for providing higher-order planning signals and the thereby necessary sensorimotor transformations [43–46].

Tract tracing studies show that the parietal and premotor areas are reciprocally connected [47–49], and most likely both are involved in coordinate transformation, decision making, and motor learning. In AIP, many cells are selective for the visual appearance of the object in addition to the appropriate movement to grasp the object [35, 36], while a reversible inactivation of AIP revealed a clear deficit in hand preshaping [50]. These studies demonstrated the functional relevance of AIP for object grasping and its role for the transformation of visual information into motor plans. In F5, many grasping neurons were found to be highly specific for a particular type of grasping movement (e.g., precision or power grip) or a particular hand orientation [40, 41]. Likewise, reversible inactivation of F5 also demonstrated its functional relevance for hand grasping [51]. These studies clearly indicate that both AIP and F5 are crucial for the generation of grasping movements. However, at this point, it is unknown how these areas interact and how the required computations are achieved.

To shed light on this fundamental problem, adaptation experiments that probe the plasticity of these brain areas might be of particular interest. In the parietal cor-

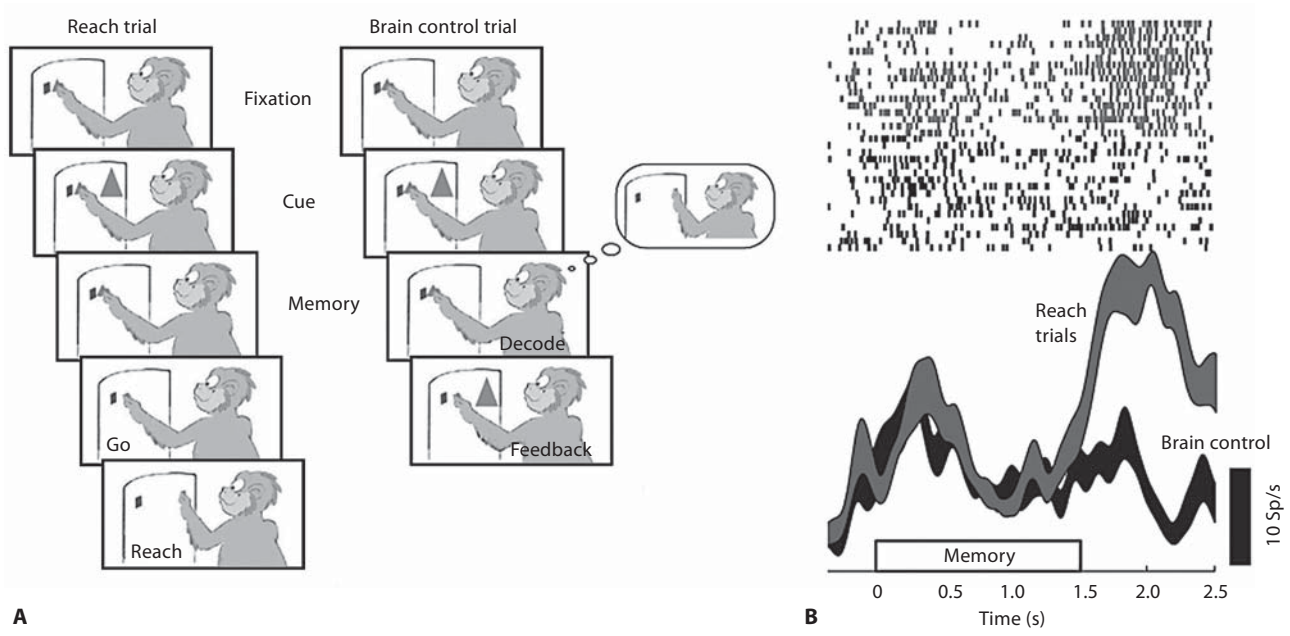

Fig. 4. Neural activity in the parietal reach region (PRR) during reach trials and brain control trials. **A** Behavioral task. Monkeys are trained in a reach task, where they first fixate and touch a central fixation spot on a touch screen (fixation). Then a visual cue indicates the location of a peripheral reach target (cue). During the following memory period, the animal continues to touch and fixate the fixation spot until a go signal appears, upon which the animal reaches to the remembered location in order to receive a juice reward. In the brain control task, the monkey plans a reach movement as in the reach task, however, neural activity during the memory period is decoded on-line by a computer (decode) and the decoded prediction of the reach is fed back to the animal (feedback). Importantly, the animal receives a reward if the decoded reach is correct, while an actual arm movement is not required (and the animal indeed does not move its arm). **B** Neural activity of an example cell of PRR. Spike rasters are shown on top of a peristimulus time histogram for reach trials and brain control trials. Activity increases at the beginning of the memory period (time zero), to which all trials are aligned. Activity stays elevated until the movement is executed (reach trials) or until the animal receives a reward (brain control trials) after the memory period for correctly decoded reach plans [modified from ref. 56].

tex, both learning and adaptation are important features in the context of sensorimotor transformations [52, 53]. Neural plasticity seems at work in the continuous fine-tuning of neural representations to keep various sensory and motor signals in register. An experiment specifically designed to disrupt this alignment is prism adaptation. Human subjects wearing vision-displacing prisms initially miss visual targets, but when provided with appropriate feedback about their errors, they recover and reach correctly within a time scale of a few minutes [54]. This suggests that a recalibration between sensory and motor coordinate frames takes place more or less continuously. A positron emission tomography study further demonstrated that prism adaptation selectively activates the parietal cortex contralateral to the reaching arm [55], which directly links this area to the adaptation effects, in agreement with its role in sensorimotor transformation.

There are some key experiments in which plasticity could be tested by perturbing the motor output signal. In the case of grasping movements, for example, activity in the areas AIP and F5 could be recorded from many permanently implanted electrodes while animals perform an instructed grasping task (e.g., precision or power grip with different hand orientations). Using statistical classification, the activity could be analyzed on-line to predict the animal's grasping intention during the task. The decoded hand grasping plan could be utilized to control a robotic hand in a real or virtual environment [56–58] (fig. 4). The robotic hand would execute the required grasping behavior instead of the animal, but the visual feedback to the animal would be realistic. By perturbing

such realistic feedback, one can investigate to what extent hand movement representations in AIP and F5 will change when the robotic command signals are systematically perturbed from the decoded signals. Such experiments can directly test the capacity of AIP and F5 for short- and long-term adaptation and might uncover functional differences between these areas.

Conclusion

This short review indicates how important the nonhuman primate studies of cortical plasticity are for understanding the full complexity and adaptability of human behavior. Cortical plasticity expresses itself through many different mechanisms. Both short- and long-term changes in the multiple representations involved in perception, cognition, and action are effected at multiple levels of synapses and circuits. These studies on structure and function reviewed above indicate how even in the nonhuman primate many questions that are central to our understanding of cortical structure and function remain unanswered. While there are well-developed computational models of plasticity (e.g., associative learning, reward learning), we have only started to understand the means by which these mathematical abstractions are actually implemented in the brain. The goal of our present work is to provide answers to some of these questions about the neural implementation of plasticity. Our integrated program of multidisciplinary research will link not only different levels of analysis such as structure, neurochemistry, high-resolution physiology in behaving monkeys, but will draw on human studies of psychophysics and brain imaging to develop as comprehensive a picture as possible of the extent to which cortical circuits modify themselves to adapt to new conditions by generating new behaviors, whether they be effected through natural or artificial means.

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Bacterial Meningitis: The Role of Transforming Growth Factor-Beta in Innate Immunity and Secondary Brain Damage

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Key Words
Chemotaxis · Leukocytes · Endothelium · Vasculitis · Inflammation

Abstract
Project 6 of the NCCR 'Neural Plasticity and Repair' focuses on mechanisms of immunity and tissue damage in autoimmune and infectious diseases of the central nervous system (CNS). In one of the subprojects, the influence of transforming growth factor-β (TGF-β) on the immune reactivity of the CNS was investigated. In mice with *Streptococcus pneumoniae*-induced meningitis, a deletion of TGF-β receptor II on leukocytes is found to enhance recruitment of neutrophils to the site of infection and to promote bacterial clearance. The improved host defense against *S. pneumoniae* was associated with an almost complete prevention of meningitis-induced vasculitis, a major intracranial complication leading to brain damage. The data show that endogenous TGF-β suppresses host defense against bacterial infection in the CNS. This contrasts with findings from other body compartments that suggested that TGF-β is a powerful chemotactic cytokine and increases microbial clearance.

Transforming growth factor-β (TGF-β) controls proliferation, differentiation, and the function of many types of cells. By these activities, TGF-β plays a pivotal role in development, tissue homeostasis and tumor formation. The multiple actions of TGF-β also influence the function of the innate and acquired immune system. In this brief review, we describe the functional role of TGF-β in bacterial meningitis.

Bacterial Meningitis

At present, approximately 1.2 million cases of bacterial meningitis are estimated to occur annually worldwide resulting in 135,000 deaths. Bacterial meningitis is now a top 10 infectious cause of death worldwide and about half of the survivors have neurological sequelae of the disease. Despite antimicrobial agents and intensive care medicine, mortality and morbidity have remained high. With the introduction of *Haemophilus influenzae* conjugate vaccines in the United States and Western Europe, *Streptococcus pneumoniae* and *Neisseria* meningitides have become the major causes of bacterial meningitis in these regions. The mortality rate associated with pneumococcal meningitis is about 20–35% [1] versus 15–30%

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for *Listeria monocytogenes*. An analysis of 87 consecutive cases that were treated in the Department of Neurology, Klinikum Grosshadern in Munich showed intracerebral complications to occur in 75% with seizures (28%), arterial cerebrovascular complications (22%), venous complications (10%), diffuse brain edema (29%), and hydrocephalus (16%) [2].

Clearance of bacteria in the central nervous system (CNS) which depends on a functional phagocyte system – polymorphonuclear neutrophils (PMN) and monocytes/macrophages – is limited due to low complement-mediated opsonic activity in the cerebrospinal fluid (CSF) and low complement C4 and C3 concentrations [3]. In meningitis, inflammation is seen in the meninges and leads to parenchymal damage in the brain and spinal cord, as evidenced by endothelial injury, increased vascular permeability, brain edema and neuronal damage. Bacterial cell wall components and toxins (released during antibiotic-induced or bacterial autolysis) enter into the CSF compartment and reach the interstitium of the CNS by paravascular fluid circulation. Besides the infectious pathogens, vascular damage and neurotoxicity are also caused by the host defense system. Recognition of microbial constituents is mediated through pattern recognition receptors of which Toll-like receptors (TLRs) are key participants [4]. TLRs recognize structural motifs referred to as pathogen-associated molecular patterns on the pathogens, a process initiating activation of PMN, monocyte-macrophages and natural killer cells. As a consequence, innate immunity results in the production of chemokines and proinflammatory cytokines [e.g. tumor necrosis factor (TNF-α), interleukin (IL)-1 and IL-6], which initiate endothelial cell damage, recruitment of PMN and monocytes into the meninges and CNS tissue, activation of both astrocytes and microglia cells, and neuronal damage. TLR2 recognizes the pneumococcal cell wall molecule lipoteichoic acid whereas TLR4 acts as a ligand for the pneumococcal cytotoxin pneumolysin [references in ref. 5]. Mice that lack either TLR2, TLR4 or their downstream adaptor protein myeloid differentiation factor 88 show reduced CSF PMN pleocytosis, less inflammation but severely impaired bacterial clearance [5]. The production of TNF-α, IL-1 and of the PMN chemokines MIP-2 and KC was significantly diminished [5]. Recent studies using anti-IL-6 antibodies and IL-6 gene knockout mice showed IL-6 to inhibit migration of PMN into the CNS but to promote vascular permeability, brain edema formation and rise in intracranial pressure [6]. In the breakdown of the blood-brain barrier nitric oxide plays a decisive role by modulating adhesiveness of PMN through inhibition of β_2-integrin expression. Impaired nitric oxide synthesis in mice with a deletion of the inducible nitric oxide synthase (iNOS) results in aggravated CSF pleocytosis [7]. The extent of the inflammatory process is limited by counterregulatory cytokines, namely IL-10 and TGF-β, which deactivate neutrophils and macrophages. These cytokines interfere with production of neurotoxic molecules by phagocytes, which include radical oxygen intermediates, nitric oxide and proteases such as matrix metalloproteinases (MMP). MMP8 and MMP9 are upregulated in the CSF in meningitis, MMP9 concentrations being higher in patients with secondary brain damage than in those who show complete recovery [8]. Besides MMP9, also high levels of the nitric oxide-induced oxidant peroxynitrite in the CSF are associated with an unfavorable outcome [9]. One of the pathways of oxidant-induced CNS damage includes poly(ADP ribose) polymerase, its deletion in mice improving the clinical score of meningitis [10].

