International Graduate School of Neurosciences
Ruhr Universität Bochum

Expression and Function of Connexins
During Schwann Cell Development in The Mouse

Doctoral Dissertation
Jing Li

Thesis advisors:
Prof. Dr. med. Rolf Dermietzel
Jun. Prof. Dr. Carola Meier

Department of Neuroanatomy and Molecular Brain Research
Medical Faculty, Ruhr-University Bochum

Bochum October 2005
To my parents
and Huadong
For their love, and constant support
Acknowledgments
I take this opportunity to thank all those who have contributed to my thesis.

First of all, I would like to thank my thesis advisor Prof. Dr. R. Dermietzel for giving me the opportunity to perform this work in the Department of Neuroanatomy and Molecular Brain Research. I am also deeply grateful to Prof. Dr. R. Dermietzel and Jun. Prof. Dr. C. Meier for their guidance.

I gratefully acknowledge International Graduate School of Neuroscience (IGSN) for the financial support.

I also would like to thank Prof. Dr. K. Willecke and J. Eiberger (Institute of Genetics, Division of Molecular Genetics, University of Bonn) for their kindly contribution of the Cx29LacZ transgenic mice.

Thanks also to Dr. G. Zoidl and Ms. M. Kremer for their donation of primers and siRNAs, Aiden Haiglikia for the dye transfer experiments, Mrs. M Lübbecke-Schumacher and Mrs. L. Janota for the technical assistance, Ms. U. Becker for her uncountable support.

I am very thankful to all the graduate students in the laboratory: Arundhati, Bianca, Mahboob, Kerstin, Stephanie, Andrew and David for creating the nice research atmosphere and unconditional support.

My special thanks to Prof. Dr. K.J. Jessen and Prof. Dr. R. Mirsky as well as the people in the Department of Anatomy and Developmental Biology in University College London for their consistent support and valuable suggestions.

Here I am especially indebted to:

Hans-Werner Habbes, Annegrit Schlichting, Monika Birkelbach and Sabine Schreiber-Minjoli for their unreserved support, daily encouragement and friendship

Dr. Dorothee. Krause-Finkeldey for her uncountable help as well as her goodwill

Dr. Elisabeth. Petrasch-Parwez for her expertise helps in morphology and scientific discussions
Figures and Tables

Figure 1. Schematic depiction of Schwann cell development ........................................... 3
Figure 2. Schematic representation of neural crest cell migration pathways and their derivatives .......................................................... 7
Table 1. Some distinct properties of NCC, Schwann cell precursor and Schwann cell .......... 11
Figure 3. Gap junction channel and connexin protein topography ................................ 17
Figure 4. Construct of the Cx29LacZ transgenic mice .................................................. 26
Table 2. List of Primers ..................................................................................................... 33
Figure 5. PCR analysis to genotype Cx29LacZ transgenic mice ...................................... 34
Table 3. List of antibodies ................................................................................................. 38
Figure 6. Schwann cell lineage progression in vitro ....................................................... 47
Figure 7. PT-PCR analysis of connexin mRNA expression in neural crest cells ............. 49
Figure 8. mRNA expression of connexin 43, 29 and 32 in the Schwann cell lineage ....... 50
Figure 9. Protein expression of connexin 43 and 29 in early Schwann cell lineage ...... 52
Figure 10. Immunostaining of connexin 43, 29 and 32 in immature Schwann cells ...... 54
Figure 11. Immunoblot analysis of connexin expression in neural crest cells ............... 55
Figure 12. Immunofluorescence and Western blot analysis of connexin29 antibodies specificity ................................................................. 57
Figure 13. LacZCx29 expression during Schwann cell development in vitro .................. 58
Figure 14. Immunofluorescent detection of neural crest neuronal differentiation and connexin expression ........................................................................ 60
Figure 15. Peripheral nerves in the mouse embryonic trunk visualized by immunohistochemistry using TuJ-1 antibody ................................................................. 61
Figure 16. Whole mount LacZ staining at E9.5 .............................................................. 63
Figure 17. LacZCx29 expression at E12 and E16 ............................................................ 64
Figure 18. Localization of Cx29LacZ in Schwann cell precursors at E12 ..................... 65
Figure 19. Localization of LacZCx29 at E14 ................................................................. 66
Figure 20. Localization of Cx29LacZ in immature Schwann cells at E16 ..................... 67
Figure 21. Expression of Cx29LacZ is detected in the nerves in other organs ............... 68
Figure 22. Development of Schwann cell precursors to immature Schwann cells in the dorsal and ventral root of the spinal nerve ......................................................... 70
Figure 23. Immunohistochemical detection of connexin31 expression on paraffin embedded transverse sections and semithin sections stained by toluidine blue at the same developmental stages ................................. 72

Figure 24. Connexin29 and LacZ localization in postnatal sciatic nerves .................. 75

Figure 25. Life phase contrast images of neural crest cells cultured with various concentration of cyclopamine .............................................. 78

Figure 26. Cyclopamine downregulates LacZCx29 expression but does not affect p75 expression ................................................................. 78

Figure 27. Cyclopamine significantly reduces the proportion of LacZCx29 expressing neural crest cells in vitro .............................................. 80

Figure 28. Sonic hedgehog promotes LacZCx29 expression one day earlier in neural crest cells and neutralizes the inhibitory effect of cyclopamine ...... 82

Figure 29. Migratory neural crest cells from neural tube explants in culture ........... 83

Figure 30. Mimetic peptides inhibit the dye coupling of neural crest cells ............... 85

Table 4. Migration index obtained from neural crest cell cultures by inhibition of connexin43 with different methods ............................................ 86

Figure 31. Mimetic peptides do not inhibit neural crest cell migration ..................... 86

Figure 32. Degradation of connexin43-ELII antibodies occurs between two and four hours in astrocyte cultures ...................................................... 88

Figure 33. Antibodies recognizing the extracellular loop of connexin43 does not significantly reduce neural crest cell migration ............................. 89

Figure 34. Proportion of hemichannels in connexin43 gap junction channels of neural crest cells ................................................................. 90

Figure 35. Silencing of connexin43 gene expression by siRNA inhibits neural crest cell migration significantly ..................................................... 91

Figure 36. BrdU incorporation in cells of neural crest cell cultures ........................ 92

Table 5. Percentage of BrdU incorporated cells in connexin43 siRNA transfected neural crest cells as well as in the non-treated cells ............................. 93

Figure 37. Connexin43 protein expression is completely suppressed by siRNA between 48 hrs and 72 hrs after transfection ......................................... 94

Figure 38. Oleamide significantly inhibits neural crest cells migration .................... 96
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Avidin-Biotin Complex</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BC</td>
<td>boundary cap</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BrdU</td>
<td>2-bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>c-JNK</td>
<td>c-Jun-amino(N)-terminal kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>cyclopamine</td>
</tr>
<tr>
<td>CTMX</td>
<td>X-linked Charcot-Marie-Tooth syndrome</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DM</td>
<td>defined supplemented medium</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>DSP</td>
<td>downstream primer</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ET-3</td>
<td>endothelin-3</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FGF-2</td>
<td>fibroblast growth factor-2</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GGF</td>
<td>glial growth factor</td>
</tr>
<tr>
<td>GJ</td>
<td>gap junctions</td>
</tr>
<tr>
<td>GJC</td>
<td>gap junction communication</td>
</tr>
<tr>
<td>Hams F12</td>
<td>F-12 Nutrient Mixtures</td>
</tr>
<tr>
<td>Hoechst</td>
<td>2, 5`-bi-1H-benzimidazole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HRG</td>
<td>heregulin</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IGF-2</td>
<td>insulin-like growth factor-2</td>
</tr>
<tr>
<td>kD</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LN</td>
<td>laminin</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NC</td>
<td>neural crest</td>
</tr>
<tr>
<td>NCC</td>
<td>neural crest Cells</td>
</tr>
<tr>
<td>NDF</td>
<td>neu differentiation factor</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NRG</td>
<td>neuregulin</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin-3</td>
</tr>
<tr>
<td>Oct6</td>
<td>octamer-binding transcription factor 6</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>p75NTR-R</td>
<td>p75 low affinity neurotrophin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PLL</td>
<td>poly-L-lysine</td>
</tr>
<tr>
<td>PN</td>
<td>peripheral nerves</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SM</td>
<td>simple defined medium</td>
</tr>
<tr>
<td>TG</td>
<td>transgenic</td>
</tr>
<tr>
<td>USP</td>
<td>upstream primer</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
# Table of contents

Acknowledgements .................................................................................................................. i
Figures and Tables .................................................................................................................. ii
Abbreviations ....................................................................................................................... iv
Abstract .................................................................................................................................. 1

1. Introduction ....................................................................................................................... 3
   1.1 Development of the Schwann cell lineage ................................................................. 3
       1.1.1 Neural crest cells ................................................................................................. 4
       1.1.2 Schwann cell precursors .................................................................................... 10
       1.1.3 Immature Schwann cells ................................................................................... 13
       1.1.4 Mature Schwann cells ....................................................................................... 13
       1.1.5 Progression of the Schwann cell lineage ......................................................... 15
   1.2 Gap junction communication and Gap junction proteins ...................................... 15
   1.3 Gap junction proteins in glial cells in the nervous system ........................................ 18
       1.3.1 Expression of connexin32 in Schwann cells ..................................................... 19
       1.3.2 Expression of connexin43 in Schwann cells ..................................................... 20
       1.3.3 Expression of connexin29 in Schwann cells ..................................................... 21
       1.3.4 Expression of other connexins in Schwann cells ............................................. 22
   1.4 Expression of connexins during embryogenesis ....................................................... 22

2. Aim of the study ................................................................................................................. 24

3. Materials and Methods ................................................................................................... 25
   3.1 Animals ....................................................................................................................... 25
   3.2 Substratum for Cell culture ....................................................................................... 26
   3.3 Media ......................................................................................................................... 27
       3.3.1 Defined supplemented medium (DM) ................................................................. 27
       3.3.2 Simple defined medium (SM) ............................................................................ 28
       3.3.3 Hybridoma medium ......................................................................................... 28
       3.3.4 Astrocyte medium ............................................................................................. 28
   3.4 Neural tube explantation and neural crest cell culture ............................................ 29
   3.5 Schwann cell precursor and embryonic immature Schwann cell cultures ............. 29
   3.6 Postnatal Schwann cell culture and immunopanning ............................................ 30
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.7 Astrocyte culture</td>
<td>31</td>
</tr>
<tr>
<td>3.8 Reverse transcription polymerase chain reaction (RT-PCR)</td>
<td>32</td>
</tr>
<tr>
<td>3.9 Genotyping and PCR</td>
<td>33</td>
</tr>
<tr>
<td>3.10 Immunofluorescence staining (IF)</td>
<td>34</td>
</tr>
<tr>
<td>3.11 Immunohistochemistry (IHC)</td>
<td>36</td>
</tr>
<tr>
<td>3.12 Western blotting</td>
<td>37</td>
</tr>
<tr>
<td>3.13 Light microscopy and semithin sections</td>
<td>39</td>
</tr>
<tr>
<td>3.14 LacZ staining</td>
<td>39</td>
</tr>
<tr>
<td>3.15 Analysis of regulation of connexin29 expression</td>
<td>40</td>
</tr>
<tr>
<td>3.16 Dye coupling</td>
<td>41</td>
</tr>
<tr>
<td>3.17 Analysis of neural crest migration</td>
<td>41</td>
</tr>
<tr>
<td>3.17.1 Mimetic peptides of connexin43</td>
<td>42</td>
</tr>
<tr>
<td>3.17.2 Antibody recognizing the extracellular loop of connexin43 (Cx43-ELII)</td>
<td>42</td>
</tr>
<tr>
<td>3.17.3 Gene silence with small interfering RNA (siRNA) of connexin43</td>
<td>43</td>
</tr>
<tr>
<td>3.17.4 Oleamide</td>
<td>44</td>
</tr>
<tr>
<td>3.18 Statistical analysis</td>
<td>45</td>
</tr>
<tr>
<td>4. Results</td>
<td>46</td>
</tr>
<tr>
<td>4.1 Connexins expression during Schwann cell development</td>
<td>46</td>
</tr>
<tr>
<td>4.1.1 Connexins mRNA expression during Schwann cell development</td>
<td>48</td>
</tr>
<tr>
<td>4.1.1.1 Neural crest cells express mRNA transcripts of connexin 26, 29, 43 and 45</td>
<td>48</td>
</tr>
<tr>
<td>4.1.1.2 Expression of connexin mRNAs in the Schwann cell lineage</td>
<td>49</td>
</tr>
<tr>
<td>4.1.2 Connexin protein expression during Schwann cell development in vitro</td>
<td>50</td>
</tr>
<tr>
<td>4.1.2.1 Connexin expression in the early Schwann cell lineage</td>
<td>51</td>
</tr>
<tr>
<td>4.1.2.2 Connexin expression in the late Schwann cell lineage</td>
<td>53</td>
</tr>
<tr>
<td>4.1.2.3 Western blot analysis of connexin expression in neural crest cells</td>
<td>54</td>
</tr>
<tr>
<td>4.1.2.4 Specificity of connexin29 antibodies</td>
<td>56</td>
</tr>
<tr>
<td>4.1.2.5 LacZ reporter gene expression in transgenic mice during</td>
<td>58</td>
</tr>
<tr>
<td>Schwann cell development</td>
<td>58</td>
</tr>
<tr>
<td>4.1.2.6 Differentiation of neural crest cells to a neuronal fate does not trigger expression of connexin36</td>
<td>59</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.1.3 Spatial expression of connexin29 in vivo</td>
<td>60</td>
</tr>
<tr>
<td>4.1.3.1 LacZCx29 expression in whole mount stained embryos during</td>
<td>62</td>
</tr>
<tr>
<td>Schwann cell development</td>
<td></td>
</tr>
<tr>
<td>4.1.3.2 Localization of Cx29LacZ in Schwann cell precursors</td>
<td>64</td>
</tr>
<tr>
<td>4.1.3.3 Localization of Cx29LacZ in embryonic immature Schwann cells</td>
<td>67</td>
</tr>
<tr>
<td>4.1.3.4 Maturation of Schwann cell precursors between</td>
<td>69</td>
</tr>
<tr>
<td>dorsal and ventral root</td>
<td></td>
</tr>
<tr>
<td>4.1.3.5 Investigation of connexins other than connexin29 in the</td>
<td>71</td>
</tr>
<tr>
<td>dorsal root of the spinal nerve</td>
<td></td>
</tr>
<tr>
<td>4.1.3.6 Postnatal expression of connexin29 in sciatic nerves</td>
<td>74</td>
</tr>
<tr>
<td>4.2 Regulation of connexin29 expression</td>
<td>76</td>
</tr>
<tr>
<td>4.2.1 Cyclopamine downregulates expression of LacZCx29 in neural crest</td>
<td>76</td>
</tr>
<tr>
<td>cells</td>
<td></td>
</tr>
<tr>
<td>4.2.2 Sonic hedgehog antagonizes the effect of cyclopamine on the</td>
<td>80</td>
</tr>
<tr>
<td>expression of LacZCx29 and triggers earlier LacZCx29 expression in</td>
<td></td>
</tr>
<tr>
<td>neural crest cells</td>
<td></td>
</tr>
<tr>
<td>4.3 Function of connexin43 in the migration of trunk neural crest cells</td>
<td>83</td>
</tr>
<tr>
<td>4.3.1 Function of mimetic peptides in neural crest cell migration</td>
<td>84</td>
</tr>
<tr>
<td>4.3.2 Impact of antibodies recognizing the extracellular loop of</td>
<td></td>
</tr>
<tr>
<td>connexin43 in neural crest migration</td>
<td>87</td>
</tr>
<tr>
<td>4.3.3 Inhibition of neural crest cell migration by gene silencing of</td>
<td></td>
</tr>
<tr>
<td>connexin43 with siRNA</td>
<td>90</td>
</tr>
<tr>
<td>4.3.4 Inhibition of neural crest cell migration by oleamide,</td>
<td></td>
</tr>
<tr>
<td>a chemical gap junction blocking agent</td>
<td>95</td>
</tr>
<tr>
<td>5. Discussion</td>
<td>97</td>
</tr>
<tr>
<td>5.1 Connexin43 expression during Schwann cell development in vitro</td>
<td>98</td>
</tr>
<tr>
<td>5.2 Connexin29 expression during Schwann cell development in vitro</td>
<td>99</td>
</tr>
<tr>
<td>5.3 Connexin32 expression during Schwann cell development in vitro</td>
<td>100</td>
</tr>
<tr>
<td>5.4 Cellular localization of connexins in vitro</td>
<td>101</td>
</tr>
<tr>
<td>5.5 Expression of other connexins in neural crest cells</td>
<td>102</td>
</tr>
<tr>
<td>5.6 Localization of connexin29 in embryonic stages of Schwann cell</td>
<td>103</td>
</tr>
<tr>
<td>development</td>
<td></td>
</tr>
<tr>
<td>5.7 Localization of connexin29 in the nervous system</td>
<td>106</td>
</tr>
<tr>
<td>5.8 Connexin31 expression in peripheral sensory and spinal motor</td>
<td>107</td>
</tr>
<tr>
<td>neurons</td>
<td></td>
</tr>
</tbody>
</table>
5.9 Sonic hedgehog regulates connexin29 expression *in vitro* ...................... 108
5.10 Connexin43 is involved in neural crest migration .................................. 111
6. Summary ........................................................................................................ 115
7. Reference ........................................................................................................ 116
Curriculum vitae ................................................................................................. 137
Abstract

In the mouse, Schwann cells generate from multipotent trunk neural crest cells (NCC) at around embryonic day (E) 9.5, transiting two intermediate stages of Schwann cell precursors (E12) and immature Schwann cells (E16), and subsequently develop into myelinating and non-myelinating Schwann cells postnatally. Gap junction channels are electrical synapses for direct intercellular communication. Expression of multiple gap junction proteins (connexin, Cx), including Cx32, Cx43, Cx29, Cx26, and Cx46 (after nerve injury), has been reported in myelinating Schwann cells. Mutations or deletion of the Cx32 gene in Schwann cells are responsible for the X-chromosome linked Charcot-Marie-Tooth (CMTX) syndrome in humans. This finding has brought attention to the importance of connexins in glial cells.

