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Identification of regeneration-associated genes after central and peripheral nerve injury in the adult rat

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Abstract

Background: It is well known that neurons of the peripheral nervous system have the capacity to regenerate a severed axon leading to functional recovery, whereas neurons of the central nervous system do not regenerate successfully after injury. The underlying molecular programs initiated by axotomized peripheral and central nervous system neurons are not yet fully understood.

Results: To gain insight into the molecular mechanisms underlying the process of regeneration in the nervous system, differential display polymerase chain reaction has been used to identify differentially expressed genes following axotomy of peripheral and central nerve fibers. For this purpose, axotomy induced changes of regenerating facial nucleus neurons, and non-regenerating red nucleus and Clarke's nucleus neurons have been analyzed in an intra-animal side-to-side comparison. One hundred and thirty five gene fragments have been isolated, of which 69 correspond to known genes encoding for a number of different functional classes of proteins such as transcription factors, signaling molecules, homeobox-genes, receptors and proteins involved in metabolism. Sixty gene fragments correspond to genomic mouse sequences without known function. *In situ*-hybridization has been used to confirm differential expression and to analyze the cellular localization of these gene fragments. Twenty one genes (~15%) have been demonstrated to be differentially expressed.

Conclusions: The detailed analysis of differentially expressed genes in different lesion paradigms provides new insights into the molecular mechanisms underlying the process of regeneration and may lead to the identification of genes which play key roles in functional repair of central nervous tissues.

Background

Lesioned axons of the peripheral nervous system (PNS) have the capacity to undergo successful regeneration leading to complete functional recovery. In marked contrast, lesioned axons of the central nervous system (CNS) do not demonstrate any functionally significant regeneration. An important aspect underlying the opposing outcome of injured PNS and CNS neurons are the different molecular programs initiated in the axotomized neuronal cell bodies [1,2]. One important step for the eventual success or failure of a neuron to regenerate a severed axon is the expression of important regeneration-associated genes [2,3]. To date, there is currently relatively little information available concerning the molecular and genetic programs which control such behavior.

In recent years, gene screening methods such as the DD-PCR technique have greatly facilitated the identification of differentially expressed genes. Pardee and co-workers were the first to demonstrate that combinations of arbitrary primers with anchored cDNA primers can be successfully applied to generate gene fragments from the total mRNA of one cell type [4,5]. In the following years various modifications have been developed and several research groups have successfully used this technique to investigate complex biological problems, e.g. during the development of tumors, neuropathies, endocrinology or ischemia [4–6]. In the neuroscience field, several reports have demonstrated that important regeneration-associated genes can be identified by the DD-PCR technique [7–11]. However, we are still far from understanding the precise mechanisms involved in the process of regeneration because only a restricted number of molecules have been examined in detail and the existence of other important as yet unidentified molecules is very likely.

In our present investigation, we have applied DD-PCR to analyze the gene expression pattern of regenerating and non-regenerating populations of neurons. For this purpose we have used the model of spinal cord hemisection as a non-regenerating lesion paradigm and transection of the facial nerve as a regenerating lesion paradigm. The retrograde reactions of axotomized regenerating neurons of the facial nucleus and non-regenerating neurons of the red and Clarke's nucleus can be investigated following these lesions. Both lesion models are well established and have been the basis for a number of investigations to analyze the cellular and molecular mechanisms underlying the process of regeneration. After transection of the facial nerve, a sequence of events takes place which usually leads to axonal elongation, re-innervation and functional recovery within a few weeks (for review see [12]. This approach has allowed numerous investigators to study retrograde changes of axotomized neurons of the facial nucleus which could be correlated with successful regeneration [13–17]. One important advantage of this model is that the blood brain barrier is not broken down after nerve transection. This avoids additional involvement by invading inflammatory cells [18]. By this lesion paradigm a number of classes of molecules have been investigated, including cytokines, transcription factors, adhesion molecules, signaling molecules etc. [13–17,19]. The differential expression of such molecules has been observed quite early (several hours to a few days) after a lesion and usually lasts for approximately two weeks [13,14,16,17,20]. The hemisection model leads to axotomy of contralateral rubrospinal neurons rostral to the lesion and also ispilateral Clarke's nucleus neurons caudal to the lesion. Both types of neurons are located in anatomically well defined nuclei and have been the used for a number of studies to analyze retrograde reactions of CNS neurons [2,21–25]. Both types of neurons do not spontaneously regenerate. Rubrospinal neurons atrophy after axotomy but a subpopulation of Clarke's nucleus neurons degenerate after axotomy [2,21,26–28]. Nonetheless, both types of CNS neurons demonstrate regenerative "capacities". They can be "rescued" by neurotrophic factors and their axons can regenerate into growth permissive environments transplanted into the lesion site [22,27–30]. Both types of CNS neurons also show similar molecular responses with the up-regulation of regeneration-associated genes, such as GAP-43 and c-jun [1,24,31]. These responses support the notion of an intrinsic regenerative capacity (for review see [32]. Since most of these molecular events take place at early after injury and peak at approximately one week, we decided to chose the 7 day post lesion time point for the differential screening.

We have isolated 180 differentially expressed bands which corresponded to 135 different gene fragments of which 69 showed high homology to known sequences and 60 showed homologies to different regions of different mouse chromosomes. Genes with known sequences could be classified into major groups belonging to transcription factors, signal transduction pathways, homeobox-genes, receptors, molecules involved in protein and lipid metabolism, cytoskeletal and miscellaneous molecules. In situ-hybridization has been performed to prove the differential expression *in vivo*. These results contribute to elucidate the molecular mechanisms underlying the process of regeneration.