TGF-β and Immune Response

TGF-β is part of the TGF-β superfamily, with additional members including bone morphogenetic proteins, activins and growth differentiation factors. From the three homologous TGF-β isoforms in mammals, it is TGF-β_1 that is predominantly expressed in cells of the macrophage lineage such as microglia, whereas TGF-β_2 and TGF-β_3 are produced by astrocytes and neurons [11, 12]. TGF-β is secreted as a homodimer noncovalently bound to the latency-associated protein. TGF-β latency-associated protein may complex latent TGF-β-binding protein-1 via disulfide bonds. The active molecule needs to be released from latency-associated protein to mediate its functions via TGF-β receptor I (TGF-βRI) ALK-5 and TGF-β receptor II (TGF-βRII). The latter binds TGF-β with high affinity (fig. 1). In the case of TGF-β_2, binding to this receptor requires the presence of TGF-βRIII, a membrane-bound betaglycan. Signaling is initiated upon binding of TGF-β dimers to the tetrameric ALK-5 and TGF-βRII, which leads to activation of TGF-βRI and thereafter phosphorylation of intracellular SMAD2 and SMAD3 [for review, see ref. 13]. TGF-β regulates T-cell survival, inhibits perforin and Fas ligand expression on CD8 T cells and converts $CD4^+$, $CD25^-$ T cells into FoxP3 expressing regulatory T cells. Expression of a dominant negative form of TGF-βRII in $CD4^+$ cells blocks TGF-β signaling in these cells and is associated

with an uncontrolled $CD4^+$ T-cell-mediated inflammatory reaction with vasculitis and perivascular cell infiltrates [14, 15].

Role of TGF-β in Innate Immunity in Bacterial Infections

Cells of the mononuclear phagocyte system and PMN mediate the innate immune response in bacterial infections. In *S. pneumoniae* infection, phagocytosis is mainly mediated by complement which interacts with CR3 and in part with CR1, CR2 and CR4. Neutrophil activation during phagocytosis of microbes leads to respiratory burst and production of radical oxygen intermediates by the NADPH oxidase of phagocytic cells. Protective microbicidal activity is mediated by radical oxygen intermediates and granule proteases including elastase and cathepsin G [16]. Activation of PMN and monocyte-macrophages results in the secretion of chemokines and cytokines, which recruit further phagocytes into the tissue and boost the inflammatory reaction. Recognition and uptake of apoptotic cells lead to TGF-β production by phagocytes, a process which depends on the presence of phosphatidyl serine in the cell membrane [17]. The classical view is that TGF-β counteracts the inflammatory response.

TGF-β suppresses: (1) the interferon-γ-induced macrophage activation including the CIITA-dependent induction of class II MHC antigens; (2) the production of expression of the proinflammatory cytokines IL-1β, IL-6 and TNF-α in activated microglia; (3) the production of MMP and of chemokines (MIP-1α, MIP-2) which are important in migration and chemoattraction of phagocytes to the site of infection; (4) the formation of oxidative response and thereby killing of intracellular bacteria; (5) the expression of the scavenger receptors CD3α and SR-A and of CD14; (6) myeloid differentiation factor 88-dependent TLR signaling, FcRI and FcRIII; (7) iNOS expression, thereby no production by phagocytes, and (8) IL-1-induced signaling by enhancing production of the IL-1 receptor antagonist.

These effects of TGF-β prevent both recognition and degradation of bacteria and interfere with microbe-induced proinflammatory activation of phagocytes [18–28]. Moreover, TGF-β inhibits lipopolysaccharide-induced septic shock in the mouse [29]. The effect of TGF-β also includes down-regulation of CD14 and binds the lipid A moiety of lipopolysaccharide, lipoteichoic acid and mycobacterial lipoarabinomannan. It is of note that

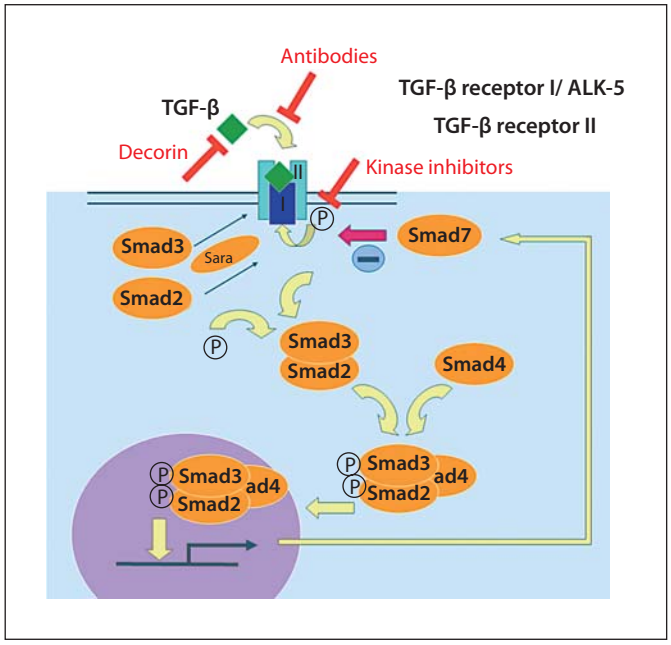


Fig. 1. TGF-β signaling pathway and potential targets of inhibition. The activated TGF-βRI/II complex recruits and phosphorylates the transcription factors Smad2/3, which bind Smad4 and translocate into the nucleus where they activate the transcription of target genes. Inhibitory Smad7 prevents activation of Smad2/3 by TGF-βRI. Disruption of the signaling pathway by anti-TGF-β and anti-TGF-β receptor antibodies, TGF-β decoy molecules and TGF-β serine/threonine kinase blockers.

CD14 activates the c-Jun N-terminal kinase, which is involved in expression of TNF-α, a major mediator of septic shock [21]. TGF-β inhibits TNF-α and MIP-2 production through the crosstalk between mitogen-activated protein kinase, specifically ERK-dependent inhibition of p38 mitogen-activated protein kinase caused by up-regulation of MKP-1 [30].

The picture of the function of TGF-β on cells of the macrophage lineage changes when analyzing the effect of the cytokine on peripheral blood monocytes. TGF-β is chemotactic for monocytes and activates the cells to express adhesion molecules (LFA-1, VLA-3, VLA-5) and Fc-γRIII, and to secrete IL-1 and TNF-α [for review, see ref. 31]. Thus, TGF-β deactivates tissue macrophages but activates blood monocytes. Similar to monocytes, TGF-β is also a potent chemoattractant for PMN. The complexity of actions of TGF-β on PMN, however, becomes evident when TGF-β is tested on endothelial cells. TGF-β inhibits its migration through TNF-α-activated endothelial cells in vitro and down-regulates E-selectin and VCAM-1 ex-

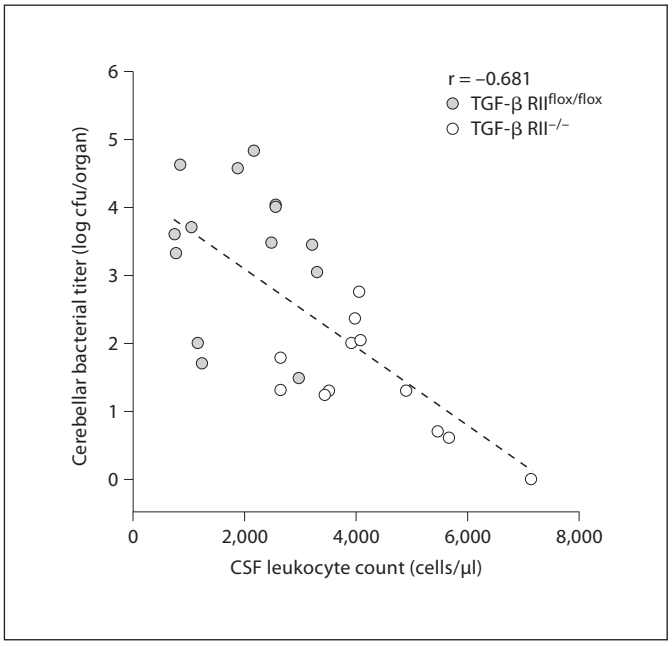


Fig. 2. High numbers of leukocytes in CSF and low bacterial titers in the CNS of *S. pneumoniae*-infected phag-TGF-βRII$^{-/-}$ mice. The higher CSF leukocyte numbers are associated with reduced cerebellar bacterial titers, indicating an improved clearance of bacteria in phag-TGF-βRII$^{-/-}$ mice.

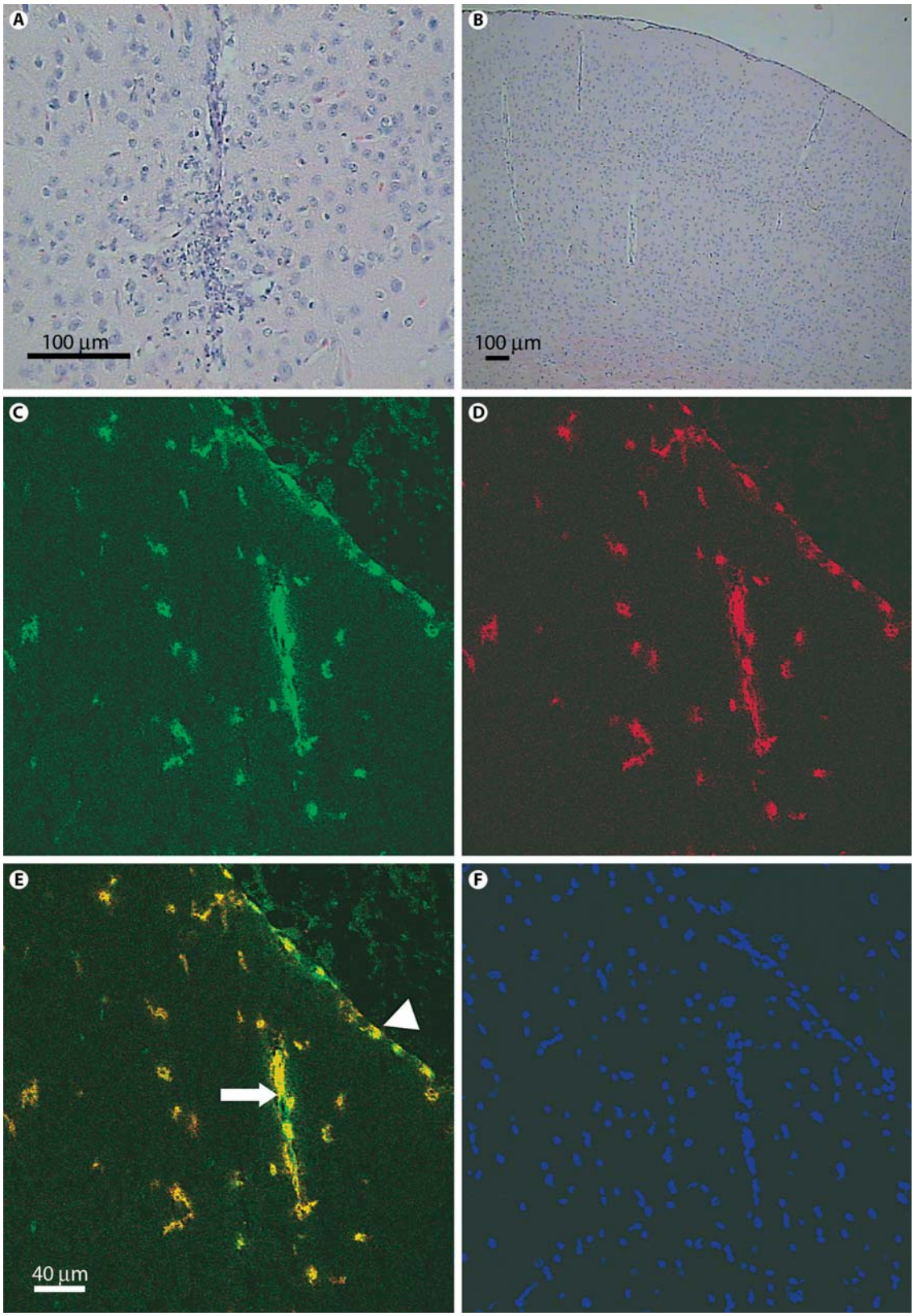
pression [32, 33]. Mice with double-deficient P- and E-selectins display a severe impairment of PMN influx into the meninges [34].