This study focused on the expression and function of connexins during Schwann cell development in the mouse. Our data provided evidence for a successive protein expression pattern of connexins during Schwann cell development in vitro. Expression of Cx43 commenced at the NCC stage (E9.5). The onset of Cx29 expression paralleled differentiation of NCC into Schwann cell precursors (E12), while expression of Cx32 protein appeared when the lineage developed into myelinating Schwann cells (i.e. postnatal day (P) 4).

The spatial expression pattern of Cx29 was investigated in vivo using wild
type and Cx29LacZ transgenic mice. At E12, expression of Cx29LacZ was restricted to Schwann cell precursors in the ventral roots of spinal nerves as well as in peripheral nerves. In contrast, the dorsal root ganglion (DRG) and the dorsal root were devoid of Cx29LacZ. However, at E16, Cx29 expression emerged in dorsal roots as well as in cells within the DRG, albeit less abundant than in ventral roots, pointing to a possibly differential regulation of Cx29 expression in ventral and dorsal roots. The regulation of Cx29LacZ expression was therefore studied, focussing on the effect of sonic hedgehog (Shh, an instructive signal for ventral patterning of the neural tube) in vitro using Cx29LacZ transgenic animals. Sonic hedgehog preponed LacZCx29 expression in NCC by twenty-four hours, whereas its antagonist cyclopamine decreased expression level in NCC cultured for 3 days, which corresponds to the Schwann cell precursor stage in vivo. Thus, Shh has been identified as a potential regulator of Cx29 expression.

As one function of Cx43 in NCC might be migration, this effect was examined by different approaches. Inhibition of Cx43 gene expression using siRNA as well as by blocking gap junction communication using oleamide resulted in a significantly decreased rate of trunk NCC migration. These results indicate that expression of Cx43 and gap junctional communication in trunk NCC has an important influence on their migration.
1. Introduction

1.1 Development of the Schwann cell lineage

Schwann cells are glial cells of the peripheral nervous system (PNS), providing trophic support for sensory and motor neurons. Myelinating Schwann cells, which form the myelin sheath around axons, are essential for electrical insulation and saltatory conduction. In vertebrates, most Schwann cells develop from trunk neural crest cells (NCC) at embryonic day (E) 9.5 in the mouse. During development, NCCs have to undergo two distinct transitions to become mature Schwann cells. These are the formation of Schwann cell precursors, and the formation of immature Schwann cells. Postnatally, cells reversibly differentiate into myelin-forming and non-myelin-forming mature Schwann cells (reviewed by Jessen and Mirsky, 1998, 2002 and 2005) (Fig. 1).

Figure 1. Schematic depiction of Schwann cell development

In the mouse, NCCs migrate out at the boundary between neural tube and epidermis at E9.5, one of the derivatives being Schwann cell precursors, which are present in peripheral nerves at E12-13. After an abrupt transition within a period of two days,
immature Schwann cells form at E16. At around birth, they develop into mature Schwann cells with differentiation of myelinating and subsequently non-myelinating Schwann cells (adapted from Jessen and Mirsky, 1999).

1.1.1 Neural crest cells

The neural crest (NC), first described in chick embryos by Wilhelm His in 1868, refers to a group of embryonic ectoderm cells detaching from the dorsolateral margins of the developing neural tube in vertebrates. After a phase of extensive migration, NCCs give rise to various derivatives e.g. most cells of the PNS, endocrine cells, melanocytes, as well as smooth muscles cells. In addition, cranial connective tissues, cartilages, and bones as well as the enteric nervous system are derived from cranial NCC in mammal (Weston, 1982; Le Douarin et al., 1988; Smith-Thomas and Fawcett, 1989; Anderson, 1997; Basch et al., 2004).

The induction of NC starts at the early gastrula stage and continues until closure of the neural tube. The Wnt signal pathway has been documented to act as an inducer in NC formation (Dunn et al., 2000; Brault et al., 2001). In mice, deletion of both Wnt-1 and Wnt-3a genes (both expressed in the dorsal neural tube) is related to broad deficits in NC derivatives (Ikeya et al., 1997). In contrast, overexpression of either Wnt-1 or Wnt-3a in Xenopus leads to a marked increase of NCC numbers (Saint-Jeannet et al., 1997). Other signals like sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs) are also considered to be involved in NC induction. Sonic
hedgehog which is secreted by the notochord and floor plate, as well as BMPs which are expressed by the overlying dorsal ectoderm and roof plate, are the signals for dorsal-ventral patterning of the neural tube, i.e. Shh for ventral patterning and BMPs for dorsal patterning (Wilson and Maden, 2005). In addition, studies in avian have shown that Shh is able to suppress NCC formation at open neural plate levels \textit{in vitro} (Liem et al., 1995) as well as in \textit{vivo} (Selleck et al., 1998). The role of BMP4 and BMP7 in NC induction is tightly linked to the induction of the neural plate; however, their effect is not consistent in different studies (Liem et al., 1997; Selleck et al., 1998; Huang and Saint-Jeannet, 2004). Moreover, fibroblast growth factors (FGFs) and the Notch/Delta signaling pathway are also considered to be involved in the NC induction (Mayor et al., 1997; Cornell and Eisen, 2000; Monsoro-Burq et al., 2003; Glavic et al., 2004). Transcription factors expressed at the neural plate border are largely involved in the formation of NC. Spokony et al. (2002) have demonstrated in \textit{Xenopus} that Sox9, a member of the SoxE subgroup of high-mobility-group (HMG)-containing transcription factors, is required for cranial NC formation. Moreover, studies in avian have demonstrated that Sox9 is sufficient to induce a number of NCC properties in chick neural tube cells (Cheung et al., 2003), and that absence of Sox9 leads to apoptosis of trunk NCC in the mouse (Cheung et al., 2005). Furthermore, Cheung et al. (2005) have demonstrated that the zinc finger transcription factor Slug/Snail, a direct
target of Wnt signaling (Vallin et al., 2001), in the presence of Sox9 induces an epithelial-mesenchymal transition in neural epithelial cells, which indicates NC induction. Overexpression of Slug/Snail leads to excess production of cranial NCC in avian embryos (del Barrio and Nieto, 2002). The paired-homeodomain transcription factor-3 (Pax3), which has been implicated in the regulation of cell adhesion, apoptosis, migration as well as cell fate decisions (Mansouri et al., 2001; Wiggan and Hamel, 2002), is considered as another transcription factor to be involved in the regulation of NC induction. In humans, mutations of Pax3 result in the Waardenburg syndrome, a dominant disorder of pigmentation defects and deafness, which results from multiple deficits in NCC derivatives (Tassabehji et al., 1992).

Prior to neural tube closure in mammals and following neural tube closure in amphibians and birds, NCCs begin to migrate out progressively along the anterior-posterior axis of the developing embryo. The migratory pathways of the NCC were first investigated by Weston (1963) using radio-labeled cells in the chick, and have later been confirmed by Le Douarin and Smith (1988) using a chick/quail chimera system as well as by Serbedzija et al. (1990) using in vivo DiI dye injection in the mouse. Two comparable streams of migratory NCC, i.e. the ventral and dorsolateral pathways, were identified in avian as well as in mammalian development. The ventral pathway is characterized by NCC migrating anterior of or
through the somites. These NCCs give rise to sensory neurons in the dorsal root ganglia (DRG), autonomic neurons, and peripheral glia including Schwann cells as well as chromaffin cells of the adrenal medulla. The dorsolateral pathway comprises cells that travel between ectoderm and somites and these cells differentiate exclusively into pigment cells of the skin.

![Diagram of neural crest cell migration pathways and their derivatives](image)

**Figure 2. Schematic representation of neural crest cell migration pathways and their derivatives**

Neural crest cells, which are committed to the ventral pathway, give rise to neurons and glia in the PNS, and to endocrine cells; NCC following the dorsolateral pathway restrictedly differentiate into melanocytes of the skin (adapted from Principle of Neural Science, 4th edition, Academic publication, Editors: Kandel, Schwartz and Jessell, page 1046).

In addition to their migratory property, NCCs are a stem cell population that can give rise to a variety of derivatives as discussed above. The differentiation potential at the single NCC level has also been widely
investigated *in vivo* and *in vitro*. Some studies have proposed a stochastic hypothesis that individual NCCs are multipotent and capable of generating multiple derivatives. Evidence of this hypothesis came from *in vivo* microinjection studies using the fluorescent dye lysinated rhodamine dextran (LRD) in avian (Fraser and Bronner-Fraser, 1988 and 1991); and from immortalization studies on NCC expressing v-myc (Rao and Anderson, 1997) *in vitro*. Other studies using clonal culture analysis of mammalian and avian NCC *in vitro* have suggested that NCCs are composed of a heterogeneous population of cells, some of which are pluripotent and able to generate numerous cell types, whereas others may constitute a predetermined population, and, therefore, be committed to a restricted fate (Baroffio et al., 1988; Stemple and Anderson, 1992; Ito et al., 1993; Henion and Weston, 1997; reviewed by Le Douarin and Dupin, 2003).

Differentiation of NCC to the various derivatives is regulated by a number of extracellular signals. *In vitro* studies have revealed that transforming growth factor-beta (TGF-β) induce differentiation of NCC into smooth muscle cells, sensory and adrenergic neurons (Shah et al., 1996). Bone morphogenetic proteins 2 and 4, which are members of the TGF-β family molecules, have been demonstrated to trigger the generation of autonomic neurons *in vitro* (Shah et al., 1996) as well as *in vivo* (Schneider et al., 1999). However, it is still not clear which signal(s) instructively regulates
the gliogenesis from NCC. *In vitro* studies have indicated that β-neuregulin (β-NRG), also known as heregulin (HRG), acetylcholine receptor inducing activity (ARIA), glial growth factor (GGF), sensory and motor neuron-derived factor (SMDF) or Neu differentiation factor (NDF), might promote Schwann cell fate by blocking neuronal differentiation of NCC (Shah et al., 1994). Moreover, in mice, which are deficient of β-NRG or its specific receptors erbB2 or erbB3, Schwann cell generation is impaired. However, the absence of Schwann cells was shown to be an indirect effect, due to the deficiency of peripheral neurons (Meyer et al., 1997; Riethmacher et al., 1997; reviewed by Garratt et al., 2000). These data indicate that β-NRG is not essential for the initial differentiation of NCC to cells of the Schwann cell lineage.

Recently, a group of cells named boundary cap (BC) cells has been detected using chick/quail chimera (Niederlander and Lumsden, 1996). These cells originate in the NCC, but are arrested at the prospective entry/exit points of the spinal cord, and are considered to be involved in the regulation of ingrowth of sensory axons into the spinal cord as well as maintaining the integrity of sensory axons into the spinal cord (Golding and Cohen, 1997; Vermeren et al., 2003). Interestingly, these cells also migrate out and give rise to nociceptive neurons and satellite cells within the DRG, as well as to Schwann cell precursors in dorsal roots and some of the Schwann cells in ventral roots (Maro et al., 2004)
1.1.2 Schwann cell precursors

At E12 in the mouse, the main anatomical structures of peripheral nerves have been formed. At this stage, the axons of peripheral spinal nerves already project into the limbs, and Schwann cell precursors, which differentiate from NCC as a distinct developmental cell type, have associated with the nerve fibers (Dong et al., 1999). The mechanism of how the migrating NCC join the outgrowing axons and become part of the peripheral nerves is still not clear, one assumption being that the axons come across NCC individually, and, subsequently, arrest them via specific adhesive interactions or indicatively secreted molecules (Mirsky and Jessen, 1996).

Schwann cell precursors are located both inside and at the edge of nerves, surround large groups of axons of similar sizes and divide the nerves into territories. At this time, peripheral nerves are compact, and characterized by the absence of fibroblasts and blood vessels. In addition to their flattened phenotype in vitro, Schwann cell precursors are characterized by a number of properties and marker proteins, mostly distinct of those of mature Schwann cells (Table 1) (Dong et al., 1999; reviewed by Jessen and Mirsky 1999 and 2005).
Neural crest cells | Schwann cell precursors | Schwann cells
--- | --- | ---
flattened, extensive cell-cell contacts *in vitro* | flattened, extensive cell-cell contacts *in vitro* | bi- or tri-polar *in vitro*
paracrine survival | β-NRG promotes survival | autocrine survival
multipotent, high motility | high motility | low motility
associate with ECM | associate with axons | associate with axons
L1 + | L1 + | L1 +
p75+ | p75 + | p75 + *
erbB3 + | erbB3 + | erbB3 +
Sox10 + | Sox10 + | Sox10 +
P0, PMP22 – | P0, PMP22 + | P0, PMP22 +
GAP43 – | GAP 43 + | GAP43 + *
Dhh – | Dhh + | Dhh +
BFABP – | BFABP + | BFABP +
S100 – | S100 – | S100 +
O⁴ – | O⁴ – | O⁴ +
GFAP ? | GFAP ? | GFAP + *

Table 1. Some distinct properties of NCC, Schwann cell precursor and Schwann cell

+ indicates expression, – indicates no expression. ECM, extracellular matrix; L1, adhesion molecule; erbB3, β-NRG tyrosine kinases receptor; Sox10, a transcription factors in sex-determining gene SRY; p75, low affinity nerve growth factor receptor, GAP43, growth associated protein 43, and GFAP, glial fibrillary acidic protein, which are labelled by *, are expressed restrictedly in non-myelinating Schwann cells, however, expression of GFAP has not been characterized yet in mouse embryonic Schwann cells. Protein zero (P0), and peripheral myelin protein 22 kDa (PMP22) are myelin proteins. Dhh, desert hedgehog; BFABP, brain-fatty acid binding protein; S100, Ca²⁺ binding protein; O4, lipid antigen (adapted from reviews of Jessen and Mirsky, 1999, 2002 and 2005).
The transmembrane type III isoform of the β-NRG in the neuregulin family is considered as the crucial axonal signal for survival and proliferation of Schwann cell precursors. Moreover, it is also the instructive extracellular signal for lineage progression from Schwann cell precursors to immature Schwann cells. When Schwann cell precursors are dissociated from embryonic peripheral axons, they undergo programmed cell death in a defined serum-free medium within 20 hrs \textit{in vitro}, while this cell death can be rescued by conditioned medium from DRG neuron cultures. Furthermore, this neuron-derived signal also promotes the conversion of Schwann cell precursors to immature Schwann cells on schedule (Jessen et al., 1994; Dong et al., 1995; reviewed by Jessen and Mirsky, 1999 and 2002). In mouse and chick, β-NRG expression is found in sensory and motor neurons during the time when Schwann cell precursors assemble along the spinal nerves (Loeb et al., 1999). Moreover, ablation of β-NRG or its receptors in the mouse leads to Schwann cell precursor absence (Meyer et al., 1995 and 1997; Riethmacher et al., 1997). Correspondingly, Schwann cell precursors highly express the specific β-NRG receptor erbB3 in chick embryos (Ciutat et al., 1996). In addition, β-NRG also plays important roles at later stages during Schwann cell development (see chapter 1.1.4). In addition to β-NRG, there are other extrinsic signals which are important for Schwann cell precursor survival and maturation as well. Fibroblast growth factor-2 plus insulin like growth factor-1 (IGF-1) support Schwann
cell precursor survival *in vitro* in rat (Woodhoo et al., 2004). Endothelin-3 (ET-3) promotes Schwann cell precursor survival as well; while it negatively regulates the transition of Schwann cell precursors to immature Schwann cells (Brennan et al., 2000).

### 1.1.3 Immature Schwann cells

Within the course of two days during development, i.e. between E14 and E16 in the mouse, all Schwann cell precursors convert to immature Schwann cells with various changes in their morphology and the molecular expression pattern (Table 1). Immature Schwann cells establish autocrine survival signals which include insulin-like growth factor-2 (IGF-2), neurotrophin-3 (NT-3), platelet-derived growth factor-BB (PDGF-BB) (Meier et al., 1999), leukemia inhibitory factor (LIF) (Dowsing et al., 1999), and lysophosphatidic acid (LPA) (Weiner and Chun, 1999). This conversion from paracrine survival, i.e. dependence on axonal signals, to autocrine survival, points to an essential biological meaning, i.e. that Schwann cells can independently survive and, thus, provide support for axonal regeneration after nerve injury.

### 1.1.4 Mature Schwann cells

Around birth, peripheral nerves (PN) contain collagen fibers and blood vessels, within PN the endoneurial space is formed, and fibroblasts surround axons and immature Schwann cells. Those immature Schwann
cells, which will differentiate into myelin-forming Schwann cells, form one-to-one contact with large-diameter axons. Shortly after birth, they start to ensheathe the axon spirally to finally form a multilamellar myelin sheath. In contrast, non-myelin forming Schwann cells wrap a number of small diameter axons individually into the furrows of the cytoplasm, therefore preventing axon-to-axon contact.

Myelination is regulated by various signals and by interaction between neuron and myelin-forming cells. It is known that the myelination process is regulated by some growth factors. β-NRG and NT-3 are found to inhibit myelination (Bermingham-McDonogh et al., 1997; Chan et al., 2001; Zanazzi et al., 2001; Michailov et al., 2004). In contrast, brain-derived neurotrophic factor (BDNF), acting via its low affinity receptor p75, enhances myelin formation in vitro and in vivo (Chan et al., 2001; Cosgaya et al., 2002). Moreover, myelination is also associated with the activation of the transcription factor Krox20, the octamer-binding transcription factor 6 (Oct6), and phosphatidylinositol 3-kinase (PI3K) signalling (Bermingham et al., 1996, Topilko et al., 1997; Maurel and Salzer, 2000), whereas the c-Jun-amino(N)-terminal kinase (c-JNK) pathway, as well as two other transcription factors, Egr2/Sox2 and Pax3, are functioning as inhibitors of myelin formation (Parkinson et al., 2004; Le et al., 2005).
1.1.5 Progression of the Schwann cell lineage

The regulation of Schwann cell lineage generation and progression has been studied in detail. For growth factors, β-NRG and ET-3, as discussed above, play important roles in Schwann cell lineage. Moreover, various transcription factors have been involved during Schwann cell development as discussed above as well. However, Sox10 is the only gene known to be crucially important for gliogenesis. In Sox10-deficient mice early peripheral glia are missing, in contrast, sensory neurons within the DRG are initially generated in normal numbers, although they die later, which is considered to be due to the absence of peripheral glia (Britsch et al., 2001; Paratore et al., 2001; Sonnenberg-Riethmacher et al., 2001). However, it is unlikely that Sox10 plays a role for NCC differentiation to the glial cell lineage, since it is initially expressed in all migrating NCC, and, moreover, mutation of Sox10 also results in deficiencies of the enteric nervous system and pigmentation (Britsch et al., 2001).