Results

Identification of regeneration-associated genes

We have performed radioactive DD-PCR using a set of arbitrary primers labeled with ^{35}S-dATP (Table 1). This technical approach is more sensitive compared with non-radioactive DD-PCR (e.g. loading PCR products on agarose gels visualized by ethidium bromide). Even gene fragments with low expression could be identified (Fig. 1).

Table 1: Set of different arbitrary primers used for DD-PCR

HAP25	AAG CTT TCC TGG A
HAP26	AAG CTT GCC ATG G
HAP27	AAG CTT CTG CTG G
HAP28	AAG CTT ACT ATG C
HAP29	AAG CTT AGC AGC A
HAP30	AAG CTT CGT ACG T
HAP31	AAG CTT GGT GAA C
HAP32	AAG CTT CCT GCA A

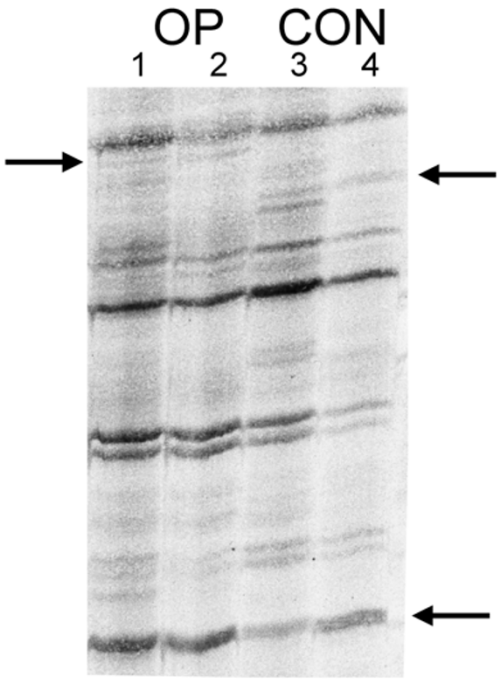

Figure 1
Detection of differentially expressed genes after facial nerve transection Radioactive labeled DD-PCR fragments after electrophoretic separation using a polyacrylamide gel and exposition to an x-ray film. Gel image illustrates that fragments with high and low expression can be identified. Lanes 1 and 2 represent the operated side. Lanes 3 and 4 represent the un-operated side (facial nucleus). By comparing operated and control side, fragments which are considered to be differentially displayed appear in both lanes for either operated or un-operated sides (arrows). Fragments indicating either up- or down-regulation have been chosen for further analysis.

For reducing the number of false-positives, duplicated DD-PCR runs were performed for each sample and PCR products were loaded onto the same gel. Only bands (fragments) which appeared to be differentially expressed in both of the duplicated lanes were used for further analysis (Fig. 1). We have thus isolated 180 fragments, 135 of which could be successfully reamplified. After sequencing and gene bank (NCBI) analysis, we have obtained 135 differentially expressed genes. Sixty nine of the gene fragments revealed a high homology to known genes, which encoded for proteins with a number of different functions reflecting the complexity of the process of regeneration. The known genes could be classified into several major groups (Table 2), of which a high number (39) were transcription factors, homeobox-genes and genes involved in signaling pathways or metabolism. Six gene fragments did not show homologies to sequences in the gene bank. The additional comparison with the mouse genome, which has been recently published, revealed 60 gene fragments showing homologies to different regions of several mouse chromosomes with unknown function (Table 4).

Verification of the differential expression by ISH

For confirming differential expression, we used non-radioactive ISH which allows the detection of the anatomical and cellular distribution of gene expression, thereby facilitating the interpretation of the functional role of these genes. By ISH analysis, differential expression of 21 genes could be confirmed (Table 3). This number represents ~15% of the total DD-PCR fragments and is in keeping with similar studies in other laboratories. However, it can not be excluded that the lack of detection by ISH for a certain number of genes is due to technical reasons. Sixteen genes with confirmed differential expression demonstrated either up- or down-regulation in neurons, 5 in glial cells (Table 3). The differential expression of transferrin receptor, P4-kinase, STAT-5 and connexin-43 confirm already published studies from other research groups [16,33–35]. To the best of our knowledge, this is the first demonstration of differential expression of the other genes such as the Na^+/K^+-ATPase which is down-regulated after facial nerve lesion (Fig. 2A,2B).

Table 2: DD-PCR fragments which indicated differential expression between axotomized versus non-axotomized neurons. (Accession Nr.).