Disruption of TGF-βRII on Phagocytes Leads to Improved Bacterial Clearance

To delineate and define the role of TGF-β in innate immunity to bacterial infections, we crossed TGF-βRII$^{flox/flox}$ mice with LysCre mice, thereby obtaining mice lacking TGF-βRII on neutrophils and macrophages (phag-TGF-βRII$^{-/-}$ mice). Given the chemotactic response of TGF-β on these cell types and their capacity to mediate vasospasm and vasculitis and thereby secondary brain damage, PMN recruitment in a bacterial meningitis model may be impaired, thereby mitigating secondary brain damage. However, the opposite has been observed. Upon infection with *S. pneumoniae* PMN in CSF were two- to threefold higher in the phag-TGF-βRII$^{-/-}$ mice compared to controls. The amount of bacteria in the CNS correlated with the number of PMN in the CNS and was reduced 140-fold in the phag-TGF-βRII$^{-/-}$ mice (fig. 2). Thus, in bacterial meningitis TGF-β in its active form is produced in the course of the disease and inhibits at the level of PMN their migration into the CNS, a step required for successful elimination of *S. pneumoniae*. Whether TGF-β also counteracts phagocytosis and bacterial destruction by phagocytes remains open. However, since in phag-TGF-βRII$^{-/-}$ mice a two- to threefold increase of PMN in the CSF is paralleled by a 140-fold decrease of the bacterial load, it is possible that TGF-β acts at two levels: the migration process of PMN and the phagocytosis and killing process executed by PMN.

Impairment of PMN recruitment has been suggested in the following studies: (1) PMN adhesiveness to human umbilical vein endothelial cells is inhibited by TGF-β; (2) TGF-β coinjected intratracheally with lipopolysaccharide impairs the acute neutrophilic inflammatory response, and (3) injection of TGF-β into MRL/n mice reduces the recruitment of PMN in the thioglycollate-stimulated peritoneal exudate [35–37]. A different view on the effect of TGF-β on PMN recruitment has been reached in other experimental systems: (1) TGF-β is chemotactic for PMN and monocytes in vitro; (2) mice with a targeted disruption of the SMAD 3 gene – SMAD 3 binds to TGF-β receptors and mediate its signaling – impairs the chemotactic response of mutated neutrophils towards TGF-β; (3) injection of TGF-β into knee joints of rats leads to extensive recruitment of PMN and monocytes [38–41], and (4) TGF-β induces leukocyte recruitment and improves microbial clearance when administered via intrabronchial routes to rats with *Escherichia coli* pneumonia [42]. Our studies show unambiguously that in bacterial infections of the CNS, TGF-β impairs rather than stimulates PMN recruitment to the site of infection. In this context, it is of note that TGF-β_1 is elevated in the CSF of children with acute bacterial meningitis [43]. In this study, no correlation existed between TGF-β_1 levels and cell counts in the CSF on the one hand and between TGF-β_1 levels and subsequent development of neurologic sequelae on the other. However, only 5 out of 16 patients have developed major neurological complications. Thus, the number of patients is too small to allow a definite

Fig. 3. Control mice infected with *S. pneumoniae* show widespread cortical and subcortical leukocytoclastic lesions (**A**), which are only occasionally observed in phag-TGF-βRII$^{-/-}$ mice (**B**). Immunohistology shows Gr1$^+$ (**C**), CD11b$^+$ (**D**), neutrophils (overlay **E**) in the meninges (arrowhead), in destroyed vessels (arrow) and in brain parenchyma of infected TGF-βRII$^{flox/flox}$ mice (**C–F**). **F** Nuclear stain with DAPI.


A
100 µm
B
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C
D
E
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F

conclusion. In fact, the data do show a tendency for TGF-β to be high in patients with low leukocyte numbers and high protein content in the CSF.

Bacterial Meningitis in phag-TGF-βRII$^{-/-}$ Mice: Combination of Increased PMN and Decreased Numbers of Bacteria in the CNS Prevents Secondary Brain Damage

In the experimental meningitis model which we used to delineate the function of TGF-β, mice were inoculated with *S. pneumoniae* type 3 and 24 h later treated with ceftriaxone. Two days after infection, control mice developed multifocal intracerebral cortical and subcortical leukocytoclastic vasculitis of the small veins (fig. 3A, B) and blood-brain barrier damage with increased brain albumin concentrations and intracerebral pressure [44]. In phag-TGF-βRII$^{-/-}$ mice, vasculitis was one tenth as pronounced, the rise of intracranial pressure and albumin significantly reduced, and the clinical course of the disease much less severe [44]. Thus, despite pronounced CSF pleocytosis, neither vasculitis nor secondary brain damage occur in the absence of TGF-β signaling in phagocytes, and no signs of macrophage or PMN hyperreactivity were found. Likewise, no abnormalities were detected in coagulation assays. In the light of numerous reports on deactivation of phagocytes by TGF-β it is remarkable that there are no signs of a failure to control PMN and macrophage activity when the cells are not provided with TGF-βRII signaling. In this context it is of note that in TGF-β_1 gene knockout mice, the 'spontaneous' inflammatory reaction which is observed in different organs is accompanied by increased expression of TNF-α, IL-1β and iNOS. High level of expression was associated with increased TLR4 mRNA expression, the respective receptor mediating NF-κB-dependent activation of proinflammatory cytokines and iNOS [45].

Animal studies of pneumococcal meningitis show an association of low initial CSF leukocyte counts with high bacterial titers, development of intracranial complications and unfavorable outcome [46, 47]. The rise of both the intracranial pressure and the CSF protein concentration in pneumococcal meningitis in rabbits was also observed in neutropenic rabbits [48], which suggests that secondary brain complications are generated by interaction of the infectious agents with endothelial cells and/or brain parenchymal cells. Likewise, in patients with bacterial meningitis brain damage is significantly more frequent in those patients showing a low CSF pleocytosis and high bacterial numbers [2, 49].

Future Roads to Go in the Treatment of Meningitis

The phenotype of phag-TGF-βRII$^{-/-}$ mice observed upon infection with *S. pneumoniae* gives rise to the hope that, by blocking TGF-β effects on innate immunity in the CNS, the clinical course of disease may be improved and the risk of secondary brain damage diminished. Different strategies can be envisaged: inactivation of TGF-β by TGF-β-binding proteins such as decorin, by antibodies against TGF-β and TGF-β receptors, by inhibition of TGF-β signaling via serine/threonine kinase blockers and by overexpression of Smad7 (fig. 1). Thus, as a first step before going into clinical studies, antibodies to TGF-β, or to its receptors or small molecules which interfere with the kinase activity of the receptor, will be studied for their effectiveness in experimental bacterial meningitis.

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Repair of the Injured Spinal Cord

A Joint Approach of Basic and Clinical Research

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Key Words
Spinal cord injury · Nogo-A · Axonal regeneration · Functional recovery · Neural plasticity · Animal models · Clinical trials

Abstract
The myelin protein Nogo-A is a potent inhibitor of neurite outgrowth in the central nervous system, thus contributing to the incapacity of fiber tracts in the adult spinal cord to regenerate after injury. In this review we report on a joint approach of different research groups to develop a therapy applying anti-Nogo-A antibodies to the injured spinal cord. While basic researchers took the initiative to provide means of neutralizing the inhibitory effect of Nogo-A and demonstrated enhanced fiber growth, regeneration and functional recovery both in rodent and primate models, clinical groups and rehabilitation engineers have sought to translate this novel strategy into a clinical setting.

Introduction

The aim of the project 'Spinal Cord Repair' of the NCCR 'Neural Plasticity and Repair' initiated by the Swiss National Science Foundation in 2001 is to develop novel treatments for spinal cord injury and implement these into a clinical setting. The challenge to bring together basic researchers, clinicians and engineers from different academic institutions (Federal Institute of Technology, University of Zurich and Fribourg, Balgrist University Hospital Zurich) and industry to collaborate on distinct aspects of this project resulted in an extremely fruitful exchange of ideas and has greatly accelerated the progress of the project.

One of the basic science groups within this project has developed regeneration-enhancing treatments for increased restoration of function after spinal cord injury. Their validation has now been successful in a preclinical setting in mouse, rat and monkey models – a key prerequisite to enter clinical trials. A network of paraplegic centers in Europe and North America has been created where clinical studies can be pursued ensuring comparable diagnostic methods, treatment schedules and follow-ups. Phase I clinical trials with an anti-Nogo-A antibody treatment of acute para- and tetraplegic patients have recently been initiated.

Basic Research and Proof of Principle of Regeneration-Enhancing Anti-Nogo Antibody Treatment in Rodent Models

Several lines of evidence suggested the presence of specific inhibitory factors responsible for the nonconducive properties of central nervous system (CNS) tissue for axonal regeneration in adult vertebrates. CNS white matter

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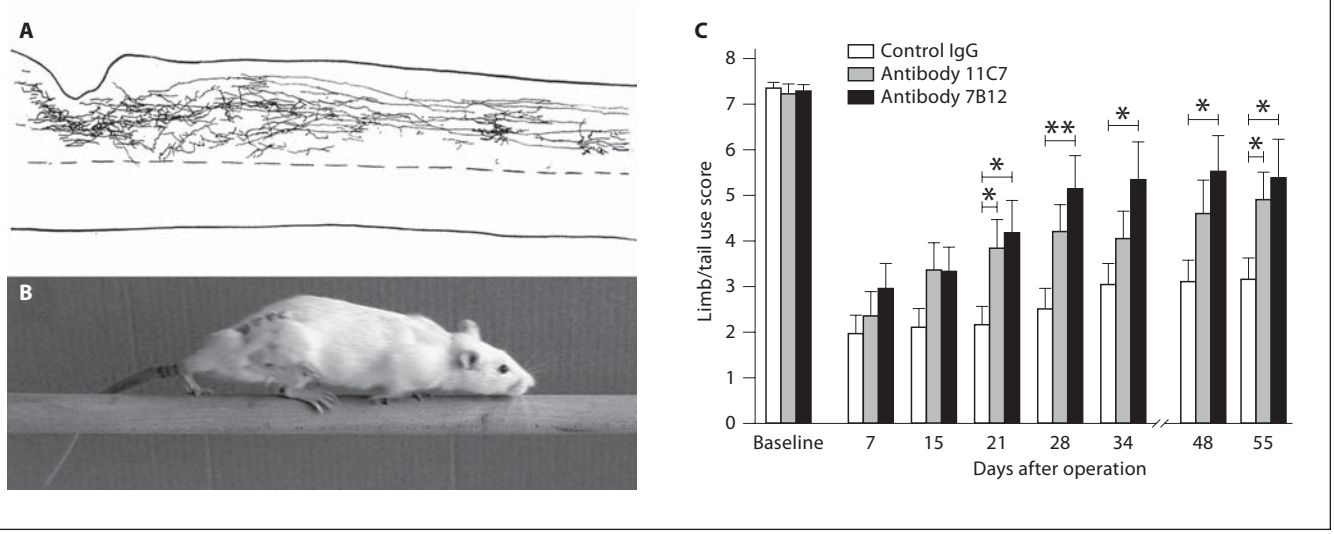

Fig. 1. Long-distance and functional regeneration after spinal cord injury in rats (**A**), and functional recovery in anti-Nogo-A antibody-treated rats after spinal cord injury (**B**, **C**). **A** Regeneration in the spinal cord: camera lucida drawing of sagittal sections of the lower thoracic spinal cord including the lesion site (left). Neutralization of Nogo-A leads to enhanced sprouting rostral to the lesion (left) and to fibers crossing the lesion on remaining tissue bridges into the caudal spinal cord and growing down the spinal cord over long distances (right to the lesion site). **B** Rat balancing over a narrow beam. **C** Anti-Nogo-A-treated animals (antibodies 11C7, 7B12) show improved performance in the swim test compared with the control IgG antibody-treated SCI rats. With permission from *Annals of Neurology*. * $p < 0.01$; ** $p < 0.001$.

contains myelin-associated neurite growth inhibitors such as Nogo-A that seem to play a crucial role in preventing regeneration of lesioned axons in adult animals [1, 2]. The same inhibitory myelin proteins probably also inhibit structural plasticity in higher vertebrates [2, 3]. Different antibodies that neutralize the inhibitory activity of Nogo-A have been developed and their regeneration-enhancing effect for CNS axons was tested both in vitro and in vivo.