1.2 Gap junction communication and Gap junction proteins

Gap junction channels are intercellular channels, which play an important role for direct communication of adjacent cells (Benedetti and Emmelot, 1965), allowing passage of water, ions (Na⁺, K⁺, Ca²⁺, etc.), as well as the exchange of small metabolites or second messengers (cyclic nucleotides, inositol 1,4,5-triphosphate, etc) (Bennett et al., 1991; Dermietzel and Spray, 1993; Bruzzone et al., 1996; Spray and Dermietzel, 1996). Gating of gap
junction channels is mediated by pH, transjunctional voltage, metabolites, phosphorylation, or can be modulated by non-specific chemical compound like oleamide as well as synthetic peptides that possess sequence homology with extracellular domain of connexins, etc (Ek-Vitorin et al., 1996; Guan et al., 1997; Dhein, 2002; Martin et al., 2005; Rivedal and Witz, 2005).

Each gap junction channel is composed of two docking hemichannels named connexons which consist of six transmembrane proteins (connexins, Cx). Each connexin protein is constituted of four hydrophobic transmembrane domains spanning the cell membrane, and two extracellular domains which are considered to be involved in homophilic coupling of hemichannels of two apposed cells as well as the on-off switch of the pore. The distance (gap) between two cell membranes is between 3.5 nm (Bennett et al., 1991; Dahl et al., 1992; Hoh et al., 1993; White et al., 1994; Zhou et al., 1997; Kwak and Jongsma, 1999; Unger et al., 1999; reviewed by Saez et al., 2003).

The sequences of the extracellular domains as well as transmembrane domains are highly conserved in different connexins. The cytoplasmic regions (intracellular loop, carboxyl as well as amino termini) of individual connexins are at relatively high variety, the variation of these sequences is assumed to be responsible for specific properties of different connexins, e.g. protein-protein interaction and presumably intracellular signal
transduction (Dermietzel, 1998a, b; Saez et al., 2003; Theis et al., 2005).

**Figure 3. Gap junction channel and connexin protein topography**

(A) Gap junction proteins cluster together at the cell surface to form intercellular channels between two docking cells. Each cell contributes one hemichannel named connexon that consists of six proteins called connexins. Two connexons span a gap of 3.5 nm between the two adjacent cell membranes and provide for cellular communication of small molecules. (B) Topological scheme of a single connexin protein showing four transmembrane domains (M1-4), two extracellular loops (EL1 and EL2), one intracellular loop (CL), as well as the carboxyl- and amino-terminal tails (Cell communication in the nervous and immune system, 2005. Meier and Dermietzel, book chapter: Electrical synapses-gap junctions in the brain, Heidelberg: Springer. In press; drawn by Ms. H. Schulze, Neuroanatomy, Ruhr University Bochum).

Connexins are a family with over 20 genes that are expressed in most types of tissues in vertebrates. The nomenclature of connexins is related to their expected molecular weight in kilo daltons (kDa), e.g. the connexin protein with a molecular weight of 43 kDa is referred to as Cx43. Connexins are grouped according to certain criteria, including amino acid sequence homology on protein level and gene structure on molecular level (Willecke et al., 2002; Sohl and Willecke, 2004).

There is also evidence that uncoupled connexons (hemichannels) can form
functional channels (Goodenough and Paul, 2003). Studies have shown the presence of connexons in the plasma membrane before they are incorporated into a junctional plaque (Musil and Goodenough, 1991). Antibodies recognizing the extracellular loop of connexin are able to block this assembly in living cells (Meyer et al., 1992). Moreover, dye transfer in cultured astrocytes has been dramatically inhibited by applying antibodies recognizing the extracellular loop of Cx43 (Hofer and Dermietzel, 1998; Contreras et al., 2002). As the gap between two docking connexons is too narrow for antibody access (Goodenough and Revel, 1971), this indicates that hemichannels are present independently as docking channels. In addition, in the catfish retina, functional Cx46 hemichannels, which are permeable for Lucifer yellow (LY) dye, have also been demonstrated (DeVries et al., 1992). Connexin45, which possesses relatively small unitary conductance for gap junction channels, has also been found capable of forming voltage, pH and calcium gated hemichannels (Valiunas, 2002). Recently, oocytes transfected with human Cx26 exhibited large membrane conductances with little voltage dependence, which suggests a relatively high open probability of these hemichannels (Rippes et al., 2004).

1.3 Gap junction proteins in glial cells in the nervous system

In the central nervous system (CNS) and PNS, multiple connexins have been found in glial cells. In the adult brain, Cx43, Cx30 and Cx26 are
expressed in astrocytes (Giaume et al., 1991; Kunzelmann et al., 1999; Nagy et al., 2001). Oligodendrocytes express Cx32, Cx47 and Cx29 (Dermietzel et al., 1989; Scherer et al., 1995; Sohl et al., 2001; Altevogt et al., 2002; Menichella et al., 2003; Odermatt et al., 2003). Astrocyte Cx26 forms heterotypic channels with Cx32 and Cx30, and Cx45 couples with Cx47 in oligodendrocytes (Altevogt and Paul, 2004). However, there is no evidence that Cx29 forms functional channels in the CNS (Altevogt and Paul, 2004; Sohl et al., 2004).

In peripheral glial cells, the importance of gap junction proteins came to front with the finding that diverse mutations or deletion of Cx32 are responsible for one of the demyelinating neuropathies in humans named X-linked Charcot-Marie-Tooth (CMTX) disease (Bergoffen et al., 1993; Scherer et al., 1998). Moreover, multiple gap junction proteins have been detected in Schwann cells.

1.3.1 Expression of connexin32 in Schwann cells

X-linked Charcot-Marie-Tooth disease was the first disease demonstrated to be related to a connexin mutation in humans. Demyelination of Schwann cells decreases the axonal conduction velocity of peripheral nerves, leading subsequently to their degeneration (Bergoffen et al., 1993; Fairweather et al., 1994). Mice lacking Cx32 also display decreased nerve conduction velocity as well as onion bulb formation in myelinating Schwann cells (Anzini et al., 1997; Scherer et al., 1998).
Schwann cells of the PNS generate myelin sheaths around peripheral axons. Connexin32 has been identified immunohistochemically at paranodal regions and Schmidt-Lanterman incisures of myelinating Schwann cells (Bergoffen et al., 1993; Scherer et al., 1995; Satake et al., 1997). In addition, Balice-Gordon et al. (1998) have detected that Cx32 and E-cadherin colocalized at these regions. By freeze-fracture replica immunolabeling, Cx32 gap junction plaques were identified ultrastructurally at Schmidt-Lanterman incisures as well as at a novel location, i.e. between the outer two myelin layers (Meier et al., 2004). Based on its location and on dye-transfer studies, Cx32 is considered to form reflexive gap junctions between myelin sheaths to provide a radial short cut for metabolites and other signals from the outermost cytoplasm to the next layer inwards and to the innermost layer, respectively (Balice-Gordon et al., 1998; Meier et al., 2004).

1.3.2 Expression of connexin43 in Schwann cells

In the mouse, expression of Cx43 has been demonstrated at Schmidt-Lanterman incisures and paranodal regions, thus being co-localized with Cx32 in these regions (Zhao et al., 1999). Its expression peaks at about postnatal day (P) 6 and decreases in the adulthood (Yoshimura et al., 1996). Furthermore, the radial diffusion of fluorescence dyes across the myelin sheath from the outer (abaxonal) cytoplasm to the inner (adaxonal) cytoplasm can also be observed by in vivo injection even in the absence of
Cx32 (Balice-Gordon et al., 1998). This evidence, therefore, points to the presence of additional connexin proteins. So far, no study on ultrastructural level of Cx43 expression has been reported.

1.3.3 Expression of connexin29 in Schwann cells

In mice, Cx29, which is the ortholog of human Cx30.2 (hCx30.2) (Sohl et al., 2001), was also detected immunohistochemically at paranodal loops and Schmidt-Lanterman incisures of Schwann cells (Altevogt et al., 2002; Li et al., 2002), and its subcellular location was determined to be the innermost, adaxonal myelin membrane (Li et al., 2002). In Schwann cells, Cx29, like Cx32, has been considered to be associated with myelin membranes. Studies have shown that in myelin-deficient rats, in which oligodendrocytes die before they produce much myelin, neither Cx29 nor Cx32 were detected in the CNS (Altevogt et al., 2002). However, recent observations also point to its occurrence in non-myelin forming Schwann cells (K. Willecke, personal communication). The function of Cx29, however, is still unclear, as Cx29 is not likely to form functional channels. In vitro studies have not detected intercellular conductance in N2A cells transfected with Cx29, or in oocytes expressing Cx29. Heterotypic gap junctional coupling of Cx29 and Cx32 has also not been observed, whereas heteromeric docking between Cx29 and Cx32 is likely to occur, at least in vitro (Altevogt et al., 2002).

The localization of Cx29 is most prominent at juxtaparanodes, pointing to
a function distinct from that of Cx32. Moreover, expression of Cx29 at the inner mesaxon and the innermost aspect of incisures was found in close approximation to potassium channel protein Kv1.2, suggesting that Cx29 hemichannels might be involved in potassium transport between axoplasm and periaxonal space (Verselis et al., 2000; Brophy, 2001; Altevogt et al., 2002).

### 1.3.4 Expression of other connexins in Schwann cells

The expression of a number of additional connexins has been reported in myelin-forming Schwann cells, one example being Cx26 is (Yoshimura et al., 1996). Moreover, upregulation of Cx46 protein after transection of peripheral nerves has also been demonstrated (Chandross et al., 1996b and 1998). However, the functions of these connexins still need to be determined.

### 1.4 Expression of connexins during embryogenesis

Previous studies have demonstrated that multiple gap junction proteins (Cx30, Cx31, Cx36, Cx43 and Cx45, etc.) are expressed during mouse embryogenesis (Davies et al., 1996; Houghton et al., 2002). The temporal-spatial pattern of their expression is highly suggestive of a role for mediating differentiation and patterning during mammalian embryogenesis (Warner, 1992; Lo, 1996). In NCC, expression of Cx43 has been described in the cardiac NCC. The gene dose turns out to be important for
extent of NCC migration (Huang et al., 1998). In mice, deletion of Cx43 results in neonatal death from pulmonary outflow obstruction (Reaume et al., 1995), whereas overexpression of Cx43 causes heart and neural tube defects (Ewart et al., 1997).
2. Aims of the study

Connexins were shown to play an important role in mature Schwann cells. In myelin-forming Schwann cells, mutations or deletion of Cx32 have been discovered to lead to CMTX disease in humans. Although expression of other connexins (Cx43, Cx29, Cx26, and Cx46 after nerve injury) has been demonstrated as well, the presence of these connexins can obviously not compensate for the deficit of Cx32. Schwann cell lineage, processing different developmental stages, differentiates into mature Schwann cells, it is therefore of interest to investigate the expression of these connexins during embryonic and postnatal development as well as to study their functions during Schwann cell development. In this study, we first investigated the temporal and spatial expression of these connexins during Schwann cell development. Secondly, in view of the distinct spatial expression pattern of Cx29, we examined the regulatory mechanisms of Cx29 expression, therefore, to elucidate the potential role of Cx29 in Schwann cell genesis as well as the lineage progression between dorsal and ventral roots of spinal nerves. Thirdly, given the predominant expression of Cx43 in NCC, effect of Cx43 on NCC migration has been investigated.
3. Materials and Methods

3.1 Animals

C57/BL6 wild type mice (Charles River Laboratories, Sulzfeld, Germany) and Cx29LacZ transgenic mice of the same strain were used in the experiments.

Cx29LacZ transgenic mice were generated and kindly provided by Prof. Dr. K. Willecke and J. Eiberger (Institute of Molecular Genetics, University of Bonn, Germany). Transgenic animals were obtained by replacing the Cx29 coding sequence by a β-galactosidase reporter gene driven by the endogenous Cx29 promoter (Fig. 4). A colony of homozygous Cx29LacZ mice was established from breeding heterozygous mice. Homozygosis was proven by genotyping the total DNA extracted from tail biopsy.

Homozygous adult wild type (WT) and transgenic (TG) mice were timemated; the day of the vaginal plug was taken as embryonic day (E) 0.5. Adult mice were killed by asphyxiation in CO₂ and decapitated. For pregnant mice, embryos at E9.5, E12, E14, and E16 were dissected from the uterus of these mice. Sciatic nerves and brachial plexus were dissected from postnatal and adult mice.

All animals were treated in accordance with the German animal protection law.
The Cx29 coding sequence of the wild type allele was replaced by the LacZ gene under the endogenous Cx29 promotor (Eiberger et al., unpublished data).

### 3.2 Substratum for cell culture

Cultured cells were grown on glass coverslips or on Petri dishes coated with different substrates. Glass coverslips (Glaswarenfabrik Karl Hecht KG, Sondheim, Germany) were washed in 95% ethanol containing 0.7% HCl under agitation overnight. After being washed three times in distilled water, coverslips were covered with 0.5 mg/ml poly-L-lysine (PLL, Sigma, Steinheim, Germany) in 10 mM Tris buffer, and incubated under agitation at room temperature for 1 hr. They were further washed in sterile distilled water for three times. Petri dishes (Becton Dickinson, Heidelberg,
Germany) were topped with 2 ml PLL (same concentration as for coverslips) per dish and incubated for 1 hr, then washed three times using sterile distilled water. After drying the coverslips/Petri dishes, they were stored under dessicated conditions until used for further coating. Laminin (LN, Sigma, Steinheim, Germany) was applied at a concentration of 10 μg/ml in Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Karlsruhe, Germany) in 30 μl drops and Fibronectin (FN, Sigma, Steinheim, Germany) was applied at a concentration of 25 μg/ml in L-15 Leibovitz (L-15, Gibco, Karlsruhe, Germany), and left on the PLL-coated coverslips or Petri dishes at room temperature for 40-60 min. After aspirating the LN or FN solution completely, cells were plated before the substrate could dry.

3.3 Media

3.3.1 Defined supplemented medium (DM)

The defined supplemented medium modified from Jessen et al. (1995) and Woodhoo et al. (2003), was used for NCC, Schwann cell precursor and embryonic immature Schwann cell cultures. A 1:1 mixture of DMEM and Ham's Nutrient Mixture F12 (Ham’s F12, Gibco, Karlsruhe, Germany) was supplemented with 100 μg/ml transferrin, 60 μg/ml progesterone, 16 μg/ml putrescine, 0.4 μg/ml tryroxine, 10.1 ng/ml triiodothyronine, 38 ng/ml dexamethasone, 16 ng/ml selenium, 0.3 mg/ml BSA, 2 mM L-glutamine (all from Sigma, Steinheim, Germany) and 100 IU/ml penicillin/streptomycin (PAA Laboratories, Linz, Austria). When the
medium was used for NCC culture, 0.5 mg/ml glucose (Merck, Darmstadt, Germany) was added, double concentrations of thyroxine and triiodothyronine were used, and dexamethasone was omitted from the medium.

### 3.3.2 Simple defined Medium (SM)

Simple defined medium was used for neonatal Schwann cell cultures according to Meier et al. (1999). Dulbecco’s Modified Eagle Medium and Ham’s F12 was mixed in 1:1 ratio and supplemented with 350 µg/ml bovine serum albumin (BSA, Sigma, Steinheim, Germany) and 100 IU/ml penicillin/streptomycin.

### 3.3.3 Hybridoma medium

Hybridoma medium contained RPMI 1640 medium (PAA Laboratories, Pasching, Germany), supplemented with 10% horse serum (Gibco, Karlsruhe, Germany), 1% sodium pyruvate (PAA Laboratories, Pasching, Germany), 1% non-essential amino acid (NEA, PAA Laboratories, Pasching, Germany), 1% L-glutamate, 100 IU/ml penicillin/streptomycin and 0.1 M β-mercaptoethanol (Sigma, Steinheim, Germany).

### 3.3.4 Astrocyte medium

Astrocyte culture medium consisted of DMEM containing 10% fetal calf serum (Gibco, Karsruhe, Germany), 1% NEA, 100 IU/ml penicillin/streptomycin and 2 mM L-glutamate.
3.4 Neural tube explantation and neural crest cell culture

The caudal trunk neural tubes were dissected from 9.5 day-old mouse embryos and dissociated in 0.1% collagenase (Cell Systems, St. Katharinen, Germany) in L-15 at 37°C for 40 minutes. After carefully removing the connective tissues and somites, neural tubes were explanted onto PLL-FN-coated 12 mm coverslips or 35 mm Petri dish for 1-1.5 hrs, then topped up with 380 µl (coverslips) or 2 ml (35 mm dishes) DM containing 10 ng/ml β-NRG (R&D systems, Abingdon, UK), 3 ng/ml FGF-2 (Peprotech, London, UK), 100 ng/ml IGF-1 (R&D systems, Abingdon, UK), 10⁻⁹ M insulin (Sigma, Steinheim, Germany), and 1 mM N-acetylcysteine (NAC, Merck, Darmstadt, Germany). For expression studies, the tubes were removed from the migrating NCC after 24 hrs, whereas for NCC migration experiments, neural tubes remained in the cultures. Some cultures were maintained for another 24-48 hrs, half of the medium was replaced every 24 hrs to maintain the same concentration of growth factors over the whole culture period. Application of additional factors, which were applied to investigate their effects on migration or differentiation, is stated in the results section.