	Clarke's and Red nucleus	**Facial nucleus**
Transcription factors	Histone acetylase (MRG15) MORF (BC 003894) C-FOS (X06791)	C-maf (AF 055376) OCT-2 (X67302) ETS-like protein (D61707) Deaconesstranscriptionfactor (409229.1) NF1(L4) (X84210) NF1-X1 (AB012235) TTF-1 (AJ 296303) JUN B (X 54686.1) C-FOS (X06791)
Homeobox genes	Hox-9 (AB027042)	HOX-1 (S76296.1) HOX-3 (S76303.1) HOX-4 (X62699.1) R1a homeobox gene (M37565)
Signal transduction	Phospholipase A2 (U 38376)	Phosphatidylinositol-4-kinase (S56508) STAT 5b (AF 234171.2) GDP-releasing protein (L10336) Phosphatase-1 binding protein (U89924) Gro (D11444)
Receptors	Androgen receptor (M23264.1) B-cell receptor ass. Protein BAP29 (X78684) T-cell receptor (AE008685)	Transferrin receptor (M58040)
Metabolism	Human esterase D (AF112219) Elongation factor-2 (AF0005761) Alkyl-dihydroxyacetone phosphate synthase (Y08826) Phosphoribolsyl transferase (X16554.1) Phosphoribosylpyrophosphate synthetase (M31084) N-acetyltransferase (L10245.1) Stearoyl-CoA-desaturase-1 (J02585) TRNA synthase (NM003191.1) TRNA-trp (N27315.1) NADH dehydrogenase subunit1. (X07479) Phosphatase 1 binding protein (MMU89924)	Elongation factor-1 (M95792) Elongation factor-2 (AF0005761) Ribosomal protein S3 (X515361.1) Ribosomal protein S6 (M29358) Ribosomal protein L7 (X57961.1) NADH-ubichinone-oxidoreductase (D86215) Pyruvat-Kinase (X05684.1) NADH dehydrogenase subunit1. (X07479)
Cytoskeletal proteins	Hax-1 (NM011826.1) Vimentin (Y077301)	Myosin light chain (S77858.1) Vimentin (Y077301)
Adhesion molecules	Putative neuronal cell adhesion molecule (AF026466) Integrin α6 subunit (X69902)	
Miscellaneous	Chaperonin (AB022158.1) Oxysterol binding protein (AB017026) ATP-sensitive inwardly rectifying K^+-channel (D86039) N-ethylmaleimide sens. factor (AF189019) Cecum cDNA (AK018679.1) Synuclein β (NM033610.2) Tubby-like protein (AJ296303) TNF converting enzyme (AF056357.1) Retrotransposon (AF000199.1) Collagen V (NM134452.1) Na+/K+-ATP'ase (β-1 Subunit) (M 14137.1) Rat clone rp32-47 (AC092531.16) Acatn gene for acetyl.CoA (AB037364.1)	Conexin-43 (NM012567) α-B-crystallin (D29960) Ferritin light chain (J02741) Chaperonin (HSP60) (U68562.1) GAP-Junction protein (BC011324.1) 3beta-Hydroxysteroid Dehydrogenase isomerase (S63167.1) ubiquitin specific protease (BC027052.1)
Unknown	4 sequences	2

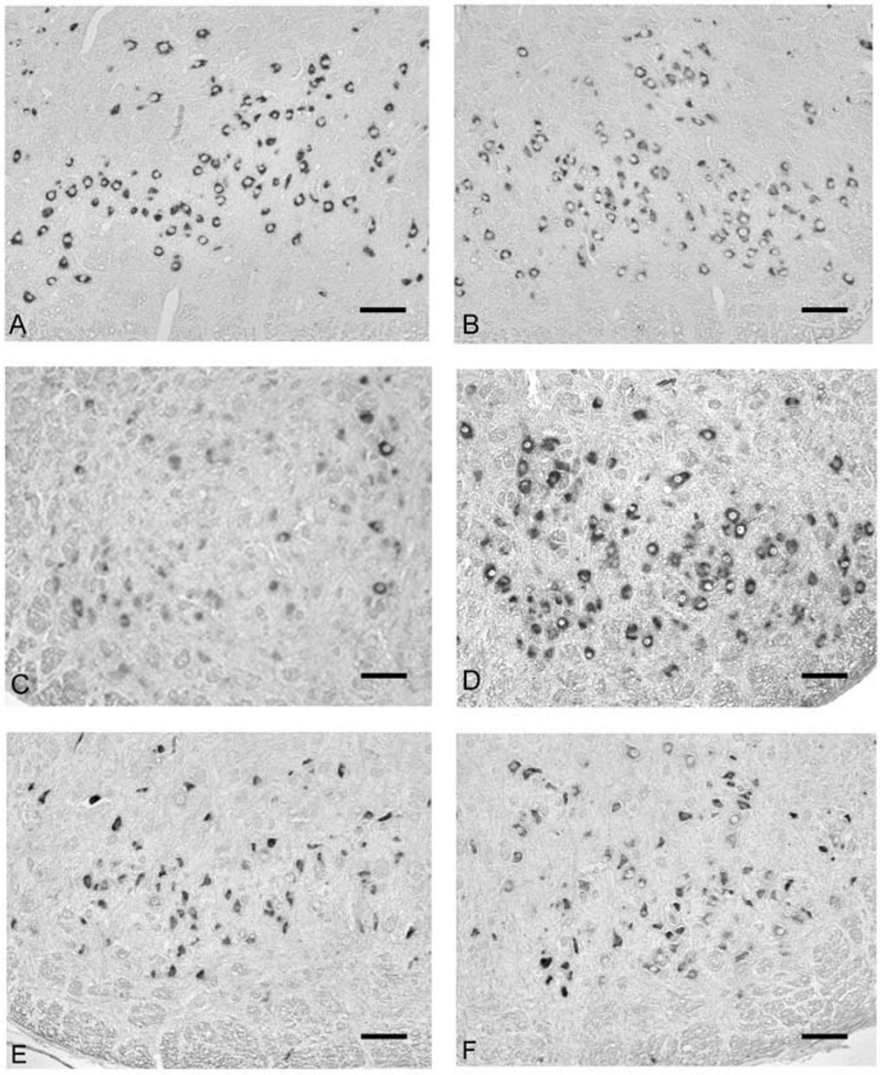

Figure 2
Differentially expressed genes within axotomized facial nucleus neurons. A,B: ISH of ATPase after unilateral transection of the facial nucleus. By comparing mRNA expression in the control (A) and operated (B) side, mRNA down-regulation could be detected within axotomized neurons. C,D: ISH of clone U27 after unilateral transection of the facial nucleus. Dramatic mRNA up-regulation could be detected within axotomized facial nucleus neurons (D) compared with the control side (C). D,E: ISH of clone U41 after transection of the facial nucleus. No differential mRNA expression could be detected in the facial nucleus neurons following injury. (A) Control and (B) operated side. Bar, 100 μm