In vivo studies were performed with adult rats lesioned at spinal cord thoracic level T8, which received anti-Nogo-A antibodies through the cerebrospinal fluid. In Nogo-A antibody-treated animals but not in control antibody-infused animals, regenerating corticospinal (CS) tract fibers were seen growing down the spinal cord (fig. 1A). These fibers exceeded a length of 8 mm and arborized profusely into the gray matter. In addition to regenerative growth of lesioned axons, sprouting and compensatory fiber growth was observed from spared, unlesioned fiber systems [3–6]. This suggested that both these phenomena – regeneration and structural plasticity – probably contribute to the often high degree of functional recovery observed in these animals. The behavioral results summarized below also suggest that the fibers growing and regenerating in the adult CNS tissue seem to be able to recognize functionally meaningful targets and to form new functional circuits.

In spinal cord-injured (SCI) adult rats, intrathecal infusion of anti-Nogo-A antibodies resulted in impressive improvements of functional recovery in particular of locomotor functions like swimming, running, or crossing horizontal ladders or beams (fig. 1B, C) [4, 6, 7]. In these studies malfunctions like pain or spasticity – both possible indicators for misled axons or erroneous connections – were consistently absent, suggesting that the new connections and circuits are formed with high specificity. Functional magnetic resonance imaging (functional MRI) was applied to study the cortical representation of forelimbs and hind limbs and their responsiveness to peripheral sensory stimulation after SCI. In contrast to control antibody-treated animals, SCI rats treated with anti-Nogo-A antibodies revealed significant cortical responses in functional MRI after hind paw stimulation, suggesting restitution of afferent spinocerebral pathways [6].

In the future, the impact of rehabilitative training on regeneration and plasticity in the spinal cord and brain (including the neocortex), and on functional recovery will be studied in adult rats. For this, a combination of specific lesion models with anti-Nogo-A antibody treatment, different tracing techniques and physical training are envisaged. In addition to behavioral analysis, these experiments allow us to study effects on fiber growth and anatomical plasticity.

Very little information is available on the distribution of therapeutic antibodies infused into the cerebrospinal fluid. We therefore studied the distribution and tissue penetration of antibodies after 7–14 days of intrathecal infusion in adult rats and monkeys [8]. Anti-Nogo-A antibodies reached the brain and whole spinal cord and penetrated deep into the parenchyma where they bound to oligodendrocytes and nerve cells. They were subsequently internalized together with the endogenous cell surface Nogo-A, leading to a down-regulation of CNS Nogo-A levels.

Nogo-A knockout mice, which can serve as a useful proof of principle for the inhibitory effect of Nogo-A on regeneration, were successfully generated. CS tract regeneration was enhanced following injury in the absence of Nogo-A [9]. To exclude a role of background genes, these Nogo-A knockout mice were successfully backcrossed into two different commonly used mouse strains (unpublished data). After spinal cord lesion, Nogo-A-deficient mice of both strains showed enhanced regenerative sprouting and long-distance regeneration as compared to wild-type mice of the same background. Surprisingly, however, one of the strains (SV129) displayed significantly higher numbers of regenerating fibers than the Nogo-A knockouts of the other strain (C57Bl/6). These results confirm that Nogo-A is an important endogenous inhibitor of axonal regeneration in the adult spinal cord; they also demonstrate that the effects of Nogo-A deletion can be modified by mouse strain-specific genes.

The reaction of neurons and CNS tissue to anti-Nogo-A antibody treatment on the one hand and the consequences of loss of Nogo-A expression in knockout animals on the other hand are being studied at the molecular level by a functional genomics approach. The aim of these experiments is to identify molecules associated with axon (re-)growth, to gain insight into signaling mechanisms involved in regeneration processes, and to find cues involved in neuronal circuit formation in the adult CNS.

Enhancement of Functional Recovery and Axonal Regrowth in Monkeys Treated with Anti-Nogo-A Antibodies after Lesion of the Cervical Spinal Cord

In order to translate the anti-Nogo-A antibody strategy from rodents to subhuman primates, monkeys have been subjected to SCI. Since the general organization of the CS system is considerably different between rodents and primates, a proof of principle in monkeys represents a crucial step before clinical application to human patients. Furthermore, the monkey model is more appropriate than rodents to test whether anti-Nogo-A antibody treatment does not generate undesired secondary effects, such as chronic pain.

Twelve adult Macaque monkeys (*Macaca fascicularis* or *Macaca mulatta*; 3.5–5 kg; 3–4 years old) were trained to perform a manual dexterity task (fig. 2B). The monkeys retrieved food pellets from 50 wells using the opposition of thumb and index, i.e. the precision grip. The number of pellets retrieved within 30 s was determined (fig. 2A). After about 2 months a subhemisection was performed unilaterally at the C7/C8 level [10–12]. The lesion completely interrupted the main CS component in the dorsolateral funiculus (fig. 2C, arrow). Immediately after the lesion, 6 monkeys were treated with an anti-Nogo-A antibody delivered intrathecally near the lesion site for 4 weeks from an osmotic pump. An inactive control antibody was infused in the other 6 monkeys. Behavioral testing was continued for 2–3 months postlesion in order to compare the extent and time course of functional recovery between the two groups of monkeys. Finally, an anterograde neuroanatomical tracer (biotinylated dextran amine, BDA) was injected in the primary motor cortex contralateral to the cervical lesion, in order to trace the CS tract and its regenerated fibers.

In all monkeys, the hand homolateral to the lesion was dramatically impaired immediately after the lesion and then, over a period of a few weeks, progressive partial recovery of manual dexterity took place. The 2 monkeys shown in figure 2A exhibited a comparable cervical cord lesion, but the anti-Nogo-A antibody-treated monkey (red) recovered faster and much more completely than the control antibody-treated animal (blue). Overall, control antibody-treated monkeys exhibited a recovery of manual dexterity that was inversely correlated to the lesion extent (fig. 2D, blue circles), whereas anti-Nogo-A antibody-treated monkeys recovered faster and almost completely, irrespective of lesion size (fig. 2D, red squares). In addition to the above-mentioned ability to grasp pieces of food with the precision grip, a better re-

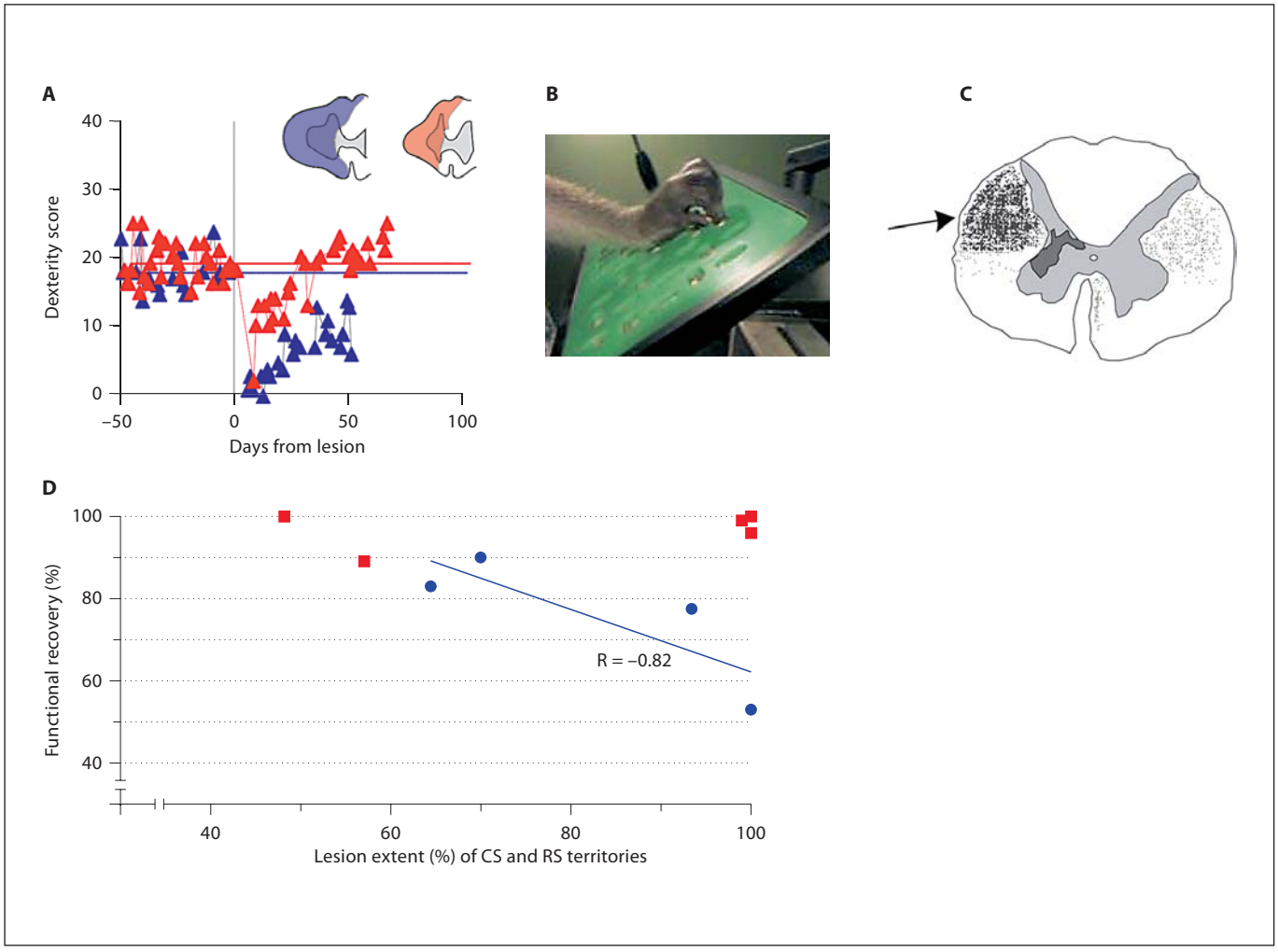

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Fig. 2. A Typical manual dexterity score (successful food pellet retrieval) derived from daily sessions of precision grip task (Modified Brinkman Board) for 2 monkeys: control antibody-treated (blue) and anti-Nogo-A antibody-treated (red). The dexterity score is high before the lesion, falls to a very low level after the spinal cord injury, and recovers to about 30–40% in the control, but to 100% of the original performance in the anti-Nogo-A antibody-treated animal. The insets (top) show the extent of the lesion on a cross section of the cervical spinal cord, for the control antibody- (blue) and anti-Nogo-A antibody-treated monkeys (red). The lesion completely interrupted the dorsolateral funiculus and covered a comparable extent of the hemicord in the 2 monkeys. **B** View of the left hand of a monkey while performing the grasping of a pellet from a well in the Brinkman Board test. **C** Frontal section of the cervical cord in an intact monkey showing the distribution of the corticospinal (CS) axons as a result of BDA injection in the right motor cortex. Each labeled CS axon is represented by a small dot. The arrow points to the main CS component, the crossed dorsolateral funiculus, which was destroyed by

covery in anti-Nogo-A antibody-treated monkeys was also observed for behavioral tasks testing other motor parameters, such as force and ballistic grasping or catching of moving objects.