3.5 Schwann cell precursor and embryonic immature Schwann cell cultures

Sciatic nerves were dissected from E12 or E16 mouse embryos, respectively, dissociated in an enzyme cocktail containing 2 mg/ml
collagenase D (Roche, Mannheim, Germany), 1.2 mg/ml hyaluronidase (Sigma-Aldrich, Steinheim, Germany) and 0.5 mg/ml soybean trypsin inhibitor (Sigma-Aldrich, Steinheim, Germany) at 37°C for 60-90 min. The enzyme reaction was stopped by adding 8 ml of DM, followed by centrifugation of the cell suspension at 1000 rpm for 10 minutes. The pellet was resuspended in DM and cells were plated at a concentration of 1000 cells/10µl on PLL-LN-coated 12 mm coverslips. After incubation at 37°C for 2 to 3 hrs, 390 µl of DM containing 10 ng/ml β-NRG and 3 ng/ml FGF-2 were added, and cells were cultured for 24 hrs.

3.6 Postnatal Schwann cell culture and immunopanning

The protocol for culture of postnatal Schwann cells was adopted from Meier et al. (1999). Neonatal mice were decapitated and sciatic nerves and brachial plexus were dissected. Upon removing of the connective tissues like the epineurium, nerves were digested in an enzyme cocktail at 37°C for 75 min. Ten ml of SM were added to end the enzyme reaction, the suspension was then centrifuged, and the cell pellet was resuspended in 6 ml SM, and filtered through a 100 µm pore size nylon mesh (neoLab, Heidelberg, Germany). Negative immunopanning of Schwann cells was performed using pan-Thy 1 antibodies derived from a T24/31 cell line (Salk Institute for Biological Research, San Diego, USA). The cell suspension was transferred to a pan-Thy 1 antibody pre-coated 90-mm² non-cell culture Petri dish (Becton Dickinson, Heidelberg, Germany) and
incubated at 37°C for 10 minutes. After shaking vigorously, the cells were further incubated for 10 minutes, the suspension containing the non-adherent cells was then moved to a second Thy 1 antibody pre-coated dish and incubated at 37°C for another 10 minutes. After the suspension was collected, centrifuged at 1000 rpm for 10 minutes, cells were resuspended in SM, and were plated onto PLL-LN-coated 12 mm glass coverslips in a drop of 2000 cells per 20µl, and topped up with 380 µl SM after incubating for 2-3 hours.

3.7 Astrocyte culture

Primary astrocyte cultures were prepared from brains of postnatal (P0–P2) Wistar rats according to Dermietzel et al. (1991). The hemispheres were dissected from rat pups. Upon removing the meninges and the choroid plexus, hemispheres were digested with 0.1% trypsin (Invitrogen, Karlsruhe, Germany) in phosphate-buffered saline (PBS) at 37°C for 30 minutes. The tissue suspension was then washed in 20 ml PBS and centrifuged at 500 x g for 7 min. The tissue pellet was then incubated in PBS containing 1% DNase I (from bovine pancreas; Serva, Heidelberg, Germany) at room temperature for 5 min. Enzyme activity was stopped by adding 20 ml DMEM containing 10% FCS and 1% penicillin/streptomycin, and the suspension was centrifuged at 200 x g for 5 min. The pellet was then resuspended in astrocyte medium, and passaged through a 60-µm pore size nylon mesh (neoLab, Heidelberg, Germany). Cells were then plated in
cell culture flask (Becton Dickinson, Heidelberg, Germany). When confluence was achieved after 4–5 days, astrocytes were trypsinozed (0.1% trypsin-EDTA in PBS) and passaged onto PLL-coated 12 mm glass coverslips at a density of 6,000 cells per coverslip.

### 3.8 Reverse transcription polymerase chain reaction (RT-PCR)

Ribonucleic acid (RNA) was extracted from cultured cells at different Schwann cell developmental stages using the absolutely RNA™ microprep kit (Stratagene, CA, USA). Total RNA from various mouse tissues was isolated using the TRIzol® reagent according to the instructions provided by the manufacturer (GibcoBRL, Eggenstein, Germany). Aliquots of 2 µg RNA were reverse transcribed to synthesize cDNA using SuperScript™ II RNase reverse transcriptase (Invitrogen, Karlsruhe, Germany). Various primers (Table 2), corresponding to different coding regions of connexin genes were used to amplify the corresponding mRNAs. Polymerase chain reaction was performed on 1-2 µl cDNA, starting with an initial denaturation step of 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 1 min, annealing at 52-60 °C for 30 sec, and elongation at 72°C for 30 sec. Samples included positive controls using cDNAs derived from various adult mouse tissue as well as negative controls, omitting the cDNA. Products were visualized on ethidium bromide-stained 1-2% agarose gels and photographically documented.
<table>
<thead>
<tr>
<th>Primer</th>
<th>sequence</th>
<th>length of amplicons</th>
<th>annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26</td>
<td>DSP5'-AGACTGGTCTTTTGGACTTTTC-3’ USP5'-AGAAGGTCGTCGATCGAAGGG-3’</td>
<td>309bp</td>
<td>55°C</td>
</tr>
<tr>
<td>Cx29</td>
<td>DSP5'-TCAAAATGGCTTCTTTTGCCTC-3’ USP5'-CTTGGAGCTTTGGCTTTTGGG-3’</td>
<td>154bp</td>
<td>55°C</td>
</tr>
<tr>
<td>Cx30</td>
<td>DSP5'-AAGTGCTTTAATGGGAAACT-3’ USP5'-AGACATGAAACTGCGCCAGAAG-3’</td>
<td>498bp</td>
<td>53°C</td>
</tr>
<tr>
<td>Cx31*</td>
<td>DSP5'-AGGACCTGGTCAAATGG-3’ USP5'-ATCATGCGGAGCATCAAACG-3’</td>
<td>186bp</td>
<td>49°C</td>
</tr>
<tr>
<td>Cx32*</td>
<td>DSP5'-GCAGGGCTGAGCATCGGTC-3’ USP5'-GCTCACAACACAACACATAGAA-3’</td>
<td>563bp</td>
<td>51°C</td>
</tr>
<tr>
<td>Cx36*</td>
<td>DSP5'-AAGCGGGAGATACCTTCCTGTC-3’ USP5'-TTAAAGAGCTGCACTCCACATCCATC-3’</td>
<td>82bp</td>
<td>60°C</td>
</tr>
<tr>
<td>Cx32</td>
<td>DSP5'-GCAGGGCTGAGCATCGGTC-3’ USP5'-GCTCACAACACAACACATAGAA-3’</td>
<td>563bp</td>
<td>51°C</td>
</tr>
<tr>
<td>Cx36*</td>
<td>DSP5'-AAGCGGGAGATACCTTCCTGTC-3’ USP5'-TTAAAGAGCTGCACTCCACATCCATC-3’</td>
<td>82bp</td>
<td>60°C</td>
</tr>
<tr>
<td>Cx32</td>
<td>DSP5'-GCAGGGCTGAGCATCGGTC-3’ USP5'-GCTCACAACACAACACATAGAA-3’</td>
<td>563bp</td>
<td>51°C</td>
</tr>
<tr>
<td>Cx36*</td>
<td>DSP5'-AAGCGGGAGATACCTTCCTGTC-3’ USP5'-TTAAAGAGCTGCACTCCACATCCATC-3’</td>
<td>82bp</td>
<td>60°C</td>
</tr>
<tr>
<td>Cx43</td>
<td>DSP5'-AATCCTCAGGTCATCAGG-3’ USP5'-GCGCAG AGCAAATCGAA-3’</td>
<td>206bp</td>
<td>55°C</td>
</tr>
<tr>
<td>Cx45</td>
<td>DSP5'-TAAATCCAGACGGAGGTCTTC-3’ USP5'-ATGCTTCACTTATGGTTGG-3’</td>
<td>443bp</td>
<td>57°C</td>
</tr>
<tr>
<td>Cx46*</td>
<td>DSP5'-GGGTCCAGGAGACCAACCG-3’ USP5'-GGAAAGGCCACAGGGTTTCCTGG-3’</td>
<td>332bp</td>
<td>58°C</td>
</tr>
<tr>
<td>Cx47*</td>
<td>DSP5'-GGAGATGACCACTATCTGGAAGACC-3’ USP5'-TCCAAACCTTCGGCAGACCG-3’</td>
<td>111bp</td>
<td>60°C</td>
</tr>
<tr>
<td>Cx29#</td>
<td>DSP5'-ATAGGGCCAGAGCTTGGAG-3’ USP5'-TCCAAGTTCACCTCGCAACACCG-3’</td>
<td>555bp</td>
<td>58°C</td>
</tr>
<tr>
<td>LacZ</td>
<td>DSP5'-CTCTTTGGCTATTACCGACAGG-3’ USP5'-TGATGTTTGGGATCAAATTTTGGG-3’</td>
<td>394bp</td>
<td></td>
</tr>
<tr>
<td>18S*</td>
<td>DSP5'-CGAAACCTCCGACTTTTGTTCTC-3’ USP5'-GAGGTGAAATTCTTGGACG-3’</td>
<td>93bp</td>
<td>60°C</td>
</tr>
</tbody>
</table>

Table 2. List of Primers

DSP: downstream primer; USP: upstream primer. Primers indicated with * were provided by Dr. G. Zoidl; primers of Cx32 indicated with ※ were provided by Ms. M. Kremer; primers of Cx29LacZ indicated with # were provided by Prof. Dr. K. Willecke.

3.9 Genotyping and PCR

Tail biopsies (c a. 1 cm pieces) were incubated in 450 µl Laird buffer (0.1 M Tris, 5 mM EDTA, 0.2 M NaCl, and 10% SDS) with 0.25 mg/ml Proteinase K (Roche, Mannheim, Germany) at 55°C overnight. Eppendorf tubes were inversed, cooled down to 20°C, and centrifuged at 13,000 rpm
for 5 min. Supernatants were then transferred to a clean tube, 500 µl isopropanol were added to each tube, and samples were centrifuged at 13,000 rpm for 10 min. Deoxyribonucleic acid (DNA) pellets were washed with 1 ml 70% ethanol, vortexed briefly, and centrifuged for another 10 min. Pellets were then air dried for 20 min and dissolved in 50 µl 10 mM Tris (pH 7.6). Polymerase chain reaction was performed starting with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at 58 °C for 30 sec, and elongation at 72°C for 30 sec.

Figure 5. PCR analysis to genotype Cx29LacZ transgenic mice
DNA was extracted from tail biopsies; genotypes of the mice was analyzed by PCR. Samples from wild type animals revealed a band at 555bp, from transgenic tissues at 394bp, and DNA from heterozygous animals resulted in both amplification products.

3.10 Immunofluorescence staining (IF)
Immunofluorescence staining was performed on teased sciatic nerves and cultured cells. For teased nerve preparations, sciatic nerves were dissected, the epineuria were removed, and axon bundles were teased onto Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and dried for 30 min. Cells on coverslips, and teased nerves were fixed in absolute ethanol at
-20°C for 20 min, or in 4% paraformaldehyde (PFA) at room temperature for 10 min, and then permeated in 100% methanol for 10 min (for p75NTR-R and S100β staining), and subsequently washed in PBS. After blocking with 10% normal goat serum (NGS) containing 0.1% Triton in PBS (blocking solution) for 1 hr, various primary antibodies (Table 3) were diluted in blocking solution and incubated at 4°C overnight. After washing in PBS, non-specific binding sites were blocked in 2 mg/ml bovine serum album (BSA, PAA Laboratories, Linz, Austria) in PBS for 30 minutes. Alexa-488/568-conjugated secondary antibodies were used at a dilution of 1:2,000 and incubation was performed at room temperature for 2 hrs. After washing in PBS, coverslips were mounted in Prolong® Antifade medium (Molecular Probes, Leiden, NL) onto glass slides. Immunofluorescence was documented by either fluorescence microscopy (Zeiss, Göttingen, Germany), or by confocal laser scanning microscopy (Zeiss, LSM 510 inverted confocal microscope, Göttingen, Germany).

In some experiments, double staining was carried out using two primary antibodies. Immunocytochemistry was either performed simultaneously with both antibodies when a combination of monoclonal and polyclonal antibodies as well as the same fixation protocol was used, or successively, when two antibodies of the same species or different fixation protocols were used.
3.11 Immunohistochemistry (IHC)

Immunohistochemistry was performed on paraffin-embedded or cryoconserved tissues.

For paraffin embedding, embryos were fixed in 4% PFA overnight, washed three times in PBS and stored in 70% ethanol. The embryos were then dehydrated through graded ethanol, cleared in xylene, and infiltrated with Paraplast paraffin using an autotechnikon (Leica, Germany). Paraffin sections (12-16 µm) were collected onto Superfrost Plus slides and dried overnight at 40°C. Sections were then deparaffinized in xylene, and rehydrated through descending ethanol.

For cryosection, sciatic nerves and embryos were dissected, immediately embedded in tissue freezing medium (Jung Tissue Freezing Medium, Leica, Nußloch, Germany), and frozen in liquid nitrogen. Embryos after whole mount LacZ staining were cryoprotected overnight at 4°C in 30% sucrose in PBS. After being washed in PBS for 6 hrs, embryos were imbedded in same tissue freezing medium. Twelve-µm-thick horizontal cryosections were obtained, mounted onto Superfrost Plus slides, and air dried for 1hr. Sections were then fixed in absolute ethanol at -20°C (20 minutes) or 4% PFA at room temperature (10 minutes).

After washing in PBS and blocking for 1 hour in blocking buffers, various primary antibodies (Table 3) were incubated overnight at room temperature. After washing with PBS, endogenous peroxidase activity was
blocked with 3% H₂O₂ in methanol (for cryosection tissues) or in PBS (for paraffin embedded tissues) for 10 min. Biotinylated horse anti-mouse or goat anti-rabbit secondary antibodies were incubated overnight before ABC kit (Vector Laboratories, Burlingame, USA) was incubated for four to six hours. Peroxidase reaction was performed by incubation with the substrate 0.5 mg/ml 3’-diaminobenzidinetetrahydrochloride (DAB, Sigma, Germany). Some sections were counterstained with methylgreen (Sigma). Tissues were then dehydrated through ethanol, cleared in xylene, and mounted in Entellan (Merck) and photographed using Olympus BH 2 binocular phase contrast microscope (Olympus, Japan).

3.12 Western Blotting

Sciatic nerves from adult WT and TG mice as well as NCC cultured for 3 days were homogenized with Laemmli lysis buffer (0.25 M Tris, 1.92 M Glycine and 1% SDS in aqueous solution). Fifteen µg of protein per lane was loaded onto 12.5% sodium dodecylsulfate polyacrylamide gels and electrophoresis was carried out at 100 V for 20 min, followed by another 60 min at 150 V. Proteins were subsequently blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany) at 160 mA for 1 hr. After blocking the non-specific binding sites with 0.5% blocking reagent (Roche, Mannheim, Germany) in PBS containing 0.1% Tween20 (Acros Organics, NJ, USA), the membrane was incubated with primary antibodies overnight at 4°C. Membranes were then washed with 0.1%
Tween20 in PBS, again blocked for 1 hr, followed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham PB Inc, Freiburg, Germany) diluted at 1:2000 for 1 hr. The color reaction was detected using the ECL chemiluminescence system (Amersham PB Inc) according to the instructions of the manufacturer.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Dilutions</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rabbit anti-p75NTR-R</td>
<td>1:100</td>
<td>Promega</td>
</tr>
<tr>
<td>rabbit anti-S100ß</td>
<td>1:4000, 1:8000</td>
<td>Dako</td>
</tr>
<tr>
<td>mouse anti-ß tubulin-III</td>
<td>1:500, 1:1200</td>
<td>Covance</td>
</tr>
<tr>
<td>anti-Cx29 rabbit</td>
<td>1:100, 1:300</td>
<td>Zymed</td>
</tr>
<tr>
<td>rabbit anti-Cx31</td>
<td>1:100, 1:300</td>
<td>Zymed</td>
</tr>
<tr>
<td>rabbit anti-Cx43</td>
<td>1:100</td>
<td>Zymed</td>
</tr>
<tr>
<td>mouse anti-BrdU</td>
<td>1:100</td>
<td>Chemicon</td>
</tr>
<tr>
<td>mouse ED1</td>
<td>1:100</td>
<td>Serotec, Germany</td>
</tr>
<tr>
<td>anti-Cx29 rabbit</td>
<td>1:100</td>
<td>Dr. Altevogt, Boston, USA</td>
</tr>
<tr>
<td>anti-Cx29 guinea pig</td>
<td>1:100</td>
<td>Dr. Altevogt</td>
</tr>
<tr>
<td>affinity purified polyclonal anti-Cx32/Cx43CT</td>
<td>1:100</td>
<td>Hofer, 1998</td>
</tr>
<tr>
<td>affinity purified polyclonal anti-Cx43ELII</td>
<td>1:100</td>
<td>Dr. Meier, Dr. Krause-Finkeldey and Habbes</td>
</tr>
<tr>
<td>Secondary antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>biotinylated horse anti-mouse IgG</td>
<td>1:500</td>
<td>Vector Laboratories, UK</td>
</tr>
<tr>
<td>biotinylated goat anti-rabbit IgG</td>
<td>1:1000</td>
<td>Vector</td>
</tr>
<tr>
<td>Alexa488/560-conjugated IgG</td>
<td>1:2000</td>
<td>Molecular probes, Leiden, Netherlands</td>
</tr>
</tbody>
</table>

Table 3. List of antibodies
3.13 Light microscopy and semithin sections

Tissue preparation for histology was carried out as described by Petrasch-Parwez et al. (2004). Pregnant mice were deeply anesthetized using Narcoren (Ketolar, 50 mg per 100 g body weight) and transcardially perfused with a solution of 2.5% glutaraldehyde and 1.5% formalin in 0.1 M sodium phosphate buffer (pH 7.4) at 38°C for 30 min. Fixation was followed by a ten-minute perfusion with 0.15 M saccharose in 0.1 M phosphate buffer at room temperature. The embryos were dissected out of the uterus, postfixed in 4% osmium tetroxide for 2 hrs, washed in PBS, dehydrated in ascending concentrations of ethanol, and embedded in Araldite (Serva, Heidelberg, Germany). Embryos were transversely cut and prepared for semithin sectioning. Cross-sections of 0.75 µm thickness were cut using a Leica Ultracut UCT ultramicrotome (Leica, Germany). Semithin sections were stained with 1% Toluidine blue and 30% saccharose, pH 9.2, and photodocumented using an Olympus BH 2 binocular phase contrast microscope.