Table 3: Genes with confirmed up-(↑) or down-(↓)regulation

Regulated in the facial nucleus	Regulated in the Red or Clarke's nucleus
c-maf (neuronal↑)	B cell receptor (neuronal↓)
Connexin-43 (glial↑)	Hox-9 (neuronal↓)
Ef-2 (neuronal↑)	Na^+/K^+-ATPase (neuronal↓)
Ferritin light chain (neuronal↑)	Vimentin (glial↑)
oct2 (neuronal↑)	
P4 kinase (neuronal ↓)	2 novel sequences (neuronal↑)
Ribosomal protein S3 (glial↑)	
Ribosomal protein S6 (glial↑)	
Ribosomal protein L7 (glial↑)	
SCD-1 (neuronal↑)	
STAT-5 (neuronal↑)	
Transferrinreceptor(neuronal↑)	
3 novel sequences (neuronal↑)	

Table 4: List of DD-PCR fragments matching with the mouse genome (sequences can be obtained on request from the authors)

clone	Chromosome (region)	Homology (%)	Accessionnr. (NCBI)	Clone	Chromosome (region)	Homology (%)	Accession Nr. (NCBI)
U1	12 (C3)	80		**U31**	4 (D1)	76	
U2	X	86	AL773583.9	**U32**	X (A3.3)	70	
U3	11 (B1.3)	71		**U33**	X (D)	65	
U4	16 (C3.3)	84		**U34**	2	86	AL845542.9
U5	11	86	AL772225.5	**U35**	19 (C1)	80	
U6	9 (F1)	83		**U36**	1 (A5)	76	
U7	X	86	AL691418.9	**U37**	1 (C1)	81	
U8	10 (A2)	75		**U38**	10 (A3)	78	
U9	18 (E3)	79		**U39**	8 (E2)	80	
U10	16 (A3)	78		**U40**	10 (B4)	78	
U11	11	89	AL663083.5	**U41**	15 (D2)	75	
U12	3 (H1)	73		**U42**	9 (F4)	68	
U13	18 (B3)	85		**U43**	1 (C1)	74	
U14	9 (A5.2)	79		**U44**	16 (C1.2)	73	
U15	5	85	AC122862.4	**U45**	4	87	AL645625.15
U16	12 (F2)	78		**U46**	10 (A3/A4)	82	
U17	X (F5)	82		**U47**	4 (B3)	82	
U18	16 (A1)	72		**U48**	2 (H3)	77	
U19	14 (C2)	82		**U49**	15 (A2)	83	
U20	3 (A2)	75		**U50**	13 (A3.3)	78	
U21	19 (B)	82		**U51**	11 (B1.1)	85	
U22	15 (D2)	75		**U52**	X	84	AL772348.2
U23	18 (B3)	85		**U53**	9	85	
U24	13 (A5)	87		**U54**	12 (B1)	85	
U25	7	90	AC126435.3	**U55**	2	96	AL591430.8
U26	4	87	AL645625.15	**U56**	18	86	AL669931.9
U27	14 (C2)	82		**U57**	16	83	AC090121.37
U28	3 (A2)	74		**U58**	8 (A2)	98	
U29	1 (G2)	82		**U59**	7 (F4)	83	
U30	2 (C1)	76		**U60**	13 (B2)	89	

To facilitate the selection of genes for future functional analysis, we have performed comparative studies in a number of different lesion paradigms. ISH analysis of the gene expression in regenerating and non-regenerating models allows the identification of those genes which are either exclusively regulated in a regenerating or a non-regenerating system or show a different regulation pattern between the two systems. To date, we have compared the expression pattern of a few selected genes in different models. In particular, all gene fragments matching with the mouse genome (Table 4) have been analyzed by ISH in our different lesion paradigms. Four of those gene fragments were exclusively regulated in one lesion model. All 3 gene fragments which have been identified using the facial nucleus model such as clone U27 showed dramatic up-regulation in the axotomized facial nucleus neurons (Fig. 2B,2C), but not in axotomized red or Clarke's nucleus neurons. One gene fragment which has been obtained after spinal cord lesion is up-regulated in axotomized red nucleus neurons, but not in facial or Clarke's nucleus neurons after injury (data not shown). One gene fragment (Clone U41), which has been obtained from the Clarke's nucleus after spinal cord hemisection, showed a different regulation pattern between regenerating and non-regenerating neurons. No differential expression could be observed within axotomized facial nucleus neurons (Fig. 2E,2F). In contrast, a dramatic mRNA up-regulation of clone U41 could be detected within axotomized neurons either of the Clarke's nucleus (Fig. 3A,3B) or red nucleus (Fig. 2D,2E,2F). These results demonstrate that clone U41 is exclusively regulated in non-regenerating CNS neurons.

Discussion

Injured CNS axons do not demonstrate any significant regeneration, whereas axotomy of PNS axons can lead to functional recovery (for review [36]). The underlying molecular mechanisms are only partially understood. Peripheral nerve injury induces changes in the molecular program of the injured neurons which include up-regulation of the immediate early genes (e.g. c-Jun) followed by regeneration-associated genes such as GAP-43 (for review [12]). Some similar reactions have also been observed in axotomized CNS neurons, but this response is rather "short-lived" and does not lead to any functional regeneration [2,24,31].