A detailed anatomical investigation of the transected CS tract showed that application of antibodies neutralizing Nogo-A led to a significant increase in BDA-labeled CS axonal arbors and CS axonal swellings caudal to the lesion, as compared to control antibody-treated monkeys. Rostral to the lesion, the anti-Nogo-A antibody treatment induced a reduction of the incidence of axonal retraction bulbs, attenuation of CS axonal dieback and increased sprouting of CS fibers. Very few CS axons truly regenerated by recrossing the lesion; instead, anti-Nogo-A antibody treatment enhanced collateral sprouting, which gave rise to BDA-labeled axons seen growing around the lesion medially in the gray matter. The fibers then elongated into the denervated gray matter territory caudal to the lesion. The presence of CS axonal arbors caudal to the lesion correlated with the level of functional recovery, suggesting that the regenerative sprouting of the CS tract contributed to the recovery of manual dexterity. Very importantly, the monkeys treated for 4 weeks with the anti-Nogo-A antibody did not show any sign of chronic pain or change of general behavior, neither with their mates within the colony in the animal room nor with respect to the experimenters. The monkeys were in good health as indicated by a stable body weight postlesion during the infusion of the antibody. This study in monkeys extended the findings with anti-Nogo-A antibody therapy in rodents to primates, paving the way for clinical application to human SCI patients.

the lesion in the present study. **D** For 9 monkeys, the plot shows the relationship between the extent of the spinal cord lesion (abscissa) and the degree of functional recovery (ordinate). A functional recovery of 100% means that the postlesion score came back to the level of the prelesion score. The extent of the lesion was expressed in percent of the zone in the white matter of the hemicord including the dorsolateral funiculus (main CS tract component) and the rubrospinal (RS) tract. In control antibody-treated monkeys (●), the functional recovery was inversely correlated to the lesion extent ($r = -0.82$); in contrast, all the anti-Nogo-A antibody-treated animals (■) reached 90–100% of recovery.

Fig. 3. Assisted treadmill training within a driven gait orthosis (Lokomat). Subjects are suspended in a harness. Body weight support as well as treadmill speed can be adapted to the patient's ability. The orthosis is equipped with sensors to provide feedback of the subject's performance to the patient and the therapist. Different patient-cooperative control strategies are implemented to enhance training success (picture courtesy of Hocoma AG, Volketswil, Switzerland).

Clinical Issues

To document the changes at the neurological and functional level after human spinal cord injury and therefore provide a historical control group for future clinical trials, a European multicenter study (EM-SCI) was initiated in 2001 [13]. In this study, standardized assessments covering neurological, functional and electrophysiological aspects are done at defined time points after the injury. Additional protocols monitoring pain, bladder function and acute care treatment will be included in the near future. New tests for voluntary function of the legs covering the whole rehabilitation period and neurophysiological methods for the assessment of impaired function of specific spinal pathways are underway. Analysis of the currently more than 700 patient data allows a better stratification of patient subgroups and a more precise prediction of outcome. This will help recognize even small improvements in the recovery of functions and to monitor any significant effect of a new treatment.

If such a new treatment could produce axonal regeneration in the lesioned human spinal cord the spinal circuits below the lesion should be preserved. This is especially true for clinically complete SCI patients where only regeneration approaches may succeed. Recently, signs of decreased neuronal function in the lower spinal cord were found in chronic complete SCI patients; they were restricted to the specific motor behaviors affected by the injury, e.g. locomotion [14]. The affected spinal neuronal circuits seem to be at least partially different from spinal reflex circuits as the latter do not show such decreased function [15]. Possible preventive effects of early motor training combined with pharmacological interventions (e.g. L-dopa) are being evaluated. Earlier results have already shown that loading and hip position afferents are essential to stimulate the spinal neuronal circuits involved in locomotion [16].

Long-term training studies with chronic incomplete SCI patients showed that these patients profit from an assisted locomotor training for their mobility [17]. Robotic devices for gait (Lokomat®, fig. 3) and arm function (ARMin) with novel training and feedback strategies have been introduced and are expected to enhance the positive output of assisted training in patients with SCI [18, 19].

New insights into the integrity of spinal circuits will be provided by studies applying high-resolution MRI techniques (functional MRI, diffusion tensor imaging) to the injured spinal cord [20]. Although very challenging, this approach holds great promise for the assessment of spared tract anatomy, residual spinal cord function, and the recovery of spinal pathways in a noninvasive manner.

Electrophysiological recordings at the level of the lesion have been shown to serve as an objective diagnostic tool to assess the integrity of autonomic afferent nerve pathways and to distinguish between central and peripheral lesions [21]. In SCI patients, synchronized activation and inactivation of the autonomic and somatic pathways was shown to be necessary for appropriate urine storage and coordinated bladder voiding. The chronology of bladder dysfunction has been assessed, providing the basis for conditional neuromodulation in patients with SCI [22]. This is a promising treatment for bladder dysfunction, which is a more confining issue for a majority of SCI patients than the walking impairment.

In conclusion, the concerted efforts of basic scientists working with rat and monkey models of SCI as well as cell biological and molecular biological tools on the one hand, and clinical researchers including neurorehabilitation specialists and engineers on the other hand in the NCCR project 'Spinal Cord Repair' have led to the full translation of basic science findings into a clinical trial: in close collaboration with Novartis Pharma in Basel, a phase I clinical trial in humans with anti-Nogo-A antibodies was started in the spring of 2006.

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New Technologies and Concepts for Rehabilitation in the Acute Phase of Stroke: A Collaborative Matrix

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Key Words
Stroke · Rehabilitation · Functional electrical stimulation · Arm robot-assisted therapy · Virtual reality · Mirror neuron

Abstract
The process of developing a successful stroke rehabilitation methodology requires four key components: a good understanding of the pathophysiological mechanisms underlying this brain disease, clear neuroscientific hypotheses to guide therapy, adequate clinical assessments of its efficacy on multiple timescales, and a systematic approach to the application of modern technologies to assist in the everyday work of therapists. Achieving this goal requires collaboration between neuroscientists, technologists and clinicians to develop well-founded systems and clinical protocols that are able to provide quantitatively validated improvements in patient rehabilitation outcomes. In this article we present three new applications of complementary technologies developed in an interdisciplinary matrix for acute-phase upper limb stroke rehabilitation – functional electrical stimulation, arm robot-assisted therapy and virtual reality-based cognitive therapy. We also outline the neuroscientific basis of our approach, present our detailed clinical assessment protocol and provide preliminary results from patient testing of each of the three systems showing their viability for patient use.

Introduction

Stroke results in several neurological impairments which often severely reduce patient ability to perform activities of daily living (ADL) in both the short and long term. To individual patients, however, the assessments of impairments performed by attending physicians may be less important than maintaining or restoring premorbid daily-life functions. This is particularly true for upper extremity function and especially for skilled tool use. Constraint-induced movement therapy is a well-accepted, evidence-based approach for the chronic stage following a stroke [1, 2]. However, the optimal type of therapy for arm and hand function in the acute stage is still unclear, although a variety of treatment concepts has been defined [3].

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Evidence from animal trials suggests that early initiation of therapy favorably influences efficacy of rehabilitation. In the early post-stroke stages the brain already shows adaptive plasticity in within-system pathways [4, 5], and the brain displays elevated sensitivity to rehabilitative experience. Also, there is evidence of a correlation between early initiation of rehabilitation and better functional outcome as assessed by the Barthel index [6]. Other critical factors for sensorimotor therapy to induce long-term brain plasticity and improve functional outcomes are that the therapy is intensive [7], highly repetitive [8], task-oriented [9] and rewarded. This has been shown in a longitudinal study where therapy was applied early in a repetitive, task-oriented scenario to significantly improve long-term functional outcomes [10, 11]. Other experiments with healthy animal and human subjects suggest that repetitive task-oriented exercise alone will not increase cortical plasticity; rather, some degree of motor learning is required [12, 13] such as that experienced by stroke patients undergoing rehabilitation.

These threads of evidence all point towards the need to develop arm and hand therapies for acute-phase stroke patients that are intensive, repetitive and oriented towards ADL. The best methods and technologies to be used are as yet unknown and likely to vary between patients. In this study we are applying new rehabilitation technologies developed in close collaboration with clinical, engineering and computer science groups interconnected in a 'Rehabilitation Technology Matrix' within the Swiss National Center for Competence in Research in 'Neural Plasticity and Repair'.

Treatment Rationale and Goals

The primary goal of our approach is the multimodal reactivation of sensorimotor mechanisms that are part of the disrupted motor program by stimulation of undamaged regions which project directly or indirectly to sensorimotor areas. At the lowest level, we achieve this by providing the afferent proprioceptive feedback to the central nervous system (CNS) that would be present during normal active movement execution, thus closing the motor control loop. At a higher level, we also aim to recruit motor planning and execution areas by embedding the movements in task-oriented scenarios. At the highest level, we can also stimulate motor planning areas by directing patient attention to a task and encouraging conceptual rehearsal of intended movements [14]. Simultaneously, we activate the action recognition system through visual input simulating the desired movement to provide feedback consistent with correct movement execution. Bilateral training using these techniques has also been shown to increase activation in the motor cortex during the post-stroke acute phase [15] in contrast to the chronic phase, where constraint-induced therapy is the appropriate choice. Coupled bimanual coordination theory postulates that learning involves development of coordinative structures as the centrally linked upper extremities function together in solving motor tasks [16].

The main secondary goal of our approach is to increase activity of the paretic limb through early, intensive and rewarded training of daily living functions, thereby motivating the patient to regain functional independence. The technologies we are deploying can play a key role in this process by replacing the physical strength of the therapist, providing for semiautomatic, objective performance evaluation, and/or enabling partially or completely unsupervised training. Additional benefits of this training regime include the elimination of nonuse patterns of the affected limb through regular daily activity, prevention of compensatory maladaptive strategies, and avoidance of secondary acquired abnormal movements.

New Approaches – Technology Overview

Conventional physiotherapy uses the decades-old method of peripheral manipulation performed by therapists, possibly with the help of mechanical devices or supports. The new methods we are investigating in this study – functional electrical stimulation (FES), exoskeleton arm robot (ARMin) therapy and cognitive virtual-reality (VR)-based therapy – build on this methodology by assisting the therapist with the manipulation and measurement processes, and providing new possibilities for engaging the patient's peripheral nervous system and CNS (PNS/CNS). The three systems mainly differ from each other in the primary methods used to stimulate the PNS/CNS, ranging from peripheral manipulation (ARMin) through direct surface peripheral muscle stimulation (FES) to CNS stimulation (cognitive VR). These differences are summarized in table 1. In the following sections we describe each of the technologies in more detail.

Functional Electrical Stimulation

FES applies bursts of high-intensity electrical pulses via surface (transcutaneous) electrodes to create action poten-

Table 1. Comparison of rehabilitation technologies

	FES	ARMin	Cognitive VR
Movement control	system and patient, assistance possible, help-arm support	system and patient robot arm support	patient only table arm support
Movement range	whole upper limb	proximal (hand planned)	whole upper limb
Data collection	data glove	force/torque and position sensors on robot arm	digital compass, accelerometer, visual tracking, data glove
Task type	real daily activities unilateral	games and real daily activities unilateral	games and simulated daily activities bilateral
Task evaluation	subjective human assessment	objective software-based	objective software-based
Nervous system stimulation	specific external muscle stimulation plus observation of own arm (unilateral) afferent proprioception	peripheral limb manipulation plus observation of target stimuli afferent proprioception	central bilateral (virtual action observation via mirror neurons) afferent proprioception if patient able to move
Unit cost	low	high	low

tials in stimulated nerves, which cause muscle contractions. The Compex Motion stimulator [17, 18] can be programmed to generate any arbitrary stimulation sequence that can be controlled and regulated. Each stimulator has four output channels; up to four channels (muscle groups) can be stimulated at a time. The stimulation sequences are stored on readily exchangeable memory chip-cards.

With this system we artificially generate muscle contractions required to perform a reaching and grasping task in subjects who have lost voluntary control of these muscles. As different stroke patients present with different disability, to perform a reaching and grasping task we program the Compex Motion stimulator according to the patient's individual needs with regard to their lost or preserved motor function, respectively. Electrode placement for elbow and finger extension is illustrated in figure 1.

Since we include patients with severe paresis a help-arm is used to partially balance the force of gravity on the arm. For hemiplegic patients, a typical combination of stimulated muscles is as follows: anterior deltoid muscle, triceps, extrinsic finger extensors, and extrinsic finger flexors. For tetraplegic patients, normally only distal muscles are stimulated, i.e. finger extensors, finger flexors, and thumb adductor. Depending on the decision of the therapists agonistic and antagonistic muscles can be stimulated. In principle, the method can be applied to subjects with severe spasticity. In order to overcome spasticity we apply pulses that have short pulse durations and therefore preferentially activate efferent nerves and not the afferents that trigger hypertonic antagonist muscles [19]. However, we start with the FES training in the very acute state, in which the subjects have not yet developed severe spasticity.