3.14 LacZ staining

Beta-galactosidase staining was performed on cells and tissues from Cx29LacZ TG mice. Embryos at E9.5 were stained whole mount, while staining of E12 and E16 embryos was performed in bisection. Embryos were fixed in 4% PFA in 0.1M PBS (Ca^{2+}/Mg^{2+} free, Cambrex, Belgium) containing 2 mM MgCl₂ and 5 mM EGTA at 4°C for 1-3 hrs; cells on
coverslips were fixed for 15 minutes at room temperature. After washing with 0.1 M PBS containing 2 mM MgCl₂, 0.01% sodium doxycholate (Riedel-Dehaen, Germany) and 0.02% Nonidet P-40 (Roche), tissues were stained with 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) in same washing buffer at 37°C for 16-24 hrs. Embryos were then stored in 70% ethanol, photographed by analySIS (Soft Imaging System, Germany) or embedded for cryosection. Cells on coverslips were mounted on glass slides with Prolong® Antifade medium.

LacZ expression or β-galactosidase activity, visualized by X-gal staining, represents the expression of Cx29. LacZ staining reveals transcription and translation of the β-galactosidase gene, therefore, the localization is mainly restricted to the nuclei of the cells.

3.15 Analysis of regulation of connexin29 expression

Regulation of Cx29 expression by sonic hedgehog (Shh) was investigated by application an amino terminal peptide of Shh (R&D System) and its antagonist, cyclopamine (CP, Toronto Research Chemicals, Inc, ON, Canada). Various concentrations of CP (5-40 µM/ml) and Shh (2 µg/ml-2 ng/ml) were applied to NCC cultures obtained from TG mice and cultures were maintained for 48-72 hrs. After LacZ staining, cells were incubated with 5 µg/ml Hoechst dye (22358, Sigma) in PBS for 15 min to reveal total cell numbers.

For quantitative analyses of the effect of CP on LacZCx29 expression after
72 hrs in culture, the percentage of LacZ-positive cells of total NCC was determined in cultures treated with 7 µM of CP as well as in control cultures by counting cell numbers at 40x magnification. In each culture, three individual microscopic fields were randomly selected for counting and the mean was calculated for each culture. The means of all cultures was used to analyze the difference between CP-treated and control cultures.

3.16 Dye coupling

The coupling of NCC was examined by microinjection of Lucifer yellow dye (LY, Molecular Probes). Twenty-four-hour NCC cultures, incubated with 100 mM mimetic peptides of Cx43 for 2 hrs and 3 hrs respectively, were transferred to phenol red-free L-15 medium (Gibco). Individual NCC were microinjected with 5% wt/vol Lucifer yellow, using a patch pipette in the whole cell recording configuration (Axon 200-B patch clamp amplifier). The resistance of the recording electrodes was 2 MΩ. Dye transfer was monitored for a period of 10 min, and the LY diffused cells were photographed using a Zeiss Axioskop with FITC filter (Zeiss, Göttingen, Germany).

3.17 Analysis of neural crest cell migration

Studies of the function of Cx43 on NCC migration were investigated by different methods and quantified by determination of a migration index.
The migration index was defined as the migration area of NCC (µm²) divided by the perimeter of the neural tube (µm) according to Huang et al. (1998) using Metamorph software (Universal Imaging Corporation, PA, USA). In the migration experiments, NCC were maintained in the cultures for 72 hrs except in the experiments using antibody recognizing Cx43 extracellular loop (Cx43-ELII), here, NCC were cultured for a period of 24 hrs in the presence of the antibodies. All the data reported was obtained from three independent experiments.

3.17.1 Mimetic peptides of connexin43

After neural tubes were plated on glass coverslips, a mimetic peptide (amino acid sequence: ESAWGDEQSAFRC), which recognizes the second extracellular loop of Cx43, as well as a randomized peptide (amino acid sequence: WFSRAEEQGSDCA) were diluted at a concentration of 100 mM and cells were incubated for 72 hrs. Peptides were applied to the cultures every 4 hrs between 8am and 12pm. Cells were then fixed, followed by Hoechst dye staining to reveal total cell numbers.

3.17.2 Antibody recognizing the extracellular loop of connexin43 (Cx43-ELII)

The affinity purified antibody used in these experiments was produced using the Cx43-mimetic peptide as antigen, and therefore, recognizes the extracellular loop of Cx43. After neural tube explants were plated on glass
coverslips, cultures were incubated with Cx43-ELII antibodies at a concentration of 20 µg/ml for 24 hrs, with an application of new antibodies every 3 hrs. Cells were then fixed and stained with Hoechst dye to determine the total number of migrating NCC.

In some experiments, NCC cells were incubated with Cx43-ELII antibodies for 24 hrs, rinsed in PBS, fixed and stained with fluorescence-conjugated secondary antibody for 2 hrs and then examined by immunocytochemistry using Cx43-CT antibody for double labeling of hemi-channel presence (Cx43-ELII) and Cx43 expression (Cx43-CT).

3.17.3 Gene silence with small interfering RNA (siRNA) of connexin43

After neural tubes from E9.5 were plated on coverslips, siRNAs of Cx43 (nucleotide sequence sense: GCCCUUAGCUAUCUGUGGAAUUU; anti-sense: AUCCACGAUAGCUAAGGGCUU) and Cx36 (sequence is not provided by company) (designed by Dharmacon, USA) were transfected into the migrating NCC at the concentration of 10 nM using siLentFect™ Lipid reagent according to the instructions provided by the manufacturer (BIO-RAD, CA, USA). After cultured for 24 hrs, the media were moved, and NCC cultures were washed by L-15 medium, topped up with DM and cultured for another 48 hrs, for allowing gene knockdown processing. Non-treated and reagent-containing controls were cultured in parallel for a period of 72 hrs. Cells were then fixed, labeled with Hoechst dye.

In some experiments, cell proliferation was monitored. Neural crest cell
cultures were incubated with 10 µM 5-Bromo-2’-deoxyuridine (BrdU, Roche) for 2 hrs before fixation with absolute methanol at -20°C for 10 min. Cells were then washed in MEM-Hepes buffer and fixed in 2 M HCl at room temperature for 20 min. After being rinsed in MEM-Hepes buffer and washed in PBS, cells were neutralized in 2 M sodium borate for 10 min and incubated with monoclonal anti-BrdU antibody (Chemicon, USA) at a 1:100 dilution in PBS/0.1% triton for 1 hr. Cells were then washed in PBS and incubated with monoclonal Alexa-488 conjugated secondary antibody for 30 min and further stained with Hoechst dye. Proliferating NCC were counted at 20x magnification and percentages were statistically analyzed; in each culture two microscopic fields were randomly selected for counting.

In other experiments, after NCC were cultured for 24 hrs and 48 hrs respectively, immunostaining using the Cx43-CT antibody was performed to detect the time course of inhibition of Cx43 protein expression.

3.17.4 Oleamide

Oleamide (Sigma) is one of the non-specific chemical gap junction and hemichannel blockers. Neural crest cell cultures were incubated with 50 µM of oleamide and maintained for three days; every 24 hrs the compound in the medium was renewed using the same concentration. Cultures were stained with Hoechst dye for determination of the total number of cells.
3.18 Statistical analysis

Results were obtained from three independent experiments (unless otherwise stated) and shown as mean ± standard error of the mean (SEM). Statistical analysis of data was done by Student’s t-test and significant differences between samples was estimated at P < 0.05, and indicated by an asterisk (*) in figures.
4. Results

4.1 Connexins expression during Schwann cell development

Cultured cells maintained for a period of 24 hrs are called +1, i.e. neural crest cells cultured for 24 hrs are called E9.5+1; Schwann cell precursors cultured for 24 hrs are called E12+1; cells cultured over a period of 72 hrs are named +3, and so on.

To confirm the Schwann cell lineage progression in vitro, firstly, immunocytochemistry was performed using marker proteins for different, distinct developmental stages. As the low affinity nerve growth factor receptor p75 has been demonstrated to be expressed in migrating NCC as well as in differentiated embryonic cells of the glial lineage (Rao and Anderson., 1997; Bannerman et al., 2000; Abzhanov et al., 2003), it was used as a NCC (E9.5) and Schwann cell precursor (E12) marker in this study. The Ca²⁺-binding protein S100 was used as an immature and mature Schwann cell marker. Neural crest cells, which were cultured for 24 hrs (E9.5+1), expressed p75 predominantly in the cytoplasm (Fig. 6A). After NCC were cultured for 3 days, p75 immunosignal was detected in the cytoplasm of the cells as well, whereas the morphology had strikingly changed to a flattened shape (Fig. 6B), which resembles Schwann cell precursor morphology (Jessen and Mirsky, 1998 and 1999). When NCC were cultured for 7 days in the presence of β-NRG, the majority of them differentiated to immature Schwann cells with bi- or tri-polar morphology.
and expressed S100 (Fig. 6C). Furthermore, when sciatic nerves, which were dissected from E12, were dissociated and cells were cultured for 4 days in the presence of β-NRG, the morphology of cells changed from flattened to a bi- or tri- polar phenotype, and cytoplasmic S100 expression was also observed (Fig. 6D). This lineage progression in vitro corresponds to the studies of Schwann cell development in rat (Jessen et al., 1994, Dong et al., 1995); however, in the mouse development occurs 2 days earlier.

Figure 6. Schwann cell lineage progression in vitro

Neural tubes were dissected out of 9.5 days embryos and explanted onto coverslips for NCC culture. Within 24 hrs, a number of NCC had migrated out of neural tube. NCC were then fixed and examined by immunocytochemistry, immunosignal of polyclonal p75 antibodies was observed in the cytoplasm of E9.5+1 (A). Some NCC were maintained in the culture for 3 and 7 days respectively, in the presence of β-NRG, and examined by immunocytochemistry. After being cultured for 3 days, NCC changed to Schwann cell morphology and expressed p75 as well (B), whereas after 7 days in cultures, they expressed S100 and had converted to Schwann cell morphology (C). Direct conversion of immature Schwann cells from Schwann cell precursors was also observed when Schwann cell precursors were cultured in the presence of β-NRG for 4 days (D). Scale bar: 20 µm.
4.1.1 Connexins mRNA expression during Schwann cell development

4.1.1.1 Neural crest cells express mRNA transcripts of connexin 26, 29, 30, 43 and 45

To investigate which connexins are expressed in multipotent NCC, RT-PCR analysis was performed on cDNA obtained from total RNA extracted from NCC cultured for 24 hrs and 72 hrs. Different primers corresponding to sequences of Cx26, Cx29, Cx30, Cx31, Cx32, Cx36, Cx43, Cx45, Cx46 and Cx47 (Table 1) were used in RT-PCR for amplification of the targeted connexins. The expression of connexin transcripts in the experiments was proven by consistent RT-PCR amplicons at least from 3 different samples. Transcripts of Cx26, Cx29, Cx43 and Cx45 were highly expressed in E9.5+1, whereas Cx30 mRNA was expressed at a very basal level. Later, in E9.5+3, the expression of Cx30 was not detectable any more, and Cx26 mRNA was reduced to basal levels. Transcripts of Cx29, Cx43 and Cx45 were still expressed at high levels in E9.5+3 (Fig. 7a), while no RT-PCR products of Cx31, Cx32, Cx46 and Cx47 could be detected in cultured NCC (Fig. 7b). The presence of Cx36 mRNA in NCC is still ambiguous, as from four different samples only one yielded an amplicon of 82bp (data not shown). Thus, RT-PCR analysis revealed that mouse trunk NCC expressed transcripts of Cx26, Cx29, Cx30, Cx43 and Cx45.
Figure 7. RT-PCR analysis of connexin mRNA expression in neural crest cells

In NCC cultured for one day (E9.5+1), expression of Cx26, Cx29, Cx43 and Cx45 transcripts was visualized at high intensity, whereas expression level of Cx30 was lower. When cultured for 3 days (E9.5+3), NCC were still expressing Cx29, Cx43 and Cx45 at high levels. Expression of Cx26 was downregulated and Cx30 mRNA was no longer present. PCR amplification of Cx31, Cx32, Cx46 and Cx47 mRNAs did not result in any product at either time point. + denotes positive control (tissues from brain for Cx26, Cx29, Cx30, Cx45, and Cx47, skin for Cx31, liver for Cx32, heart for Cx43, and lens for Cx46). - denotes negative control (omitting cDNA from samples), and 18S rRNA primers were used for standardizing the total amount of mRNA.

4.1.1.2 Expression of connexin mRNAs in the Schwann cell lineage

Connexin mRNA expression was further investigated at various stages of the Schwann cell lineage, i.e. in Schwann cell precursors (E12), immature Schwann cells (E16), as well as early postnatal stages, P1 (non-myelin Schwann cells), P4 (initiating myelin-forming Schwann cells) and adult Schwann cells (myelinating). Cells of all stages were cultured for 1 day (+1). Connexin43 and Cx29 transcripts were present throughout Schwann cell development, i.e. in Schwann cell precursors, immature Schwann cells and mature Schwann cells. In contrast, Cx32 mRNA expression
commenced in embryonic immature Schwann cells at basal levels and increased strikingly postnatally (Fig. 8). Transcripts of Cx26, Cx30, Cx31, Cx46 and Cx47 were not detected at any developmental stage (data not shown). Data on Cx45 mRNA expression were inconsistent in Schwann cell precursors, only one out of five samples yielded an amplicon, and Cx45 mRNA was not detectable at later stages (data not shown). Therefore, mRNAs of Cx29 and Cx43 were expressed in the cells of the defined Schwann cell lineage (from Schwann cell precursor, E12, onwards), whereas transcripts of Cx32 commenced in embryonic immature Schwann cells (E16).

![Figure 8. mRNA expression of connexin 43, 29 and 32 in the Schwann cell lineage](image)

**Figure 8. mRNA expression of connexin 43, 29 and 32 in the Schwann cell lineage**

Connexin29 and Cx43 transcripts were amplified from cDNAs at different Schwann cell developmental stages, whereas Cx32 mRNA presence was detected in discrete amounts in embryonic immature Schwann cells and increased to high levels postnatally.

### 4.1.2 Connexin protein expression during Schwann cell development

*in vitro*

In these experiments, NCC and Schwann cell precursors have been
defined as early Schwann cell lineage, whereas Schwann cells from E16 onwards are referred to late Schwann cell lineage.

**4.1.2.1 Connexin expression in the early Schwann cell lineage**

Protein expression of connexins in cells of the early Schwann cell lineage was investigated by immunocytochemistry focusing on the detection of Cx43 and Cx29. The p75 protein, used as a marker for NCC and Schwann cell precursors, was expressed in the cytoplasm at E9.5+1 (Fig. 9A), E9.5+3 (Fig. 9D) and E12+1 (Fig. 9G). The immunosignal of Cx43 was detected predominantly in the cell membranes at E9.5+1 (Fig. 9B). After three days in culture (E9.5+3), typical punctate pattern of Cx43 immunolabeling was observed, however, the signal intensity was reduced compared to the cells cultured for one day. In addition, diffused immunopositivity was detected in the cytoplasm, as well as some clustered fluorescent signals in the cytoplasm, possibly reflecting staining of the Golgi apparatus (Fig. 9E). Schwann cell precursors (E12+1), showing flattened morphology, and were also labeled with Cx43 in the cytoplasm. However, the immunosignal was detected at low level (Fig. 9H).

Expression of Cx29 protein *in vitro* was also examined by immunocytochemistry using various polyclonal antibodies against Cx29. There was no immunoreactivity of the Cx29 antibodies in NCC cultured for one day (Fig. 9C). Expression of Cx29 protein commenced in NCC after three
days in culture (Fig. 9F), which corresponds to the Schwann cell precursor stage at E12 in vivo. Expression of Cx29 was also shown in Schwann cell precursors dissected from E12 nerves and cultured for one day (Fig. 9I). The immunofluorescence was also predominantly localized in the cytoplasm.

Figure 9. Protein expression of connexin 43 and 29 in early Schwann cell lineage
A-C: E9.5+1; D-F: E9.5+3; G-I: E12+1. p75 was used as a marker of early stages of the cell lineage. Scale bar: 20 µm.
4.1.2.2 Connexin expression in the late Schwann cell lineage

Schwann cell precursors convert to immature Schwann cells, which express the marker protein S100, a Ca\(^{2+}\)-binding protein, and change to a bi- or tri-polar morphology in culture. In Figure 10, intense immunosignal of the S100 antibody was detected in the cytoplasm of the embryonic immature Schwann cells (Fig. 10A) as well as of postnatal Schwann cells (Fig. 10E, I). Connexin43 protein expression was detected in embryonic immature Schwann cells in the cytoplasm (Fig. 10B). At postnatal day 1, Schwann cells expressed Cx43 at basal levels (Fig. 10F), whereas at P4, intensity of the Cx43 immunosignal slightly increased (Fig. 10J).

Cytoplasmic immunofluorescence of Cx29 continued to be observed at high intensity in immature Schwann cells at three different time points during development (Fig. 10C, G, K).