In our present study, we have investigated the gene expression pattern of regenerating facial nucleus neurons and non-regenerating red and Clarke's nucleus neurons after axotomy using the DD-PCR method. This allows the identification of similarities and differences in the molecular reactions of the lesioned PNS and CNS neurons. The lesion paradigms chosen for the present investigation are widely acknowledged and popular models of PNS and CNS injuries. Since a large number of molecules are known to be differentially expressed at approximately 7 days after injury [1,13,16,17,24,31,37], we have chosen this time point for our DD-PCR analysis. Subsequent ISH analysis has been applied to prove the differential expression of these genes *in vivo*, revealing the anatomical and cellular localization. Since the identification of the cellular localization of the gene of interest facilitates the interpretation of its functional role, ISH represents the ideal tool for the verification of differential expression.

Sixty nine gene fragments showed high homologies to known gene sequences. Most of the identified genes could be divided into different groups belonging to transcription factors, homeobox-genes, genes involved in signaling pathways or metabolism, receptors and cytoskeletal molecules (Table 2). The group of miscellenous genes encoded for a variety of proteins with specific functions. The high number of different genes related to different groups associated with the process of regeneration reflects the complexity of this issue. In a similar, very extensive study, using micro array analysis, Costigan and co-workers identified a vast number of differentially expressed genes within axotomized sensory neurons after sciatic nerve transection [7]. Similar to our results, these genes encode for different classes of proteins.

However, we were surprised not having detected genes encoding for neuropeptides or transmitters. It is likely that this is simply due to chance. The screening was halted when we had obtained 180 differentially expressed mRNAs. We believe that, had we performed an even more extensive screen, we would indeed have identified mRNAs corresponding to neuropeptides or transmitters.

We have identified several transcriptions factors in this investigation. This is not a major surprise, because the protein synthesis is largely regulated by gene transcription. Transcription factors are proteins which may recognize specific binding sites of a number of genes, regulating their activation [38]. A number of studies have already demonstrated that transcription factors such as c-jun or STAT-3 play a significant role for the processes of regeneration and degeneration [3,16,39–41]. In our investigations, c-maf and Oct-2 are induced in axotomized facial nucleus neurons. The transcription factor Oct-2 is belonging to the POU family and is induced in sensory neurons after sciatic nerve transection [42]. It is known that the expression of Oct-2 is influenced by the nerve growth factor (NGF) and might therefore be affected by the removal of target derived NGF following axotomy. This up-regulation of Oct-2 has been suggested to induce the phenotypic changes involved in the neuronal response to injury [42]. C-maf is known to be expressed during embryogenesis and is involved in cell differentiation. No data are available for an involvement in the process of regeneration.

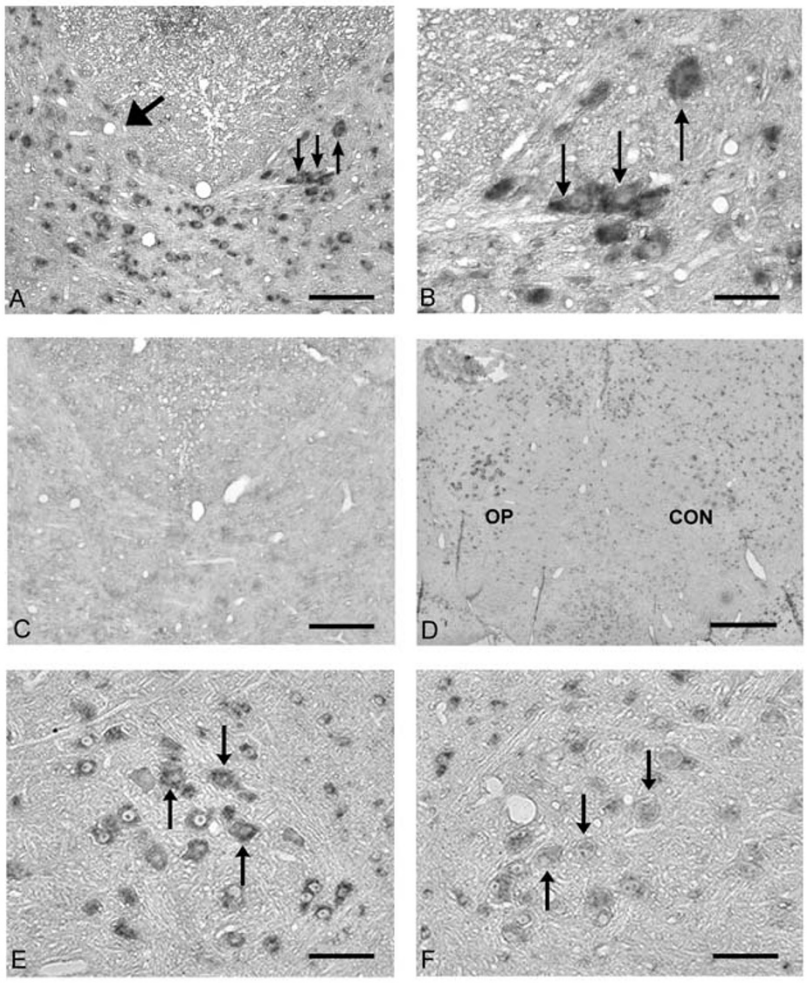

Figure 3
Differentially expressed genes within red and Clarke's nuclei. Non-radioactive ISH illustrates the differential expression of clone U41 seven days after spinal cord hemisection. A: Dramatic up-regulation of mRNA of clone U41 within axotomized Clarke's nucleus neurons (small arrows) ipsilateral and caudal to the lesion. Clarke's nucleus neurons of the control, non-operated side (large arrow) show only very faint expression. Bar, 100 μm B: High magnification of axotomized Clarke's nucleus neurons (arrows) demonstrates increased mRNA expression of clone U41. Bar, 20 μm C: Negative control for ISH using a sense probe. Bar, 100 μm D: Low magnification of the midbrain at the level of red nucleus. Up-regulation of clone U41 has been detected within axotomized red nucleus neurons, contalateral to the lesion (OP). Red nucleus neurons of the control side (CON) show only faint expression. Bar, 400 μm E: High magnification of red nucleus contralateral to the lesion reveals increased mRNA expression of clone U41 within axotomized neurons. Bar, 100 μm F: Red nucleus neurons of the control side showing weak mRNA expression. Bar, 100 μm