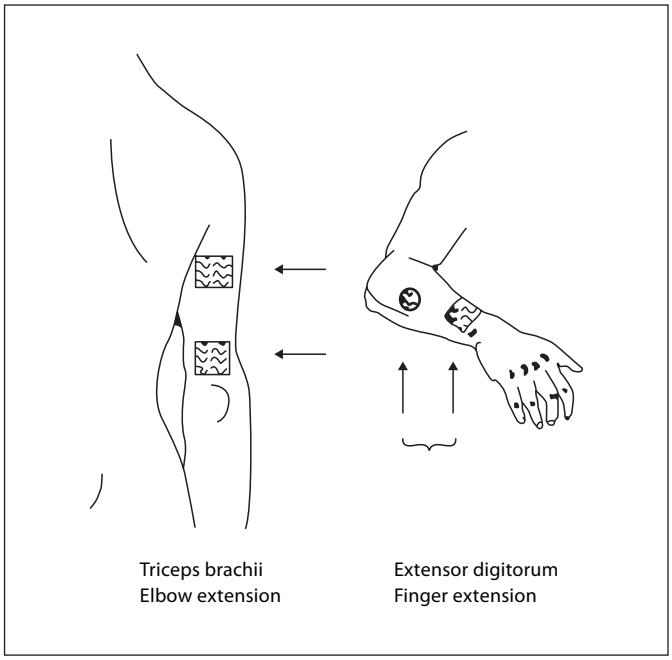

Fig. 1. Electrode placement for functional electrode stimulation to extend the elbow (left) and finger (right).

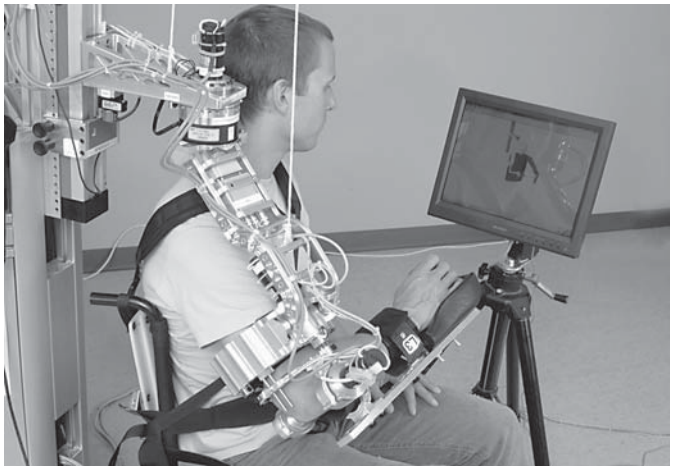

Fig. 2. ARMin robot with a healthy test subject.

Robot-Assisted Rehabilitation and Measurement (ARMin)

Rationale

Manually assisted movement training has several limitations. The training is labor-intensive, and, therefore, training duration is usually limited by personnel shortage and fatigue of the therapist, not by that of the patient. The disadvantageous consequence is that the training sessions are shorter than required to gain an optimal therapeutic outcome. Furthermore, manually assisted movement training lacks repeatability and objective measures of patient performance and therapy progress.

In contrast, with automated, i.e. robot-assisted, arm training the duration and number of training sessions can be increased, while reducing the number of therapists required per patient. Long-term automated therapy appears to be the only way to make intensive arm training affordable for clinical use. In the future, one therapist may be able to train 2 or more patients simultaneously. Thus, personnel costs can be significantly reduced. Furthermore, the robot provides quantitative measures, thus, supporting the evaluation of the rehabilitation progress.

Robot System Design

The robot is mounted to the wall with the patient sitting beneath (fig. 2). The patient's torso is fixed to the wheelchair with straps. A semiexoskeleton solution was selected for the mechanical structure of the robot. The distal part of the robot is characterized by an exoskeleton structure, with the patient's forearm and upper arm placed inside two shells moving the elbow joint. The upper arm is connected to an end effector-based structure moving the shoulder in three degrees of freedom. A six-axis force sensor and four position sensors enable the robot to work in different patient-interactive control modes. The robot is designed primarily for the rehabilitation of incomplete spinal cord-injured and stroke patients.

Therapy Modes

ARMin allows three different therapy modes: movement therapy, ADL therapy and game therapy. The goal of *movement therapy* is to prevent joint degeneration and to preserve joint mobility. In this mode, the therapist first guides the human arm together with the robot. The robot stores the movement and then repeats it with adjustable velocity. In *ADL therapy* the subject can perform different tasks such as filling a virtual glass of water, grasping it and moving it towards the mouth (fig. 3). The purpose of *game therapy* is to motivate the patient with simple games presented by an audiovisual display. In one game the user can move a virtual hand to intercept a ball which is rolling down a virtual plane (fig. 3). The robot supports the patient with just as much force as is needed. If the patient is not able to intercept the ball, the robot guides the patient's arm with an adjustable force right before interception.

VR-Based Interactive Cognitive Therapy

The VR-based interactive cognitive therapy system is based on the idea that observing an action with intent to imitate engages similar neural circuitry to that used in actually performing an action – the so-called 'mirror neuron' hypothesis [14]. Indeed, there is evidence that such observation may even induce cortical plasticity under certain conditions [20]. In a rehabilitation setting, it thus seems reasonable that a system capable of appropriately stimulating the action observation system could encourage plasticity and repair during the post-stroke acute phase.

Our interactive multimedia system uses low-cost input devices such as consumer-grade data gloves (P5 data glove, Essential Reality, New York, N.Y., USA) and digital compasses (HMR3300, Honeywell/Digi-Key Corp., Thief River Falls, Minn., USA) linked to a multi-user three-dimensional virtual environment (Torque, GarageGames, Oreg., USA) with visual and audio outputs.

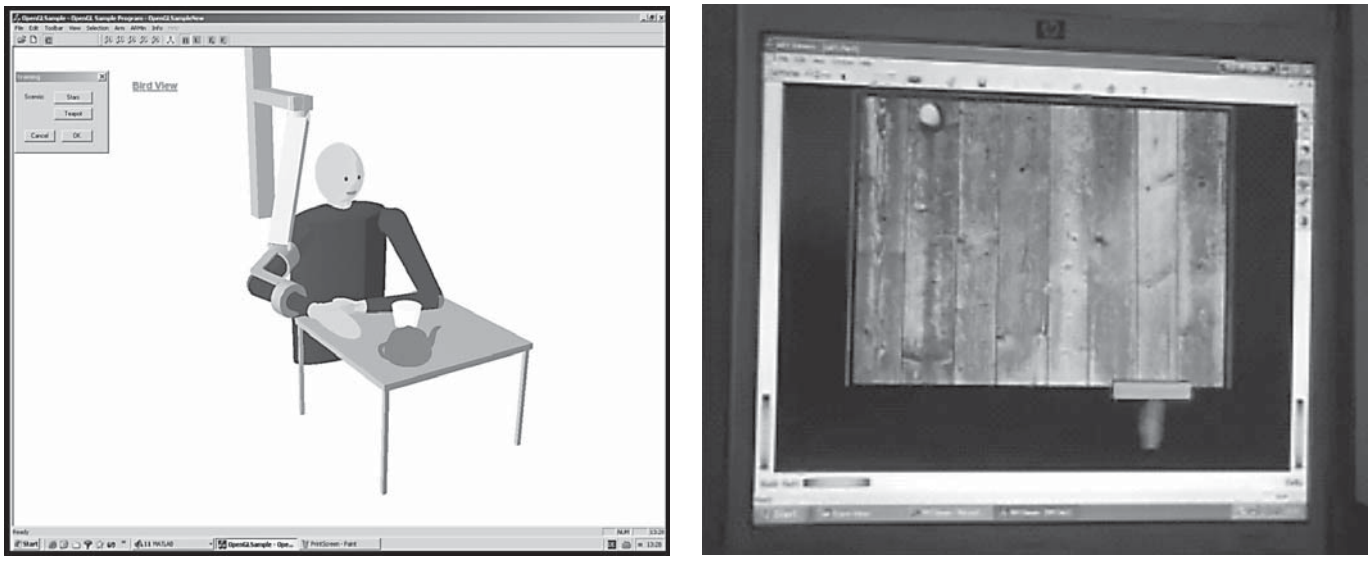

Fig. 3. Visual scenarios for ADL therapy mode (left) and game mode (right).

The system hosts a set of rehabilitation scenarios which are customizable to individual patient needs. The different scenarios provide a graded training program of reaching and grasping for each patient, with on-line quantitative feedback about patient performance for enhancing motivation and monitoring patient progress. The initial scenarios being tested, in order of increasing difficulty according to patient progress, are: (1) hitting – intercept virtual balls moving along a surface towards the patient by moving the arms; (2) catching – intercept objects, with the additional constraint of 'catching' them using the data gloves, and (3) grasping – move hands towards a virtual object, pick up the object, move it to a target location and release it.

In each scenario the patient sits in a chair with his/her arms on a table (fig. 4). The display is designed so that a three-dimensional rendering of two virtual arms appears in a similar orientation to the patient's real arms. Hand and arm movements detected by the input devices are mapped onto the movements of the virtual arms. This mapping can be adjusted by the therapist, and takes the form of scaling factors for the arm movements and/or left/right crossover mappings – i.e. the nonparetic real arm can be used to control movements of the paretic arm. The patient performs the task while simultaneously trying to imitate the actions he/she observes in the virtual arms. The control of the movements of the 'mirrored' arm can be gradually shifted from the intact arm to the paretic arm as the patient recovers, possibly accelerating further the speed of recovery.

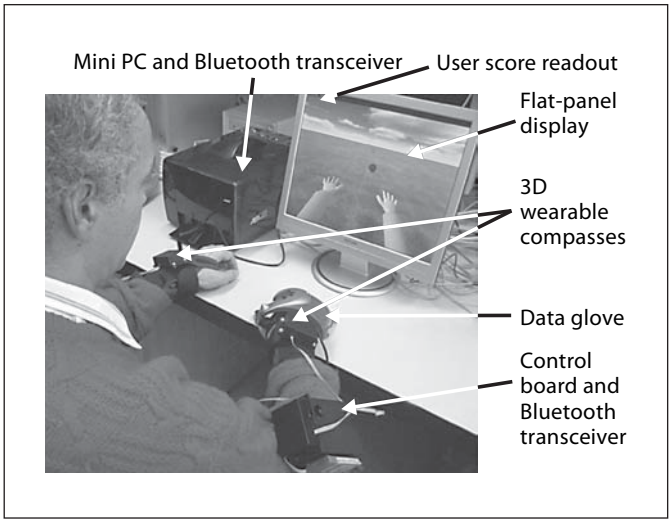

Fig. 4. VR-based cognitive therapy system.

Detailed position and event data from each game is recorded for analysis to both diagnose patient deficits and provide a record of improvement over training sessions.

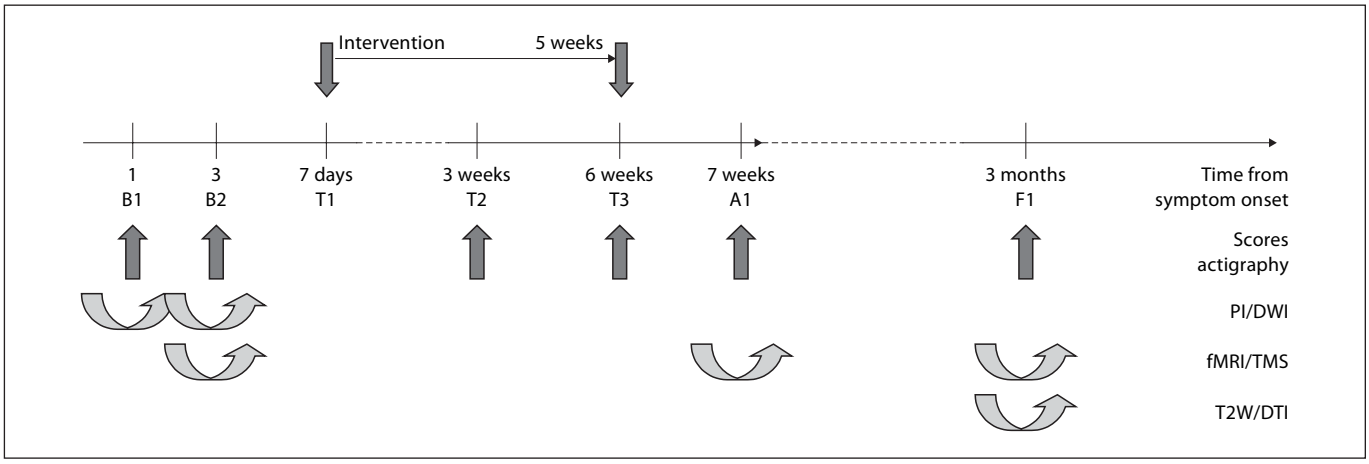

Fig. 5. Timeline of patient behavior evaluation. PI = Perfusion-weighted imaging; DWI = diffusion-weighted imaging; TMS = transcranial magnetic stimulation; DTI = diffusion tensor imaging.