To investigate protein expression of Cx32, immunofluorescence staining was performed on Schwann cells from E16, P1 and P4, which were each cultured for one day (+1). Although RT-PCR analysis had revealed expression of Cx32 mRNA in embryonic immature Schwann cells, there was no Cx32 immunosignal detected in E16 and P1 cells (Fig. 10D, H). However, in P4 Schwann cells, the presence of Cx32 protein was detected in the cytoplasm weakly (Fig. 10L).
Figure 10. Immunostaining of connexin 43, 29 and 32 in immature Schwann cells
S100 marks immature Schwann cells (A, E and I). Expression of Cx43 and Cx29 was
detected in immature Schwann cells at all three time points of immature Schwann
cells, i.e. (B+C) E16+1, (F+G) P1+1, and (J+K) P4+1, whereas the onset of Cx32
expression emerged on postnatal day 4 only. A-D: E16+1; E-H: P1+1; I-L: P4+1.
Scale bar: 20 µm.

4.1.2.3 Western blot analysis of connexin expression in neural crest
cells
We semi-quantitatively analyzed connexin expression in NCC cultured
for 3 days (E9.5+3). Western blot yielded a strong band for the marker
protein p75 at 75 kDa in NCC sample. Two bands at around 43 kDa were
detected using Cx43 antibody, pointing to different phosphorylation
forms of Cx43, whereas two lower bands at around 32 kDa were also
faintly detected. Using a Cx29 antibody, multiple bands were observed, however, a predominant band around 29 kDa was present in the sample obtained from E9.5+3 (Fig. 11). Western blot analysis was exclusively performed on E9.5+3 cell cultures. Because the number of the cells obtained from other embryonic developmental stages was very low, Western blot analysis has not been applied to those stages.

![Western blot image](image)

**Figure 11. Immunoblot analysis of connexin expression in neural crest cells**

Neural crest cells cultured for 3 days were analyzed by Western blot. p75 antibody yielded a band around 75 kDa, two bands at 43 kDa were revealed by the Cx43 antibody. The Cx29 antibody yielded several bands from same tissue; however, a band at around 29 kDa was prominent. Beta-actin was used for standardizing the protein amount in cell samples.

To summarize this part of the study, a successive expression pattern of multiple connexins has been revealed for Schwann cell development *in vitro*. Connexin43 commenced at NCC stage (E9.5); the onset of Cx29 expression coincided with the differentiation of NCC into Schwann cell precursors (E12), whereas Cx32 appeared when the myelination initiated postnatally (P4).
4.1.2.4 Specificity of connexin 29 antibodies

To exclude non-specific binding or crossreactivity of Cx29 antibodies, immunohistochemical staining and Western blot analysis was performed on WT and Cx29-deficient (LacZ TG) animals. Three polyclonal antibodies, which recognize the C-terminal loop of Cx29, were investigated (see Materials and Methods); anti-Cx29 guinea pig, and anti-Cx29 rabbit were kindly provided by Dr. Altevogt (Harvard Medical School, Boston, USA); a polyclonal anti-Cx29 antibody was available and purchased from Zymed. Immunohistochemically, an intensive fluorescent signal was detected on cryosections from WT as well as from Cx29-deficient (KO) sciatic nerves using anti-Cx29 guinea pig antibody (Fig. 12a A, D). The anti-Cx29 rabbit (Altevogt) antibody also showed fluorescence, though the signal was weaker in KO tissue than in WT (Fig. 12a B, E). Using the commercial polyclonal anti-Cx29 rabbit (Zymed) antibody, a predominant signal was present around the nerve fibers in WT tissue, however, the immunosignal was not detected in transgenic tissue (Fig. 12a C, F), pointing to a specific labeling with this antibody only.

Western blot analysis confirmed the specificity of the anti-Cx29 rabbit (Zymed) antibody, which yielded a dark band around 29 kDa in the tissue from WT animal that was not detected in the KO sample. In addition, an upper band of around 70 kDa was present in both samples suggesting crossreactivity. Although the anti-Cx29 rabbit (Altevogt) antibody
resulted in a similar band at 29 kDa in WT samples, this band was also present in sciatic nerve sample from KO mice (Fig. 12b). The antibody produced from guinea pig did not work in Western blot (data not shown).

Figure 12. Immunofluorescence and Western blot analysis of connexin29 antibodies specificity
(a) Anti-Cx29 guinea pig revealed a high cross reactivity in the Cx29KO sciatic nerves. The anti-Cx29 rabbit antibody showed low immunosignal in the knock out animal as well. Using the polyclonal antibody from Zymed, no immunofluorescence was detected in the knock out tissue. (b) Immunoblotting supported the observation from immunohistochemistry that rabbit anti-Cx29 antibodies (gift by Dr. Altevogt) detected a band at 29 kDa as well as other multiple bands which identically displayed both in the wild type and knock out tissues. In knock out tissue; the band at around 29 kDa was absent when using the commercially available polyclonal Cx29 antibody. +/+ indicates tissues obtained from wild type animals and +/- indicates tissues from Cx29LacZ knockout mice. Scale bar in a: 10 µm.

Therefore, all immunochemical data of Cx29 expression reported in this thesis were obtained using anti-Cx29 rabbit (Zymed) antibody.
4.1.2.5 LacZ reporter gene expression in transgenic mice during Schwann cell development

Connexin29 expression was further investigated in Cx29LacZ TG mice in which the Cx29 coding region had been replaced by a lacZ reporter gene driven by the endogenous Cx29 promoter (see Materials and Methods; Fig. 4).

LacZ staining was performed on cells of different stages in the Schwann cell lineage obtained from TG mice. In general, LacZ staining was shown positively in the nuclei of the majority of cells (except for E9.5+1), whereas a low percentage of cells failed to display LacZ signal, and some cytoplasmic localization could also be observed (Fig. 13). In NCC cultured for 3 days (Fig. 13B) (corresponding to Schwann cell precursors in vivo), high levels of β-galactosidase activity were observed, however, in Schwann cell precursors as well as in immature Schwann cells, the LacZ expression was revealed at lower level (Fig. 13C-F).
Figure 13. LacZCx29 expression during Schwann cell development *in vitro*

Beta-galactosidase activity under the endogenous Cx29 promotor was absent in NCC cultured for 1 day (A), whereas LacZ positivity *in vitro* commenced at E9.5+3 (B) which corresponds to the Schwann cell precursor stage *in vivo* (C), LacZ expression was further detected in immature Schwann cells at three different stages of development (D-F). Scale bar: 20 µm.

**4.1.2.6 Differentiation of neural crest cells to a neuronal fate does not trigger expression of connexin36**

Reverse transcription-PCR analysis did not consistently amplify Cx36 mRNA in cultured NCC in the presence of β-NRG. Given that Cx36 is a neuronal gap junction protein, it is possible that in the glia differentiation medium (in the presence of β-NRG), expression of Cx36 cannot be promoted. It was therefore investigated, if differentiation of NCC by bone morphogenetic protein-2 (BMP-2) might induce neuronal differentiation and, furthermore, if the derivatives would express Cx36 *in vitro*.

After 72 hrs in the presence of BMP-2 (10 ng/ml), intense immunosignal for the neuronal marker β-III tubulin (TuJ-1) was observed in the cytoplasm of a certain proportion of NCC, which also labeled elongated axon and dendrites. Although neuronal differentiation was achieved, Cx36 expression was not detected in these cultures (Fig 14).
Figure 14. Immunofluorescent detection of neural crest cell neuronal differentiation and connexin expression

(A) Hoechst staining revealed the total number of NCC in culture. (B) Monoclonal TuJ-1 antibody labeled cytoplasm as well as the axons and dendrites of differentiated neurons. (C) Merge of A and B showed that only a certain percentage of NCC displayed neuronal morphology and was positively stained with the neuronal marker. (D) Immunosignal of Cx36 antibody was absent in all cells of the same culture. Scale bar: 20 µm.

4.1.3 Spatial expression of connexin29 in vivo

As in vitro data have demonstrated the expression of Cx29 protein from the Schwann cell precursor stage onwards, the localization of Cx29 was investigated in vivo using WT and Cx29LacZ TG mice from E12 onwards.
Figure 15. Peripheral nerves in the mouse embryonic trunk visualized by immunohistochemistry using TuJ-1 antibody

Transverse paraffin sections through thoracic levels at E12 (A) and E16 (B). White arrows point to peripheral nerves and the ventral roots of spinal nerves, white arrowheads indicate the dorsal roots of spinal nerves; black arrowheads indicate the DRG, black arrows in B point to a sympathetic trunk (up) and the vagal nerve (low). Scale bar: 500 µm.

During development, as soon as neurites grow out, the prospective Schwann cells (the differentiating NCC or the Schwann cell precursors) migrate to them, and thus become incorporated into embryonic nerves (Bhattacharyya et al., 1994; Dong et al., 1994). To investigate the spatial distribution of Cx29 in peripheral glia, nerve fibers were visualized using a monoclonal TuJ-1 antibody which labels neurons and their axons (Dong et al., 1999; Maro et al., 2004). At E12, the contour of the PNS was already formed and peripheral nerves were observed to extend into the forelimb (Fig. 15A). At E16, the luminal diameter of the central canal of the spinal cord has diminished considerably compared with that observed
at E12. In addition to the presence of spinal nerves, sympathetic trunks and vagal nerves can also be observed (Fig. 15B).

4.1.3.1 LacZCx29 expression in whole mount stained embryos during Schwann cell development

LacZ staining was performed on whole mount embryos to detect sites of Cx29 expression via β-galactosidase activity. At E9.5, when the trunk NCC migrate out of the dorsal neural tube via ventral and dorsolateral pathways, heading to their various destinations, LacZCx29 expression was not detected in the trunk, neither beside the tube (Fig. 16A, white arrow) nor between the somites (Fig. 16B, black arrow). LacZCx29 expression was restricted to the developing hindbrain, located on both sides of the developing fourth ventricle (Fig. 16B, C, white arrow). At this stage, the otic vesicles (Fig. 16, white arrowhead) and optic eminences (black arrowhead) are formed, but were devoid of β-galactosidase activity.

In the trunk of E12 embryos, peripheral nerves have already grown into the limbs, and Schwann cell precursors begin to populate the axons. At this developmental stage, LacZCx29 expression was observed along the peripheral nerves as well as at the ventral root emerging from the developing spinal cord (Fig. 17A, arrow). However, no signal was detected in the DRG (Fig. 17A, big arrowhead) or along the dorsal root (Fig. 17A, small arrowhead). In the developing brain at E12, the
pigmentation of the peripheral part of the outer retinal layer is visible. Around the pigment epithelium LacZCx29 expression was present, which is likely to be due to nerve fibers from oculomotor cranial nerve (III) (Fig. 17B, black arrowhead). Moreover, LacZCx29 expression was also observed in the trigeminal (V) and spinal accessory cranial nerves (XI) (Fig. 17B, white arrowheads) as well as in the trigeminal ganglion, the vagal ganglion and, presumably, the inferior cervical ganglion (Fig. 17B, white arrows). In addition, the region with intense β-galactosidase activity (indicated with a * in Fig. 17B) might indicate some overlapped cranial ganglia of VII, IX and X.

Figure 16. Whole mount LacZ staining at E9.5
(A) The arrow indicates the neural tube, the NCC migration pathway along the neural tube lacked LacZCx29 expression. (B) Lateral overview of an E9.5 which revealed LacZCx29 expression in the hindbrain (white arrow), white arrowhead points to an otic vesicle (also in C) and black arrowhead points to the optic vesicle which were devoid of LacZCx29 expression, black arrow points to the somites, NCC migrating between them are devoid of LacZCx29 expression as well. (C) Dorsal view of an E9.5 head, β-galactosidase activity could be clearly seen on the roof of the hindbrain (arrow) beside the fourth ventricle (black arrowhead).
When the Schwann cell lineage develops to immature Schwann cells at E16, β-galactosidase activity remained high in peripheral nerves as well as in the ventral roots of spinal nerves (Fig. 17C, arrow). In addition, at this developmental stage, LacZCx29 expression commenced within the DRG (Fig. 17C, big arrowhead) as well as in the dorsal root of spinal nerves with low abundance (Fig. 17C, small arrowhead).

**Figure 17. LacZCx29 expression at E12 and E16**

(A) E12 trunk, the arrow points to a peripheral nerve with the ventral root emerging from the spinal cord, both showing β-galactosidase activity. The big arrowhead points to a DRG (no lacZCx29 expression); the small arrowhead indicates a dorsal root entering the spinal cord, which was also devoid of LacZCx29 expression. (B) Lateral image of a head at E12. LacZCx29 expression was observed around the pigmented outer retinal layer (black arrowhead), in the cranial nerves (white arrowheads) as well as in the cranial ganglia (white arrows). (C) E16, the arrow points to a peripheral nerve and the ventral root of the spinal nerve showing LacZCx29 expression, in the DRG (big arrowhead) and the dorsal root of the spinal nerve (small arrowhead), LacZCx29 expression was present at basal levels.

**4.1.3.2 Localization of Cx29LacZ in Schwann cell precursors**

Cellular localization of Cx29 was investigated in tissues from WT animals by immunohistochemistry performed on cryosections counter-
stained with methylgreen, or in tissues from TG mice after whole mount LacZ staining. Immunohistochemistry using Cx29 antibodies (Fig. 18) showed staining of the ventral root (Fig. 18B) as well as the extending peripheral nerve (Fig. 18C) in a cross section of a WT embryo at E12, whereas no signal was detected along the dorsal root of the spinal nerve or within the DRG (Fig. 18D). LacZCx29 expression combined with immunohistochemistry using TuJ-1 antibodies excluded neuronal localization of Cx29. Given that LacZCx29 expression was observed beside the axonal fibers, which were labeled with TuJ-1 antibodies, therefore, LacZCx29 expression is likely to be in peripheral glia, i.e. Schwann cell precursor at this stage (Fig. 18E-G). However, in the dorsal root of the spinal nerve as well as within the DRG, LacZCx29 expression was not present around TuJ-1 labeled neurons or axons (Fig. 18H).

Figure 18. Localization of Cx29LacZ in Schwann cell precursors at E12
Low magnification view of transverse sections through the thoracic part of (A) a WT E12 embryo immunostained with polyclonal Cx29 antibody (Zymed), and (E) a TG
embryo stained for LacZ and double labeled with TuJ-1 antibodies. Areas labeled B-D (in A) and F-H (in E) are presented at high magnification in B to D and F to H. Connexin29LacZ expression was observed in the dorsal root of the spinal nerve (B and F), as well as in peripheral nerves (C and G), whereas along the dorsal root to the spinal cord and within the DRG, no Cx29LacZ expression was detected (D and H, arrow points to dorsal roots). Scale bar: (A and E), 200 µm; (B-D and F-H): 50 µm.

The asymmetric expression of Cx29 and LacZ in the dorsal and ventral root of the spinal nerve at E12, prompted us to examine LacZ expression in an E14 embryo to trace the onset of the LacZCx29 appearance in the dorsal roots. However, an identical expression pattern of LacZCx29 was observed showing an exclusive expression in the Schwann cell lineage in the ventral roots and the peripheral nerves, but neither in the dorsal roots, nor within the DRG (Fig.19).

Figure 19. Localization of LacZCx29 at E14

(A) Low magnification view of a transverse section through the thoracic part of a LacZCx29 TG embryo after whole mount LacZ staining. (B) At high magnification, LacZCx29 expression was visible in the ventral root. (C) Dorsal root of the spinal nerve as well as within the DRG was devoid of LacZCx29 expression. Scale bar: (A), 500 µm; (B-C): 50 µm.
4.1.3.3 Localization of Cx29LacZ in embryonic immature Schwann cells

Schwann cell precursors undergo transition to immature Schwann cells over a two-day period in vivo, i.e. E14-15. At E16, all cells have converted to immature Schwann cells (Dong et al., 1999). Therefore, E16 was considered as the day when differentiation to immature Schwann cells is completed, and the spatial expression of Cx29LacZ was further investigated at this stage. Connexin29 and LacZ expression was detected predominantly at the motor exit points from the ventral horn of the spinal cord (Fig. 20B, G, arrows) as well as along the peripheral nerves (Fig. 20C, H). Moreover, within the DRG (Fig. 20D, I) and in the dorsal roots of spinal nerves (Fig. 20E, J), the signal of Cx29 immunostaining as well as β-galactosidase activity became positive at basal levels.

Figure 20. Localization of Cx29LacZ in immature Schwann cells at E16

(A and E) Low magnification of transverse sections through cervical-thoracic part of E16 embryonic trunk. At high magnification, expression of Cx29 and LacZ was detected in the exit points of spinal nerves from the ventral horn of the spinal cord (arrow in B and G) as well as in peripheral nerves (C and H). Moreover, in dorsal
roots and within the DRG, the presence of Cx29 and LacZ commenced at basal levels (D, E, I and J). WT tissues were counterstained with methylgreen, and TG embryos were counterstained with eosin. Scale bar: (A and F), 500 μm; (B-E and G-J), 50 μm.

In addition, Cx29 immunoreaction and LacZ expression were also detected in the esophagus (Fig. 21A and E) and in the trachea (Fig. 21B and F), pointing to Cx29 expression in peripheral nerves in and around these tissues. Expression of Cx29LacZ was also detected in the trunk of the tenth cranial nerve (vagal) at high intensity (Fig. 21C and G, arrow). Moreover, expression levels of Cx29LacZ in the developing spinal cord was weak, showing a more diffuse pattern compared to the high intensity of the LacZ expression at the motor exit point (Fig. 21H, arrowhead).