However, it is known that post-traumatic regenerative events in the nervous system often recapitulate some of the molecular systems that were employed during development [43]. In this context, the differential expression of homeobox-genes such as Hox-9 might recapitulate their role during embryonic ontogenesis. Homeobox-genes are important for the establishment of the specific identity of a cell and its topographic organization [44,45]. Previous investigations have revealed that the homeobox-gene, Islet-1, is down-regulated in axotomized facial nucleus neurons. Islet-1 is a key marker for motoneurons during development and this down-regulation might reflect a loss of neuronal identity or some form of de-differentiation. This might be an important prerequisite for neuronal regeneration [37].

We have also identified genes involved in signal transduction pathways and metabolism. These classes of molecules have also been identified by others using similar experimental approaches [46,47]. Specific signal transduction pathways are involved in almost every cellular process [48]. They are rapidly activated and may play key roles in the regulation of differential gene expression [49]. It is very likely that the identification of genes involved in metabolism rather reflects the functional stage of the cells than being key elements for the process of regeneration. However, ISH demonstrated the differential expression of several genes influencing protein translation, which might reflect an interesting aspect of the process of regeneration which has, to date, been poorly appreciated. In the axotomized facial nucleus, genes encoding for proteins of the small (S3 and S6) and large (L7) ribosomal subunits were up-regulated in glial cells. In axotomized neurons, a gene fragment homologue to the mouse elongation factor-2 (eEF2) showed enhanced neuronal expression. The difference to the published sequence for rat eEF2 suggests an unknown isoform. It could be hypothesized that the balance of these molecules might control the rate of protein translation leading to selective production of specific mRNA species.

One interesting molecule identified in the present investigation might be the enzyme stearoyl-CoA desaturase (SCD-1), which is dramatically up-regulated in axotomized facial nucleus neurons. One of the preferred substrates, stearoyl-CoA, is desaturated into oleoyl-CoA, which is further converted into its corresponding fatty acid: oleate. Beside its function as an energy store in the form of triacylglycerides, oleate is part of biological cell membranes and is believed to be involved in protein kinase C (PKC) dependent second messenger cascades, thereby influencing, for example, the activation of regeneration-associated genes such as GAP-43 [50,51]. By using comparative studies, we have demonstrated that SCD-1 is not induced in non-regenerating rubrospinal or Clarke's nucleus neurons suggesting a significant role for successful neuronal regeneration (Breuer et al., submitted for publication).

Furthermore, we have identified receptors and cytoskeletal molecules. The role of the transferrin and B-cell receptors being differentially expressed in the axotomized facial nucleus [33] or red nucleus respectively, remains unclear. However, it could be demonstrated that the lack of functional B and T lymphocytes led to a significant loss of axotomized facial nucleus neurons [52,53]. These results highlight that the immune system is heavily involved in regulating neuronal survival after peripheral nerve injury [54–56]. Cytoskeletal elements such as GFAP or vimentin are well known to be up-regulated in glial cells when they respond to different types of injury which reflects a typical feature of accompanying glial reactions [57,58].

Among the group of miscellenous genes, differential expression of connexin-43, ferritin light chain and the β-1 subunit of the Na/K-ATPase were identified. Connexin-43 (Cx-43) has already been reported to be up-regulated in glial cells surrounding axotomized facial nucleus neurons. The role of the induction of ferritin light chain within axotomized facial nucleus neurons remains to be elucidated. Very little is known about the involvement of iron metabolism in the axonal regeneration. However, ferritin is known to be the major iron storage protein and is a key component in protecting the brain from iron induced oxidative damage [59]. The β1-subunit of Na/K-ATPase was down-regulated in axotomized Clarke's nucleus neurons. It has been already demonstrated that the alpha 3 subunit mRNA is reduced in ventral horn neurons after spinal cord transection. This reduction could be reversed by dexamethasone, indicating that the expression of Na/K-ATPase may constitute an important mechanism by which glucocorticoids help to re-establish neuronal function after injury [60]. Furthermore, the alpha 2 and beta isoforms of Na/K-ATPase are down-regulated after sciatic nerve injury. This reduction took place in the distal segments owing to Wallerian degeneration and returned to baseline during nerve regeneration [61]. These data suggest a functional role for the process of regeneration which has to be proven by further studies.

Furthermore, 66 gene fragments did not show homologies to known sequences of the rat genome. Since the rat and mouse genomes are known to be very similar, we compared our "unknown" sequences obtained from rat RNAs with the mouse genome, which was recently published. As anticipated, we found many homologies to the mouse genome. Sixty gene fragments showed homologies to different regions on different mouse chromosomes (Table 4). It is likely that these gene fragments are related to the corresponding sequences on the rat genome, which

have not yet been identified. ISH demonstrated that 3 of those genes were up-regulated in axotomized facial nucleus neurons. One gene was up-regulated in axotomized rubrospinal neurons and one gene was down-regulated in axotomized Clarke's nucleus neurons.