Control Group

In the control group patients receive once daily basal task- and ADL-oriented physical therapy consisting of several modules such as vital (cardiopulmonary, etc.), static (posture, position, etc.), mobility (transfer, gait, etc.), and upper extremity functions. The rehabilitative effort available in the acute hospital (Neurology Department, University Hospital Zürich, USZ, Switzerland) is broadly similar to that in a rehabilitation hospital (Valens, Rheinfelden, Switzerland). The composition of the modules is the same in all participating clinics (vital: 0–5%, static: ~20%, mobility: 60–70%, upper extremity: 15–20%). One difference, however, is that the time basis in the Neurology Department averages 1.5 versus 2 h in the rehabilitation hospitals. Patients in the specific intervention groups receive daily basal task- and ADL-oriented physical therapy as described above. Additionally, concomitant therapies such as occupational therapy, logopedics, or neuropsychological therapies are offered to all patients depending on their individual needs.

Pilot Study – Treatment and Assessment Protocols

Treatment Protocol

The therapeutic interventions are carried out once a day on 5 days per week during a period of 5 weeks. Each treatment session lasts 45 min. During their stay in hospital, patients receive medical treatment, including recombinant tissue-plasminogen activator, whenever applicable. All patients receive standard physiotherapy.

Assessment Protocol

Patients who meet the entrance criteria are admitted into the trial during the first week after stroke onset. After initial clinical and functional assessment, patients are randomly allocated to either one of the experimental groups or to a control group. All procedures follow the ethical standards of the responsible institutional ethics committees. Informed consent is obtained from all patients participating in the study or from close relatives.

The timeline for patient treatment and evaluation is shown in figure 5. Clinical parameters are evaluated before (1st and 3rd day post-onset of stroke, baseline measurements B1, B2), midway (7th day, 3 and 6 weeks after stroke onset, referred to as T1, T2, T3), after the intervention period (7 weeks after stroke onset, referred to as A1), and during a follow-up, at 3 and/or 6 months after stroke onset (F1, F2).

Patient Selection

Inclusion Criteria

Stroke patients admitted to the emergency ward or stroke unit of the Neurology Department, USZ, are screened for inclusion. The diagnosis of stroke is based

on clinical history and examination and confirmed by MRI. The criteria for inclusion are: (1) diagnosis of acute ischemic brain damage in the first 48 h after symptom onset; (2) supratentorial localization of the stroke (comprising cortical as well as combined cortico-subcortical localization); (3) an obvious motor deficit of the hand with best hand function defined as Medical Research Council scale ≤3 (effort against gravity) lasting until the beginning of treatment; (4) older than 18 years of age; (5) alert and sufficient cooperation to permit full clinical examination, and (6) able to sit in a wheelchair or on a chair.

Exclusion Criteria

Patients older than 80 years of age, with a previous clinical history of stroke or a prestroke disability affecting the arm are excluded. Furthermore, pregnant women and patients with major cognitive deficits (comprehension deficits, severe depression, dementia, etc.), disturbances of basal sensibility, which may not allow testing of adequate electrical stimulation, epileptic seizures, progressive stroke, symptomatic intracerebral hemorrhage (ICH-associated increase of NIHSS >4 points), severe rheumatoid illnesses restricting joint mobility of the upper extremities, skin injuries, rash, burns, fresh scars, or inflammation on arms or hands, painful shoulder-hand syndrome, shoulder subluxation (palpatory >2 fingers), severe autonomic dysreflexia, i.e. requiring medication to treat autonomic dysreflexia, patients with metal implants, pacemakers or any other stimulation devices, prosthesis of bones or joints in the local region of treatment as well as patients with any severe medical diseases are also excluded.

Clinical (Descriptive) Assessment

At entry to the study, patient characteristics such as age, sex, side of paresis, site of lesion, type and onset of stroke as well as associated medical conditions are documented. On admission to the stroke unit at the Neurology Department, USZ (fig. 5, B1), the NIHSS [21] and the MMSE [22] are performed by clinicians involved in the routine treatment of the patients. The remaining acute neurological assessment (B1, B2) including neurological impairment – as measured by the Kunesch Score [23] – as well as a detailed sensory examination, handedness, and neuropsychological examination of each patient are performed prior to randomization. After the intervention period (A1), and during follow-up (F1), the overall outcome is assessed by the Modified Rankin Scale [24–26] in the Neurology Department of USZ.

Outcome Measures

Clinical Scales

The primary outcome is evaluated in terms of activity by means of the Chedoke Arm and Hand Activity Inventory [27]. Three secondary outcome measures are employed to follow the levels of activity (extended Barthel index [28, 29]) and participation (SF-36 [30, 31], Motor Activity Log [1, 32]). All measures meet the criteria of reliability and validity. They are assessed before randomization (B2), after referral to the rehabilitation clinic (Valens or Rheinfelden) after each treatment week and after the treatment period (A1, F1).

Behavioral Evaluation

In addition to clinical scores, we assess behavioral data to follow up the functional recovery process of the paretic upper extremity (fig. 5). In the acute stage (B2), by the end of every treatment week as well as after the treatment period (A1, F1), we also use a drawing test to follow recovery progress more closely [33]. Actigraphy recordings from the contra- and ipsilesional arm yield additional data about the amount of spontaneous motor activity on predefined days over the whole observation period [34].

Functional Brain Alterations

Neuroimaging

Functional Magnetic Resonance Imaging (fMRI). We use fMRI to study the evolution of activation patterns during the process of recovery. The dynamics of cerebral activation maps, especially their lateralization related to the course of motor recovery, showed changes during the course of motor recovery in several previous studies [35–37]. We therefore use the laterality index to quantify the amount of blood oxygenation level-dependent (BOLD) activation between the ipsi- and the contralesional hemisphere [38]. Furthermore, we compare the activation pattern of the patients in individual and group analysis with healthy subjects matched for gender, age and manual dexterity [39]. Before (B2) and after (A1, F1) intervention we measure BOLD fMRI to test the activation of motor areas for the different trained interventions. The patients have to generate with each hand isometric repetitive force pulses of 20% maximal voluntary contraction. In this

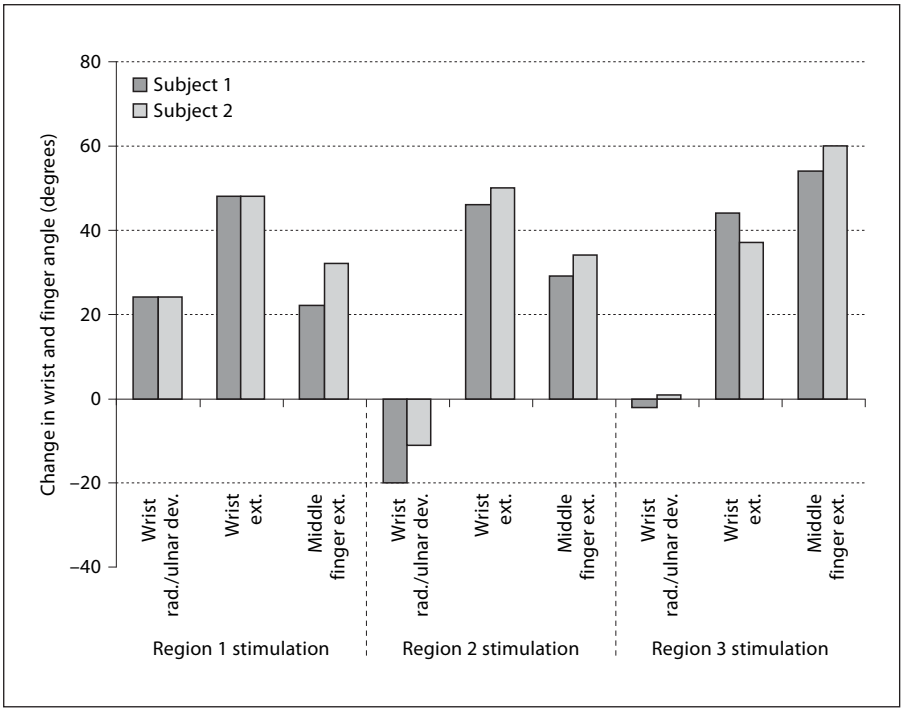

Fig. 6. Kinematic wrist and finger angular data for selective electrical stimulation of the wrist and finger extensors using three activation regions over the wrist and finger extensors. The results indicate that simultaneous activation of regions 1 and 2 produces co-contraction of radial/ulnar deviation and contraction of wrist extension with less activation of finger extension compared to the activation of region 3.

block design, including 21 s of force condition alternating with 21 s of rest (5 repetitions), the subjects are guided by color-coded feedback, where target and exerted forces are displayed on a screen in front of the subject.

Perfusion-Weighted Imaging, Diffusion-Weighted Imaging, Diffusion Tensor Imaging. In addition to fMRI, we use our regular clinical protocol comprising perfusion- and diffusion-weighted imaging. Lesion volumetry of perfusion- and diffusion-weighted imaging is based on automatic lesion outline at predefined thresholds relative to mean image intensity in the unaffected hemisphere [40, 41]. T_2 lesions are outlined manually by one of the authors.

Preliminary Results

The studies are still in progress and first results are appearing. Here, we summarize the results obtained to date for each of the three technologies being tested. Because only a few patients have been tested so far, between-subject power calculations have not yet been performed. For equivalent total amounts of training per patient, large numbers of subjects per test group ($n > 50$) may be required to achieve statistically significant results due to the high between-subject variability. However, we believe that because patient motivation to use the therapy technologies is high (as measured by user questionnaires), and patients receive our therapies in addition to normal therapy, much smaller numbers of subjects per group will be required to show improved outcomes. This testing scenario is realistic because our therapies are designed to supplement rather than replace existing therapy, with only minor increases in staff workload because of the semiautomated nature of the therapy systems. The main results we expect for each of the three technologies are improved functional recovery as measured by the ADL tests, and cortical activations that are more normal than in the control cases.

Functional Electrical Stimulation

Subjects after stroke with remaining upper limb deficits often suffer from abnormal flexion hyperactivity in shoulders, elbows and arms. It could be shown that FES can overcome these abnormal synergies in the elbow by stimulating the triceps muscle during reaching activities [19]. For achieving functional use of the hand it is also necessary to be able to overcome similarly occurring abnormal hyperactivity in the fingers.