**Figure 21. Expression of Cx29LacZ is detected in the nerves in other organs**

(A and E) Connexin29 and LacZ were expressed in and around the esophagus. (B and F) The immunosignal of Cx29 and LacZ expression were also detected in and around the trachea. (C and G) In the X cranial nerve, Cx29LacZ expression could also be observed at high levels (arrows). (D and H) In the developing spinal cord, Cx29 and LacZ expression were also present, albeit at lower level (arrows) compared to the intensity at motor exit point (arrowhead). Scale bar: 50 μm.
4.1.3.4 Maturation of Schwann cell precursors between dorsal root and ventral root

In this and other studies, the spinal nerves that innervate the limbs (sciatic nerves and brachial plexus) were used as a model for Schwann cell embryonic development in rat and mouse in vitro (Jessen et al., 1994, Dong et al., 1995 and 1999). These data demonstrated that cells of the Schwann cell lineage follow a defined developmental process, i.e. Schwann cell precursors are present from E12-13 in mouse. At E16 they convert to immature Schwann cells. However, our in vivo investigation on the localization of Cx29LacZ during the transition from Schwann cell precursors to immature Schwann cells raised the question about synchronization of lineage progression, particularly because of the differences in the onset of Cx29 expression between dorsal and ventral roots of spinal nerves. One possible explanation for the delayed expression of Cx29LacZ in dorsal roots could be that development of the Schwann cell lineage is generally delayed in dorsal roots. Therefore, we visualized Schwann cell maturation by immunohistochemical detection of the S100 protein, which is a marker protein of immature and mature Schwann cells, to investigate the time course of the conversion from Schwann cell precursors (E12) to embryonic immature Schwann cells (E16) in the dorsal and ventral roots of the spinal nerves.
Figure 22. Development of Schwann cell precursors to immature Schwann cells in the dorsal and ventral root of the spinal nerve

S100 protein was used as a marker for maturation of the Schwann cell lineage. (A) Low magnification of an E16 transverse section stained for LacZ and immunolabeled with S100 antibodies. (B) S100 immunoreaction in the immature Schwann cells in the ventral root of the spinal nerve (arrow) and at the motor exit point from the spinal cord overlapped with the intense β-galactosidase activity. (C) Immunosignal of S100 could be observed in the axon fibers within the DRG. (D) S100 expression was intensively present in the afferent root to the spinal cord. Scale bar: (A), 500 µm; (B-D), 50 µm.

Immunohistochemistry for S100 was performed on the cryosections after LacZ whole mount staining of the embryos. Figure 22 reveals that staining of S100 could be observed both in the dorsal and ventral root of the spinal nerve as well as within the DRG at E16. In the ventral root, S100 immunostained cells, which overlapped with LacZ expression, could be observed (Fig. 22B arrow). However, staining in the dorsal root and within the DRG displayed a linear pattern, thus resembling the
morphology of axons, presumptively, pointing to the localization of S100 immunoreaction in the axons (C, D). In addition, S100 expression was also detected in sensory neurons within the DRG, which has, together with the axonal expression of S100, been reported previously (Ichikawa et al., 1997; Albuersen et al., 1998). Therefore, detection of S100 expression in dorsal roots of spinal nerves and within the DRG can not unequivocally point to the progression of the Schwann cell lineage to immature Schwann cells in the dorsal root at E16.

4.1.3.5 Investigation of connexins other than connexin29 in the dorsal root of the spinal nerve

As described above, Schwann cell precursors in the dorsal root of the spinal nerve as well as within the DRG were devoid of Cx29. Therefore, we investigated which additional connexin(s) might compensate for Cx29, and thus, be located in the dorsal root. Previous studies in the mouse have reported that at E11.5, expression of Cx31LacZ was detected in boundary cap (BC) cells, which is a group of late migrating NCC (Jungbluth et al., 2002) and might therefore be the source of the connexin in the dorsal roots.
Figure 23. Immunohistochemical detection of connexin31 expression on paraffin embedded transverse sections and semithin sections stained by toluidine blue at the same developmental stages

(A, D, G and J) Low magnification of transverse sections through the thoracic part of E12 and E16 trunks. At high magnification, dorsal roots of spinal nerves were devoid of Cx31 labeling (B and E, arrows). In contrast, Cx31 expression was detected in the lateral horn of the spinal cord as well as in the nuclei of the neurons within the DRG (B and C, arrowheads) at E12, whereas at E16, immunosignal for Cx31 antibodies was observed in the cytoplasm of sensory neurons within the DRG as well as of motor neurons in spinal cord (E and F). Correspondingly, semithin sections revealed the
morbidity of the tissues from the same stages at higher resolution (G-L). At high magnification, arrow in H indicates a dorsal root at E12; arrow in K points to the afferent fibers which form dorsal roots of spinal nerves; arrowheads in H, I and K indicate neurons in the ventral horn of the spinal cord as well as within the DRG; (L) reveals the ventral horn of the spinal cord. Scale bar (A, G and J), 200 µm; (D), 500 µm; (B, C, E, F, H, I, K and L), 30 µm.

Immunohistochemistry was therefore performed on paraffin embedded embryos at E12 and E16 using a polyclonal Cx31 antibody. There was no immunoreaction with the Cx31 antibody in dorsal roots of spinal nerves at E12 (Fig. 23B, arrow) or at E16 (Fig. 23E, arrow). However, at E12, immunosignal for Cx31 was observed to be restricted to the nuclei of neurons within the DRG (Fig. 23C), and some scattered signals were also observed in the lateral horn of the spinal cord (Fig. 23B, arrowhead), which might indicate expression of Cx31 in the differentiating sympathetic neurons. At E16, protein expression of Cx31 was observed in the cytoplasm of neurons within the DRG (Fig. 23E, arrowhead) as well as in the motor neurons of the ventral horn of the spinal cord (Fig. 23F). Transverse semithin sections of E12 and E16 embryos revealed the morphology of the tissues at the same stages (E12 and E16) (Fig. 23). Cytoplasms of cells were darkly stained with toluidine blue, whereas nuclei were lightly stained. Sensory neurons in the DRG as well as motor neurons in the spinal cord showed a bigger nuclei size than the cells of the lateral horn of the spinal cord.
4.1.3.6 Postnatal expression of connexin29 in sciatic nerves

The expression of Cx29 was further investigated in postnatal Schwann cells of sciatic nerves. Sciatic nerves were dissected from P1 and P4 mice and were either teased onto slides or embedded for cryosections, and subsequently processed for immunohistochemistry and LacZ staining. At P1 and P4, Cx29 immunolabeling as well as LacZ staining was located predominantly around small nerve fibers (Fig. 24B, C, E, F), as observed on horizontal sections. The expression of the Schwann cell marker S100 displayed a similar distribution pattern (Fig. 22A, C), however, at a higher intensity. In teased peripheral nerves obtained from P1 and P4 mice, the immunosignal of the Cx29 antibodies and the β-galactosidase activity were distributed homogenously throughout the fibers (Fig. 24).

Taken together, the *in vivo* investigation of Cx29LacZ expression and localization during embryonic Schwann cell development revealed that Cx29LacZ was expressed in the ventral roots of the spinal nerves as well as in the peripheral nerves from E12 onwards, whereas in the dorsal roots and within the DRG, Cx29LacZ expression commenced at E16. S100 immunolabeling can not unequivocally illustrate if cell lineage progression to immature Schwann cells occurs simultaneously in the dorsal and ventral roots. Connexin31 expression was observed in neurons within the DRG as well as in the developing spinal cord, however, Cx31 immunolabeling was absent from cells of the Schwann cell lineage.
Figure 24. Connexin29 and LacZ localization in postnatal sciatic nerves

(A-F) Connexin29 and S100 immunoreaction as well as β-galactosidase activity on transverse cryosections of sciatic nerves from P1 and P4 animals. S100 and Cx29 immunosignals were localized around the axon fibers (arrowheads), whereas the nerve fibers themselves were devoid of staining (arrows). Beta-galactosidase activity also revealed expression of the LacZCx29 gene, however, due to the diffusion of the LacZ reaction product, single nerve fiber could not be well identified. (G-L) In teased nerves, the immunosignals of Cx29 as well as LacZ expression were present along the nerve bundles. Scale bar: (A-F), 30 µm; (G-L), 200 µm.
4.2 Regulation of connexin29 expression

Our *in vivo* data of the Cx29 spatial and temporal expression pattern revealed that in the Schwann cell lineage the expression of Cx29 commenced in the ventral roots of spinal nerves as well as in peripheral nerves earlier than in the dorsal roots and within the DRG, and these differences were observed between E12 and E16. One hypothesis is that extracellular signal(s), for instance factors like sonic hedgehog (Shh), which directs ventral patterning during development, might also be responsible for the regulation of ventral versus dorsal expression of Cx29. We therefore investigated the potential effect of Shh on the regulation of Cx29 expression *in vitro* using recombinant Shh peptide and a Shh antagonist, cyclopamine (CP).

4.2.1 Cyclopamine downregulates expression of LacZCx29 in neural crest cells

Cyclopamine was firstly examined for its potential toxic effect on NCC and, thus, applied at various concentrations of 7, 10, 20 and 40 µM. After 24 hrs in the presence of CP, NCC incubated with 7 and 10 µM CP as well as the cells in the control migrated out normally (Fig. 25A-C). However, at the concentration of 20 µM of CP, the NCC outgrowths from the neural tube explants were severely reduced, and the neural tubes started to dissociate (Fig. 25D). At a concentration of 40 µM of CP, neural tubes were completely dissociated and very few NCC were
observed (Fig. 25E). After 48 hrs, reduction of the NCC migration in the presence of 7 µM of CP could be observed (Fig. 23G). In the presence of CP at 10 µM, NCC migration was dramatically blocked and a certain percentage of cells was observed in forms of clusters floating above the living cells with round and shrinking appearance, which indicated that these cells were undergoing cell death (Fig. 25H, arrowhead). At higher concentrations, no NCC was observed in phase contrast (Fig. 25I, J).

After NCC cultures were maintained in the presence of CP for 72 hrs, the survival of cells was investigated by LacZ staining as well as immunofluorescence using the p75 antibody. Intensity of the β-galactosidase activity was inversely related to the concentration of CP (Fig. 26A-F). In non-treated cultures, LacZCx29 expression was observed in the nuclei and cytoplasm of the NCC at high intensity, whereas in the culture in the presence of 7 µM CP, β-galactosidase activity was highly reduced, and at 10 µM CP, LaZCx29 expression only occurred sparsely in scattered NCC. Concomitantly, migration of NCC was also inhibited inversely to the CP concentration in a dosage-dependent manner. In contrast, the morphology of the NCC was not affected evidently by CP, and, moreover, there was no change in the immunofluorescence intensity of p75 (Fig. 26G-I).
Figure 25. Life phase contrast images of neural crest cells cultured with various concentrations of cycloamine

(A-E) Neural crest cells from tube explants were cultured for 24 hrs in the presence of various concentrations of CP and compared to control cultures. (F-J) Outgrowth of NCC cultured for 48 hrs in the presence of CP (arrowhead in H indicates NCC undergoing cell death). Arrows point to the neural tubes. Scale bar: 5 µm.

Figure 26. Cycloamine downregulates LacZCx29 expression but does not affect p75 expression
Neural crest cells were cultured for 72 hrs in the presence of CP at different concentrations, LacZ staining and immunocytochemistry were then performed. (A-C) At low magnification, the number of the migrating NCC as well as the intensity of LacZ staining decreased corresponding to the concentration of CP. (D-F) At high magnification, cytoplasmic as well as nuclear localization of ß-galactosidase activity was highly reduced in the presence of CP. (G-I) Expression of the early Schwann cell lineage marker p75 was not affected by CP. Scale bar (A-C), 2 µm; (D-F), 20 µm; (G-I), 20 µm.

The data above revealed a strong inhibitory effect of CP on LacZCx29 expression. Therefore, the effect of CP on inhibition of LacZCx29 expression in NCC was further quantitatively analyzed in vitro. In these experiments, 7 µM of CP was applied as the constant concentration in all NCC cultures. After maintaining NCC in the presence of CP for 72 hrs, percentage of LacZCx29 positively stained NCC were counted. Only clustered LacZCx29 expression was considered being positive, scattered blue dots were not counted. In control groups, 89.89±2.40% (mean ratio) of NCC were LacZ-positive. In cultures treated with CP, the percentage of LacZ-positive cells dropped to 62.41±4.01%, which exhibited significant reduction of LacZCx29 expression in the presence of CP compared to the control (Fig. 27).
Figure 27. Cyclopamine significantly reduces the proportion of LacZCx29 expressing neural crest cells in vitro

LacZ stained NCC from CP treated cultures and untreated control cultures after 72 hrs incubation were counted at 40x magnification. In the presence of 7 µM CP, the number of LacZ expressing NCC declined significantly (mean ratio: 62.41±4.01%) compared to the control (89.89±2.40%, p values <0.05).

4.2.2 Sonic hedgehog antagonizes the effect of cyclopamine on the expression of LacZCx29 and triggers earlier LacZCx29 expression in neural crest cells

Cyclopamine as an antagonist of the Shh signaling pathway significantly inhibited LacZCx29 expression in NCC, indicating that Shh plays a role in the regulation of Cx29 expression in NCC. The direct effects of Shh on LacZCx29 expression in NCC were therefore examined in vitro.

Initially, the optimal concentration of Shh recombinant peptide was determined. At the concentrations of 2 µg/ml, 500 ng/ml, and 50 ng/ml of Shh, NCC underwent cell death within 24 hrs (data not shown). When the
concentration was decreased to 2 ng/ml, no cell death was observed, and 
NCC migrated out of the neural tube in the same manner as observed in 
control cultures. The concentration of 2 ng/ml of Shh therefore, was used 
as a defined concentration in these experiments. In addition, 2 ng/ml Shh 
plus 7 μM CP were applied in parallel cultures. After NCCs were cultured 
in the absence of Shh for 2 days, i.e. in control cultures, LacZCx29 
expression was absent (Fig. 28A). When cultured in the presence of Shh, 
expression of LacZCx29 was present in NCC cultured for two days 
(E9.5+2), which is one day earlier than observed in control cultures, 
however, staining displayed a diffuse pattern in the nuclei and cytoplasm 
of NCC (Fig. 28B). In contrast, in the presence of 2 ng/ml Shh plus 7 μM 
CP, no LacZ staining was observed at E9.5+2 (Fig. 28C). After NCCs 
were maintained in cultures for 3 days, a slight increase of β-
galactosidase activity was observed when cultured in the presence of 2 ng 
/ml Shh (Fig. 28E), while in the presence of Shh plus CP, the intensity of 
LacZCx29 expression as well as the number of LacZ-positive cells was 
identical to the control (Fig. 28D, F). Like with CP, no change in 
migration or cell morphology of NCCs was observed in the presence of 
Shh.
Figure 28. Sonic hedgehog promotes LacZCx29 expression one day earlier in neural crest cells and neutralizes the inhibitory effect of cyclopamine

Neural crest cells were cultured in the presence of 2 ng/ml Shh (B and E), 2 ng/ml Shh plus 7 µM CP (C and F) and under control conditions (A and D) for 2 and 3 days. Sonic hedgehog upregulated LacZCx29 expression after NCC were cultured for 2 days, and this effect was abolished by 7 µM CP. After being maintained in culture for 3 days, LacZCx29 expression in NCC was increased slightly in the presence of 2 ng/ml Shh, and moreover, Shh also blocked the inhibitory effect of CP on LacZCx29 expression. Scale bar: 30 µm.

In this part of the study, expression of LacZCx29 was shown to be regulated by Shh, which promoted LacZCx29 expression 24 hrs earlier (E9.5+2) than under control condition (E9.5+3) *in vitro*, whereas its antagonist, CP, significantly inhibited LacZCx29 expression and reduced the number of LacZ-positive NCCs.
4.3 Function of connexin43 in trunk neural crest cells migration

As outlined in Chapter 3.1.2.1, trunk NCC predominantly express Cx43. Previous studies have reported expression of Cx43 in cardiac NCC (Lo et al., 1997; Waldo et al., 1999). Moreover, Cx43 has been demonstrated to be involved in cardiac NCC migration (Huang et al., 1998). Therefore, we investigated if the presence of Cx43 in trunk NCC is related to cell migration as well.

In control cultures, NCCs were obtained from caudal trunk neural tube explants at E9.5. After neural tubes were explanted and cultured for 3 hrs, a number of NCC started to migrate out of the neural tubes. After 24 hrs, a group of NCC was observed around the neural tube explants, and by 72 hrs, the migration area of the NCCs was markedly enlarged (Fig. 29).

![Figure 29](image)

**Figure 29. Migratory neural crest cells from neural tube explants in culture**

After the neural tube explants were plated on glass coverslips, NCCs migrated out of the tubes rapidly. After 24 hrs (E9.5+1) in culture, a monolayer of NCCs surrounded the explants; when cultured for 72 hrs (E9.5+3), the area of the NCCs outgrowth was markedly enlarged.
4.3.1 Function of mimetic peptides in neural crest cell migration

Homophilic peptides (RD4) which recognize the second extracellular loop of Cx43, as well as randomized peptides (RD22), which contain the same amino acids but in a randomized sequence, were examined for their effects on NCC migration. Firstly, the blocking efficiency of the peptides to gap junction coupling was checked by microinjection of Lucifer yellow dye (LY). Lucifer yellow dye has a small molecular weight (457.24 Da) and can, therefore, be transmitted between cells via gap junctions. By microinjection, we could show that NCCs were highly coupled in the control cultures (Fig. 30A). However, after NCC were cultured for 2 hrs in the presence of 100 mM mimetic peptides (RD4), the coupling of NCC was strongly reduced (Fig. 30B). In addition, the coupling of the cultured NCCs in the presence of randomized peptides (RD22) was also decreased to about 70% (Fig. 30C). After maintaining NCC cultures in the presence of RD4 for 3 hrs, the coupling of NCCs increased to the level of cultures incubated with RD22 for 2 hrs (data not shown). This indicated that the inhibitory effect of peptides was transient.
Figure 30. Mimetic peptides inhibit the dye coupling of neural crest cells

Neural crest cell cultures were incubated in the presence of 100 mM mimetic peptides for 2 hrs; cultures were then transferred to phenol red free L-15 medium containing no peptides, and were examined for coupling by microinjection of LY dye. Lucifer yellow dye was transmitted to a number of NCC (in average, 21 cells) neighboring the injected cell in the control culture (A). By applying RD4 in the culture, dye coupling was effectively inhibited (B), whereas the randomized peptides also reduced the coupling of the NCC to 70% (C). (D-F) Phase contrast images of the three different cultures showed identical density of NCC. Arrows point to the injected cells.

To investigate the effect of mimetic peptides on NCC migration, long term experiments were performed. Neural crest cell cultures were incubated in the presence of 100 mM mimetic peptides for 72 hrs with application of the same dose every 4 hrs (see Methods). Mimetic peptides did not inhibit NCC migration significantly (Table 4 and Fig. 31). Compared to the randomized peptide treated group, a slightly elevated migration index was observed. However, the reduction of the migration
rate in the presence of randomized peptides was also not significant.