Although the DD-PCR technique leads to the rapid identification of a number of differentially expressed genes, a major problem lies in the selection of genes for further studies. It has to be considered, that the elucidation of the functional role of defined genes and their products is very time consuming. In the past, several other techniques such as subtractive suppression hybridization (SSH) or serial analysis of gene expression (SAGE) have been developed to analyze differentially expressed genes. Recently, even more advanced methods such as arrays and DNA chip analysis have been introduced. However, all of these techniques lead to the identification of a high number of differentially expressed genes and this leads to the limitations of what can be quantitatively and qualitatively analyzed by one research group. All new techniques do not solve the problem of identifying which genes have to be selected for further detailed analysis. Therefore, other strategies are necessary to facilitate this selection. In our studies we have decided to performed comparative studies using different lesion paradigms. Genes which have been identified by DD-PCR and have been proven to be differentially expressed using ISH will be analyzed in our different lesion paradigms. The comparison of data from regenerating and non-regenerating models facilitates the identification of those genes that are regulated in a „model-specific" manner and those that may be of general importance for the process of regeneration. By these comparative analyses, we will select genes for further investigation which are exclusively regulated in a regenerating or non-regenerating lesion paradigm or show different regulation pattern.

So far, we have compared the gene expression pattern of a few selected genes identified by the DD-PCR in all our lesion models. By this means, we have demonstrated that 4 gene fragments showing homologies to the mouse genome are exclusively regulated in one lesion paradigm. In contrast, mRNA up-regulation of one of those gene fragments could also be detected in non-regenerating neurons of the red and Clarke's nucleus, but not in regenerating facial nucleus neurons. Such molecular differences between regenerating and non-regenerating neurons could be observed for the stearoyl-CoA-desaturase. SCD-1 is induced in regenerating facial nucleus neurons, but not in non-regenerating neurons of red and Clarke's nucleus. Thus, these genes fulfill the criteria of being exclusively regulated in only one lesion paradigm, indicating the importance of the functional role in the process of regeneration. These examples will provide the impetus for detailed functional analysis (e.g. antisense or gene transfer studies) of these genes.

Conclusions

By means of the differential display technique, we have identified 135 genes belonging to different classes of molecules. For 15% of the identified genes, the differential expression could be verified by ISH. In particular, the strategy of employing differential display PCR in the comparison of regenerating and non-regenerating populations of neurons significantly facilitates the identification of genes which may a key role in the process of axonal regrowth. Our results provide insights into the molecular mechanisms underlying the final outcome of attempted nerve fiber regeneration following axotomy. In particular, genes that are exclusively regulated in a regenerating or non-regenerating paradigm, might indicate an important role in attempted tissue repair. Further studies will determine the functional significance of these molecules in the process of regeneration.

Methods

Lesion models

All surgical procedures were performed on female sprague-dawley rats (250 g) and were approved by the government of Nordrhein-Westfalen (50.203.2 AC 23,21/00) and the government of Oberbayern (211-2531-37/97).

Peripheral nerve transection

The right facial nerve was transected outside the foramen stylomastoideum under deep chloral hydrate anesthesia (350 mg/kg, i.p.). Seven days after surgery, 10 rats for each lesion model were given terminal anesthesia (chloral hydrate, 640 mg/kg i.p.). For DD-PCR, the animals were perfused via the left ventricle with sterile saline (0.9%). The brains were immediately removed and frozen in liquid nitrogen. For *in situ*-hybridization, 4 animals for each lesion model were perfused with 4% paraformaldehyde and brains were removed for subsequent paraffin embedding.

Spinal cord hemisection

Under deep chloral hydrate anesthesia (350 mg/kg, i.p.) a Th9 or C2 laminectomy was followed by lateral hemisection of the spinal cord. Seven days after injury, rats were given terminal anesthesia (chloral hydrate, 640 mg/kg i.p.). For DD-PCR, the animals were perfused via the left ventricle with sterile saline (0.9%), the spinal cords (including the lesion site) were rapidly dissected and frozen in liquid nitrogen. For each animal, serial transverse (20 μm thick) sections of the lesion site were stained with thionin to assess the accuracy and extent of the lesion. Spinal cords with confirmed lateral hemisection were processed, together with the corresponding brains, for further analysis. For *in situ*-hybridization, animals were perfused

with 4% paraformaldehyde, the spinal cords and the brains were removed and subsequently processed for paraffin embedding.

RNA isolation and cDNA synthesis

For RNA isolation, the area of axotomized and non axotomized nuclei (facial, Clarke's and red nucleus neurons) were punched out separately. The tissue was mechanically homogenized and the RNA was isolated using TriStar (Peqlab) according to the manufacturers protocol. To eliminate genomic DNA an incubation (30 min) with 20 U DNAse (Boehringer Mannheim, Germany) was performed. For cDNA synthesis, 1 μg of the RNA, 2 μl RNAsin (40 U/μl) and 175 pmol T_{11}A primer (AAG CTT TTT TTT TTT A) were incubated 10 min. at 70°C and subsequently cooled on ice. Six μl of reaction buffer (5X), 7 μl dNTP's (each 2 mM), 1 μl $MgCl_2$ (50 mM), 2 μl RNA'sin (40 U/μl, Boehringer Mannheim, Germany), 1 μl AMV (30 U/μl, Peqlab, Germany) and DEPC-water were added to a total volume of 30 μl. After an incubation of 60 min. at 42°C, the reaction was stopped by a 5 min. incubation at 94°C.