Preliminary tests were performed in 2 stroke subjects with abnormal movement patterns to selectively acti-

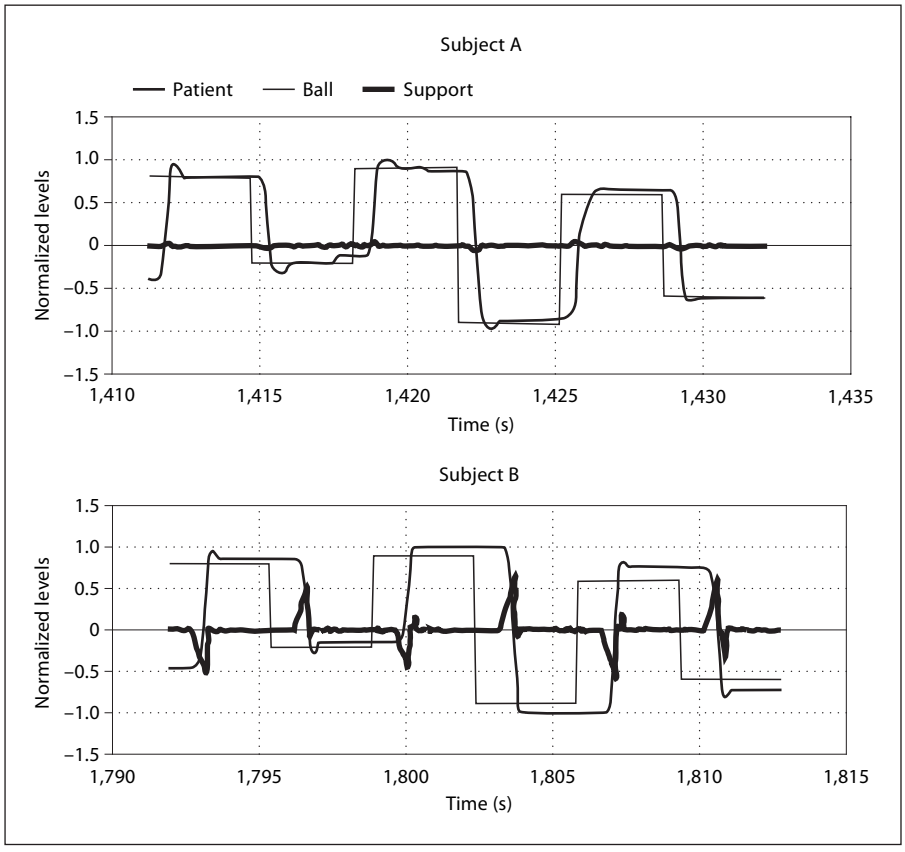

Fig. 7. Movement and force support recorded from 2 hemiplegic subjects playing the ball game.

vate wrist and finger muscles. Both subjects (female) had severe paresis of their right arm and hand, which resulted in a Fugl-Meyer score of 20/66 and 27/66. For selective activation of wrist and finger extensors by means of transcutaneous electrical stimulation three regions were found over the finger and wrist extensor muscles that resulted in differential wrist and finger movements. The goal was to activate finger extensor muscles with minimal ulnar and radial deviation in the wrist and to activate wrist extension with minimal activation of the finger extensors. This second strategy would allow stroke subjects to close their hands without compromising a natural wrist position achieved by stimulating the wrist extensors. Region 1 activated wrist extension combined with wrist radial deviation and some finger extension. Region 2 activated wrist extension combined with wrist ulnar deviation and some finger extension. Region 3 mainly activated the finger extensors with almost no ulnar/radial deviation and some wrist extension. In both subjects, activation regions could be found at moderate levels of stimulation (150 μs pulse width, 25 Hz stimulation frequency and amplitudes of 18–22 mA). To illustrate the results (fig. 6) the angular change of wrist and finger positions during selective stimulation of the three regions was measured with a P5 data glove (Essential Reality Inc.). All three regions were stimulated in consecutive order, first region 1, then region 2, and finally region 3. Each region was stimulated with the following pattern: 1 s amplitude ramp up, 5 s constant stimulation at moderate amplitude of 18–22 mA, 1 s amplitude ramp down with 1-second resting periods between patterns. Resting position before stimulation was 0° radial/ulnar deviation, 40° wrist flexion and 80° finger flexion. Stimulation of region 3 (for finger extension) showed almost no radial/ulnar deviation and more finger extension than stimulation of regions 1 and 2. Stimulation of region 1 resulted in wrist radial deviation and more index finger activation than ring finger activation. Conversely, stimulation of region 2 showed more ring finger activation than index finger activation. Both regions 1 and 2 produced more wrist extension than region 3. On the other hand, finger extension, especially for the middle finger, was partially reduced compared to stimulation of re-

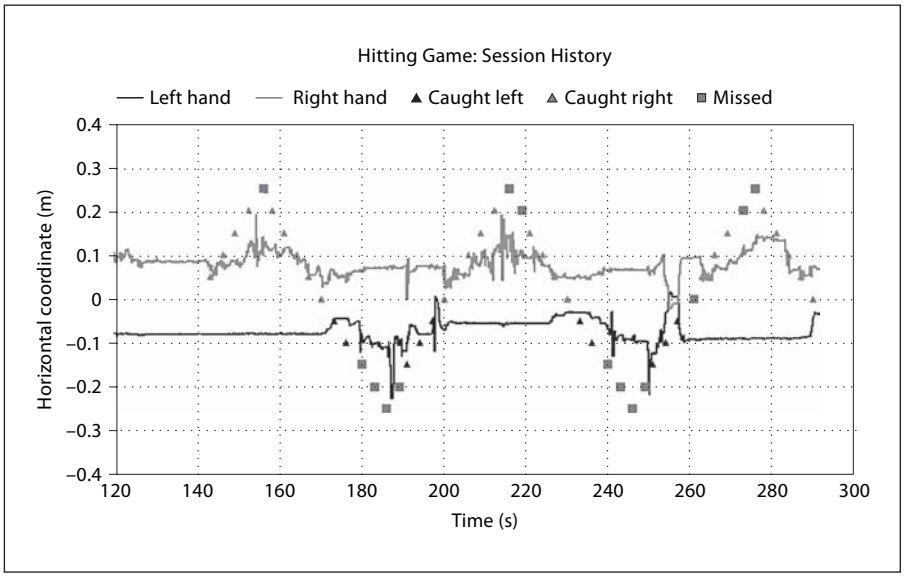

Fig. 8. Sample session history from patient 1, usability and assessment pilot.

gion 3. These results show that finger extension can be functionally stimulated to overcome paresis and flexion hyperactivity in the hands of subjects after stroke. In addition, the results indicate that a certain level of selectivity in wrist and finger extensor activation can be achieved with transcutaneous electrical stimulation.

ARMin

A pilot study with 10 healthy subjects and 5 chronic stroke patients was carried out to analyze comfort, functionality, acceptance, and whether the patients are able to perform the proposed tasks. With the 5 patients a series of sessions were performed, each including 30 min of movement therapy and 30 min of game therapy. The 5 patients used the robot for more than 30 h altogether.

The fixation of a patient in the robot takes approximately 5 min. The robot can easily accommodate subjects with body sizes between 155 and 192 cm. The robot allowed reliable trajectory recording and repetition with adjustable velocities during movement therapy. During game therapy it provided interactive support for the patient. Participants and therapists gave ARMin high grades with respect to comfort, design, and clinical usability. Although it was not the primary goal to study the therapeutic effect during the relatively short training sessions, an improvement of the patients' motor functions could be observed. From session to session the robot support decreased, while speed and range of motion increased and joint coordination improved. Thus, the patients could play games with increasing difficulty levels.

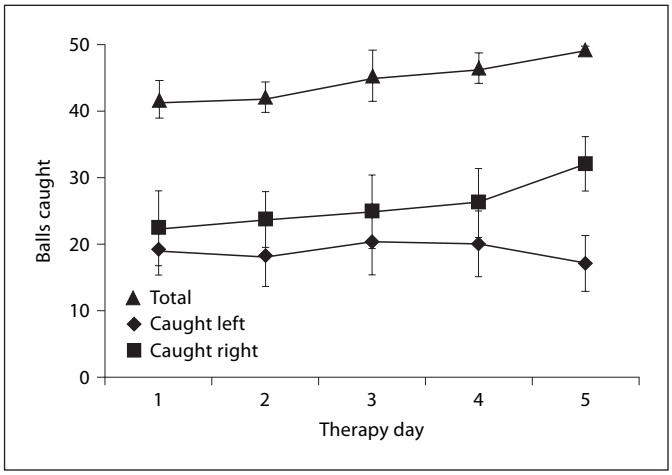

Fig. 9. VR game performance over successive therapy days for stroke patient 2 of the usability and assessment pilot starting on the 7th day after symptom onset. Each therapy session consisted of six sets of 50 balls each. Error bar = ±1 standard deviation.

Figure 7 shows the results of a game therapy performed with 2 chronic hemiplegic subjects. The subjects had to catch a visually displayed ball (fig. 3) by moving their hand towards the ball. Subject A was able to catch all balls without any robotic support, whereas subject B needed

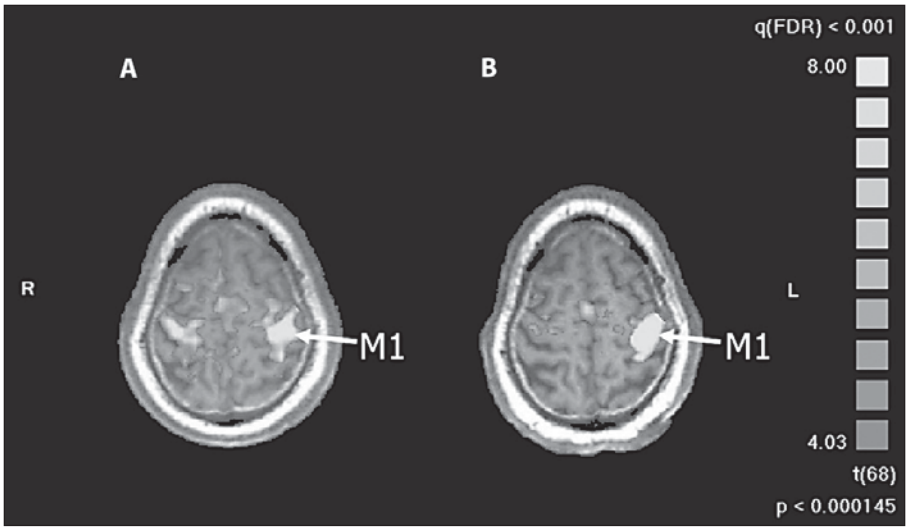

Fig. 10. Changes in cortical activation as a function of intervention: involvement of M1 during dynamic force generation with the paretic right hand. **A** Bilateral activation in the primary motor cortex (M1) before intervention. **B** Contralateral activation in M1 3 months after intervention.

some support to catch the balls. ARMin allows measuring the voluntary force of the subjects and storing it for later therapy assessment.

VR Cognitive Therapy

The VR cognitive therapy system was implemented using the hitting scenario in pilot studies on the following groups of subjects: (1) healthy subjects, mean age 29 ± 4 years (n = 19, mean standard ± deviation), usability pilot study; (2) stroke patient, age 63 (n = 1), usability pilot study, and (3) stroke patients, ages 62 and 56 (n = 2), usability and assessment pilot studies.

Both the healthy subjects and the stroke patients were able to use the system for learning how to perform the task within a short time after the commencement of the first test session. For the initial settings given (1 ball every 2.5 s, ball start location random or wave pattern), most healthy subjects and patients were able to intercept between 70 and 100% of the balls. User acceptance of the system was high (anecdotal and questionnaire responses); in particular the patients tested expressed a desire to use the system on an ongoing basis.

Figure 8 shows a plot of the data for a test run with one of the stroke patients, showing the movements of the left and right hands as well as the fates of each of the balls (caught with left hand, caught with right hand, or missed). In this example the balls appeared every 2.5 s in a wave pattern. It can be seen that most of the 'missed' events occurred towards the extremities of movement, and that a greater proportion of balls were missed on the left side than the right side. The patient's paretic side was the left side and the patient was right-handed when healthy, so the relative contribution of paresis and handedness to the left/right performance imbalance can only be assessed after further testing as the patient regains left arm function.

Figure 9 shows the performance of patient 2 from the usability and assessment pilot studies over successive therapy days. Each therapy session consisted of six sets of 50 balls each. The mean score on the last day was significantly higher than that on the first day (t test, $p < 0.01$). As the patient had reached virtually perfect performance by the fifth day, continued therapy would probably have benefited from an increase in game difficulty (increased ball speed, increased dispersion of balls, etc.).

Figure 10 shows the change in cortical activation in a patient who underwent VR cognitive therapy for 2 weeks. The patient was instructed to perform a right-handed grip strength task in the MRI scanner. The bilateral activation of M1 that was present shortly after the stroke changed significantly towards normal localized contralateral activation. The generalizability of this result, and the extent to which VR cognitive therapy contributed to this result, will be determined in future control tests with patients undergoing normal physiotherapy.

Conclusions and Outlook

Our interdisciplinary approach to the application of multiple technologies in a simultaneous study of their efficacy for stroke rehabilitation permitted a well-validated, consistent evaluation of each of our three complementary approaches to neurorehabilitation. This synchronized assessment of multiple new technologies in an extensive simultaneous clinical study is, to the best of our knowledge, a novelty in the field of neurorehabilitation. While some of our initial results from patient testing are promising, more data is required before we can make definitive statements concerning the efficacy of our different methods. The question of whether a combination of our new technologies is more effective than any single method alone is open, and should be the subject of a future study.

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