As an internal control for the content and quality of the cDNAs, the housekeeping gene cyclophillin was used, amplified by PCR (5' primer: CAA CTC TAA TTT CTT TGA CTT GCG GG; 3'primer: AGA GAT TAC AGG GTA TTG CGA G). If necessary, the amount of cDNA used for the PCR was adjusted.

Differential display PCR

All animals used for DD-PCR were sacrificed 7 days p.o. To avoid false positive results due to an unequally performed PCR, all runs were carried out in parallel (2 × operated and 2 × non operated). Radioactive DD-PCR experiments were performed using one arbitrary primer (Tab. 1) and one T_{11}-A primer (AAG CTT TTT TTT TTT A). The PCR reaction was performed in a volume of 12,5 μl and contained 1 × PCR buffer, 20 pmol/μl dNTP's (each), 0,5 μl ^{35}S-dATP (Amersham, SJ-1304), 0,2 pmol/μl primer (each), 3 mM $MgCl_2$, 5 U Taq-Polymerase (Sigma) and 5 μl cDNA. PCR was started by an initial incubation at 94°C for 2 min, followed by 48 cycles of 10 s at 91°C, 20 s at 40°C and 40 s. at 72°C. The resulting PCR products were separated using 6% polyacrylamide gels and exposed to an x-ray film (Kodak). Differentially expressed bands were cut out, extracted by an incubation in 100 μl boiling H_2O for 15 min and precipitated with ethanol. The pellet was resolved in 10 μl H_2O. To reamplify the isolated bands, a second PCR was performed in a volume of 12,5 μl, containing 1 μl isolated DNA, 1 × PCR buffer, 200 pM/μl dNTP's (each), 1 pmol/μl primer (used in the first PCR), 1,5 mM $MgCl_2$, 2,5 U Taq-Polymerase (Sigma). After successful amplification and purification (Qiaquick; Qiagen; Hilden Germany), the isolated DNA was cloned into a transcription vector (p-zero1-1, Invitrogen) and sequenced with an automatic sequencer. By comparing the sequences with known sequences in the gene bank of the NCBI (National Center for Biotechnology Information) we separated all clones which contain only bacterial- or vector-DNA. Gene fragments which did not show homologies to known sequences were addionally compared with the mouse genome http://www.ensembl.org.

In situ hybridization

Antisense and Sense RNA probes were generated for ISH, which allowed the anatomical and cellular distribution of genes identified by DD-PCR to be detected. This facilitates the interpretation of a functional role for these genes.

For PCR amplification 0.5–1 ng of the cloned DD-PCR fragments were used. Primers specific for either the T_7 or Sp_6 RNA-polymerase recognition sequence were chosen. A PCR with a volume of 12.5 μl containing 0.2 ng plasmid was performed for 28 cycles with denaturation at 94°C for 10 s, annealing at 52°C for 10 sec and elongation at 74°C for 20 s. The PCR product was purified from primers and salt using QiaQuick (Qiagen, Hilden, Germany). Transcription, using either T_7 or Sp_6 RNA-polymerase, was performed according to the manufacturers protocol (Boehringer Mannheim, Germany) using 250 ng of the respective PCR product as template.

Paraffin sections were prepared for ISH by dewaxing and rehydration in 0.1 M PBS buffer, followed by proteinase K-incubation (10 μg/ml) for 20 min at 37°C. For ISH, the Boehringer protocol for non-radioactive ISH using digoxigenin-labeled RNA probes was used. Briefly, sections were pretreated with PBS containing 0.1% Triton X-100. Hybridization was performed with 50% deionized formamide, 10% dextran sulfate, 5 × Denhardt's solution, 0.5 M Tris (pH 7.0),10% laurylsarcosine, 1% ssDNA, 0.1% tRNA (10 mg/ml), 0.1% 0.5 M EDTA (pH 8,0). Sections were incubated with 100 μl hybridization mixture containing 200 ng of digoxigenin labeled antisense or sense RNA probe at 55°C overnight. The slides were then washed extensively, treated with 2 × SSC containing 20 μl/ml RNAse at 37°. To reduce background staining, slides were washed in 2 × SSC with and without 50% formamide at 50°C in a shaking water bath.

To detect digoxigenin labeled RNA probes, slides were incubated with sheep anti-DIG-alkaline phosphatase antibody (#1093274, Boehringer Mannheim, Germany) at a dilution of 1:1000 in a humid chamber at 4°C overnight. After washing in a buffer containing 100 mM Tris-HCl (pH 9,5), 100 mM NaCl and 50 mM $MgCl_2$, the sections were incubated with the same buffer containing 0.45% nitroblue tetrazolium and 0.35% X-phosphate (5-bromo-4-chloro-3-indolylphosphate) at room temperature. The reaction was stopped after the colored signal appeared (with

no or only weak background in the sections incubated with the sense RNA probe). Sections were coverslipped with ICN aqueous mounting solution.

Authors' contribution

AS participated in carrying out the molecular studies, in the design and coordination of the project and wrote the manuscript. SB participated in carrying out the molecular studies on the CNS lesion paradigm and coordinating the experiments. JL participated in carrying out the DD-PCR, cloning experiments and sequence analysis on the CNS lesion paradigm. AB participated in carrying out DD-PCR and sequence analysis on the CNS lesion paradigm. CS participated in carrying out the in situ-hybridization experiments. KP participated in performing animal surgery and cloning experiments for both lesion paradigms. EH participated in carrying out the molecular experiments on the PNS lesion paradigm and in coordinating the project. GB participated in performing animal surgery and in drafting the manuscript. JN participated in coordinating the project. FS participated in carrying out the molecular studies on the PNS lesion paradigm and in the design of the project. All authors read and approved the final manuscript.

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