<table>
<thead>
<tr>
<th></th>
<th>Migration index</th>
<th></th>
<th>Migration index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD4</td>
<td>392.0±26.67 µm (5)</td>
<td>Cx43</td>
</tr>
<tr>
<td></td>
<td>RD22</td>
<td>369.7±48.28 µm (5)</td>
<td>Cx36</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>428.5±87.32 µm (5)</td>
<td>reagent</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>control</td>
</tr>
<tr>
<td></td>
<td>Cx43-ELI</td>
<td>258.2±21.36 µm (7)</td>
<td>oleamide</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>261.1±11.81 µm (7)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Migration index obtained from neural crest cell cultures by inhibition of connexin43 with different methods

Numbers indicate mean ± SEM. Numbers in parentheses indicate number of explants.

Figure 31. Mimetic peptides do not inhibit neural crest cell migration

Migrating NCC from E9.5 neural tube explants were incubated in the presence of mimetic peptides (RD4) as well as randomized peptides (RD22) for 72 hrs. Mimetic peptides did not inhibit NCC migration (mean ratio of migration index: 392.0±26.67 µm) significantly compared to the control (428.5±87.32 µm); RD22 also decreased the migration rate of NCC insignificantly (369.7±48.28 µm).
4.3.2 Impact of antibodies recognizing the extracellular loop of connexin43 in neural crest cell migration

Connexin43 has been described to form functional uncoupled channels (hemichannels) in astrocytes (Musil et al., 1991; Quist et al., 2000). Further studies from Hofer and Dermietzel (1998) have reported that antibodies raised against the extracellular loop of Cx43 could cause the closure of hemichannels in cultured astrocytes. Therefore, a polyclonal antibody, which was produced in rabbit using the mimetic peptide as an antigen (Dermietzel et al., 2003; produced by Dr. Meier, Dr. Krause-Finkeldey and Mr. Habbes, Department of Neuroanatomy), therefore recognizing the second extracellular loop of Cx43 (Cx43-ELII), was applied to examine its effect on NCC migration.

As antibodies might be degraded in the culture, the degradation period was tested for the Cx43-ELII antibodies using primary astrocyte cultures. Astrocytes were incubated in the presence of 20 µg/ml Cx43-ELII antibodies for various time intervals (2, 4, 6, 8 and 12 hrs, respectively), cells were then fixed and double immunostained with ED1 antibodies (labeling microglia). In the cultures incubated with Cx43-ELII for 2 hrs, the typical punctate pattern of Cx43 expression was observed around the membrane of astrocytes, occasionally overlapping with ED1 possibly indicating the phagocytosis of antibodies by microglia (Fig. 32). In cultures incubated with Cx43-ELII antibodies for 4 hrs or longer, no
signal of Cx43 was detected either in astrocytes or in microglia cells (data not shown for 6, 8 and 12 hrs cultures). This suggested that degradation of the Cx43-ELII antibody in cultures occurred between two and four hours.

Figure 32. Degradation of connexin43-ELII antibodies occurs between two and four hours in astrocyte cultures

A monolayer of primary astrocyte cultures was incubated in the presence of 20 µg/ml Cx43-ELII for two and four hours, cells were rinsed in PBS, fixed and incubated with Alexa-488 conjugated secondary antibodies (in B and E). Immunostaining using ED1 antibody (A and D) was further performed and photographed using fluorescence microscope. Immunosignal of Cx43-ELII antibody was detected after 2 hrs of incubation, but no signal was present in cultures incubated for 4 hrs. Scale bar: 20 µm.

Consequently, Cx43-ELII antibodies were applied at a concentration of 20 µg/ml every 3 hrs over a period of 24 hrs to the NCC cultures, maintaining non-treated groups in parallel as controls. Subsequently, the migration index was measured. However, applying Cx43-ELII to NCC cultures did not influence their migration significantly (Table 4 and Fig.)
Figure 33. Antibodies recognizing the extracellular loop of connexin 43 do not significantly reduce neural crest cell migration

After NCC cultures were incubated with Cx43-ELII antibodies for 24 hrs, cells were fixed and stained with Hoechst dye for estimation of total cell numbers; the migration index was determined and statistically analyzed. Connexin43-ELII antibody did not inhibit NCC migration significantly (mean ratio of migration index: 258.2±21.36 µm) compared to the mean of the control groups (261.1± 11.81 µm, p>0.10).

As Cx43-ELII antibody did not influence NCC migration significantly, the proportion of Cx43 hemichannels in NCC was investigated. After being cultured for 24 hrs, NCC were examined by double immunocytochemistry with antibodies recognizing the cytoplasmic terminus of Cx43 (Cx43-CT), which labels the total amount of Cx43 in NCC, as well as those recognizing the extracellular loop of Cx43 (Cx43-ELII), which specifically label hemichannels. Compared to the intense immunofluorescence with Cx43-CT antibodies, there were only a few punctate fluorescent signals labeling hemichannels (Fig. 34). This indicated that only the minority of Cx43 in NCC was present in form of hemichannels.
Figure 34. Proportion of hemichannels in connexin43 gap junction channels of neural crest cells

After Cx43-ELII was incubated for 2 hrs in the culture, NCC were fixed and incubated with Alexa-488 conjugated secondary antibody, Cx43-CT antibody was then subsequently incubated for double labeling. (A) NCC expressed Cx43, showing a punctate pattern of the fluorescent signal (arrows). (B) Hemichannels, which were labeled by Cx43-ELII antibody, were present in NCC in low numbers (arrows). Scale bar: 20 µm.

4.3.3 Inhibition of neural crest cell migration by gene silencing of connexin43 with siRNA

Since our investigation using peptides and antibodies directed to external loop sites of Cx43 did not provide conclusive evidence of the function of Cx43 hemichannels on NCC migration, we focused on studies which lead to either gene silencing of Cx43 or complete blocking of junctional coupling. Recently, it has been reported that Cx43 expression could be effectively down-regulated by transfection with chemically synthesized siRNA of Cx43 in human breast cancer cell lines (Shao et al., 2005). The effect of inhibiting Cx43 gene expression on NCC migration was,
therefore, investigated by using siRNA.

When transfected with Cx43 siRNA, the mean migration index of NCC was 193.6±33.01 µm, which was significantly lower than that of non-treated control groups (331.0±50.69 µm; p<0.05) using Student’s T test. Moreover, transfection with Cx36 siRNA, which could be used as a substrate control, also decreased the migration index of NCC, but to an insignificant level compared to both the reagents and to control groups (Fig. 35).

**Figure 35. Silencing of connexin43 gene expression by siRNA inhibits neural crest cell migration significantly**

Connexin43 siRNA as well as Cx36 siRNA were transfected into migrating NCC. Transfection of Cx43 siRNA into NCC, significantly inhibited NCC migration (mean migration index: 193.6±33.01 µm) compared to the non-treated group (331.0± 50.69 µm; p<0.05, Student’s T test), whereas no significant inhibition was detected between Cx43 siRNA and reagent groups (302.6±81.07 µm; p>0.05). Connexin36 siRNA also affected NCC migration (260.4±29.96 µm), but the effect was not significant compared to controls (p>0.05).
After transfection of NCC with Cx43 siRNA and culture for 72 hrs, proliferation was examined using BrdU incorporation and Hoechst staining. Hoechst dye stained the total number of NCC (presented in red), and BrdU labeled proliferating NCC are shown in green. Small interfering RNA transfected NCC showed indistinguishable BrdU incorporation amounts compared to controls. Scale bar: 10 µm

In primary cultures of astrocytes, inhibition of Cx43 gene expression using siRNA is accompanied by decreased cell proliferation (A. Turchinovich, unpublished observation). A BrdU incorporation assay was therefore performed to detect proliferation in NCC transfected by siRNA as well as in the non-treated culture, to exclude the possibility that the significance of NCC migration inhibition by siRNA resulted from an inhibition of NCC proliferation. Immunofluorescence using BrdU antibodies, detecting proliferating cells, and Hoechst dye staining of nuclei, revealing the total number of NCC, were examined in two individual experiments. Figure 36 shows that a similar percentage of NCC has incorporated BrdU in both Cx43 siRNA transfected cultures and in control cultures. Consistent with the observation from immunostaining, the percentage of proliferating NCC in siRNA transfected cultures (18.6
±0.12%) and control groups (19.5±0.22%) did not show any significant difference (Table 5). This data indicated that inhibition of Cx43 gene expression did not affect NCC proliferation.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of BrdU incorporation</th>
<th>mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture 1</td>
<td>Culture 2</td>
</tr>
<tr>
<td>siRNA-Cx43</td>
<td>18.7%</td>
<td>19.1%</td>
</tr>
<tr>
<td>Control</td>
<td>20.5%</td>
<td>19.7%</td>
</tr>
</tbody>
</table>

Table 5. Percentage of BrdU incorporated cells in connexin43 siRNA transfected neural crest cells as well as in the non-treated cells

Neural crest cultures transfected with Cx43 siRNA as well as control cultures were examined for their proliferation rate using BrdU incorporation. The number of BrdU labeled NCC was expressed as the percentage of the Hoechst dye stained total number of cells. The mean ratio of BrdU incorporation cells in siRNA treated cells was 18.6±0.12% which was not significantly different from controls (19.5±0.22%).

We further investigated the efficiency of silencing the Cx43 gene by immunocytochemistry. Neural crest cells from cultures, which were transfected with Cx43 siRNA, from those incubated with reagent, as well as from control cultures, were subjected to immunocytochemistry using the Cx43-CT antibodies. Staining was performed 24 hrs, 48 hrs and 72 hrs after transfection (Fig. 37). After 24 hrs, a predominant immunosignal for Cx43 antibody was detected at the cell membrane of NCC under all three culture conditions (Fig. 37A-C). The characteristic punctate pattern
of Cx43 was also observed after 48 hrs in the control culture (Fig. 37D), whereas the immunosignal of Cx43 from siRNA treated culture was decreased (Fig. 37F), but still visible. At 72 hrs, no Cx43 immunofluorescent signal was detected in siRNA transfected culture (Fig. 37J), however, the intensity of the immunosignal in the control was also reduced (Fig. 37H). This observation indicated that total inhibition of Cx43 expression by siRNA occurred after 48 hrs, i.e. in the last 24 hrs of the migration assay. Moreover, the lipid transfection reagent also showed perturbation of Cx43 expression at a certain level.

Figure 37. Connexin43 protein expression is completely suppressed by siRNA between 48 hrs and 72 hrs after transfection

Small interfering RNA was transfected into NCC cultures. After 24 hrs, the first group of cultures was immunostained with Cx43-CT antibody (A-C). In the control (A), Cx43 fluorescent signal was predominantly observed at the cell membrane of NCC,
whereas in the culture transfected with Cx43 siRNA (C), NCC migration was slightly reduced and the immunosignal of Cx43 antibodies was also lower than in the control. At 48 hrs, immunofluorescent staining was performed on a second group of cultures (D-F). The immunosignal of Cx43 was detected in the control as well as in the Cx43 siRNA transfected NCC, though at much lower level. At 72 hrs, no immunosignal was detected in Cx43 siRNA transfected NCC (J), whereas intensity of fluorescent signal in the control (H) was also decreased compared to the control at 24 hrs. The cultures which were only incubated with lipid transfection reagent also revealed reduction of Cx43 immunosignal at a certain level (B, E and I). Arrows point to neural tubes. Scale bar: 20 µm.

4.3.4 Inhibition of neural crest cell migration by oleamide, a chemical gap junction blocking agent

Oleamide, which is known to effectively block gap junction communication (Guan et al., 1997; Boger et al., 1998), has been examined on its effect on inhibition of NCC migration at a concentration of 50 µM according to previous study by Huang et al. (1998). Neural crest cells were cultured in the presence of oleamide for 72 hrs and the migration index was analyzed (Fig. 38). In the mouse trunk NCC cultures, oleamide reduced migration significantly (mean migration index: 128.5±10.10 µm) compared to the control (404.5±42.93 µm; p<0.005)
Figure 38. Oleamide significantly inhibits neural crest cell migration

Neural crest cells were cultured in the presence of 50 µM oleamide for 3 days. Medium was replaced every 24 hrs. The migration index of NCC markedly decreased when treated with oleamide (128.5±10.10 µm), compared to the control groups (404.5±42.93 µm; p<0.005).

Taken together, a significant inhibition of NCC migration by oleamide could be demonstrated in this part of the study. The effect of Cx43 siRNA on NCC migration was moderate, whereas Cx43 mimetic peptides and Cx43-ELII antibody did not significantly change NCC migration.
5. Discussion

Mutations or deletion of the Cx32 gene in peripheral glial cells are responsible for a peripheral pathological phenotype of the X-chromosome linked Charcot-Marie-Tooth (CMTX) syndrome in humans. The pathology is thought to be linked to the failure of diffusion of ions and small molecules across the myelin sheath, which impairs myelinating Schwann cells as well as the axons they ensheathe, subsequently leading to demyelination and axonal loss (Sander et al., 1998; reviewed by Saez et al., 2003). This finding has brought attention to the importance of connexins in glial cells.

Oligodendrocytes, which are the myelin-forming glial cells in the CNS, express Cx32 (Dermietzel et al., 1989), Cx29 (Sohl et al., 2001; Altevogt et al., 2002) and Cx47 (Menichella et al., 2003; Odermatt et al., 2003). In mouse knock out models, neither deletion of the Cx32 nor Cx47 gene alone has any significant effect on myelin formation and structure in oligodendrocytes. However, deleting both connexin genes at the same time, the effects on oligodendrocyte myelin became deleterious (Menichella et al., 2003; Odermatt et al., 2003). These findings indicate that Cx32 and Cx47 are likely to fulfil compensatory functions in maintaining myelin in the CNS. However, in the PNS, the presence of Cx43 and Cx29 does not compensate for the function of Cx32.

Immunohistochemically, Cx43 colocalizes with Cx32 at Schmidt-
Lanterman incisures as well as in paranodal loops in mice (Zhao et al., 1999). However, for Cx29, a different subcellular localization has been demonstrated in Schwann cell myelin, i.e. at the innermost, adaxonal myelin membrane (Li et al., 2002), whereas Cx32, is localized at the outermost layer of myelin (Meier et al., 2004). Moreover, Cx32 is expressed predominantly in adult Schwann cells, whereas expression of Cx43 and Cx29 has been described to appear earlier in postnatal Schwann cells and to decline to lower levels in adulthood compared to the level of Cx32 (Yoshimura et al., 1996; Altevogt et al., 2002). This might indicate that, in addition to synergistic functions of connexins in mature Schwann cells, Cx29 might possess a unique potential, like Cx43, which has already been detected to be expressed during embryonic development in the cardiac NCC and is involved in heart development as well as in NCC migration (Lo et al., 1997; Huang et al., 1998). It was therefore of interest to investigate the expression of these connexins during embryonic and postnatal development to identify their function during Schwann cell differentiation.

5.1 Connexin43 expression during Schwann cell development in vitro

Our study demonstrated that the expression of Cx43 was present from NCC throughout Schwann cell development. Immunosignal using Cx43 antibodies first appeared in cultured NCC, whereas at later stages, it seemed to be downregulated and kept at basal levels. Connexin43 is the
most widely distributed gap junction protein, which has been reported to be expressed in the two- to four-cell preimplantation stage in murines as well as in humans (Houghton et al., 2002; Bloor et al., 2004). Furthermore, our finding of abundant Cx43 expression in cultured trunk NCC is consistent with *in vivo* observations of its expression in cardiac as well as in trunk NCC (Lo et al., 1997). Given its prominent expression in NCC compared to the lower level in later stages of the Schwann cell lineage, Cx43 might be of particular importance in NCC and possibly also play a role in trunk NCC migration as has been demonstrated in cardiac NCC (Huang et al., 1998) (see discussion 5.10).

5.2 Connexin29 expression during Schwann cell development *in vitro*

In the Schwann cell lineage, a number of molecular markers have been detected and used to characterize different developmental stages. Some of them are present at all developmental stages, like Sox10, erbB3, p75, and L1 (Faissner et al., 1984; Riethmacher et al., 1997; Kuhlbrodt et al., 1998a; Hillenbrand et al., 1999; Bannerman et al., 2000). Others are reported to be expressed both in NCC and Schwann cell precursors, but downregulated in later stages, for example activator protein 2a (AP2a) (Stewart et al., 2001). The brain-fatty acid binding protein (BFABP) is present in Schwann cell precursors as well as in immature Schwann cells, but absent from NCC (Britsch et al., 2001). Protein zero (P0) and peripheral myelin protein 22 kDa (PMP22) are myelin proteins, which are
expressed from the Schwann cell precursor stage onwards, however, at basal levels, and are upregulated when myelination starts (Lee et al., 1997; Hagedorn et al., 1999; Notterpek et al., 1999). S100 and O4 proteins are present from the stage of immature Schwann cells onwards, but absent (O4) or expressed at much lower levels (S100) in Schwann cell precursors (Jessen et al., 1994; Dong et al., 1995 and 1999). In our study, we detected the onset of Cx29 expression when NCC entered the developmental stage of Schwann cell precursors and the expression was kept at prominent levels during later embryonic and postnatal stages. The presence of Cx29 has been previously revealed in myelin-forming glial cells during adulthood, i.e. in oligodendrocytes of the CNS and Schwann cells of the PNS, and, therefore, Cx29 was assumed to be associated with myelin (Altevogt et al., 2002; Nagy et al., 2003). In addition, our data have demonstrated that expression of Cx29 in the Schwann cell lineage occurs much earlier than the onset of myelination, and, in fact, commences at the beginning of the defined Schwann cell lineage (E12). Therefore, the data indicates that Cx29 can be defined as a novel marker gene for cells of the Schwann cell lineage.

5.3 Connexin32 expression during Schwann cell development in vitro

Connexin32 is the most abundant gap junction protein in adult Schwann cells (Scherer et al., 1995). During Schwann cell development, Cx32 mRNA was detected from embryonic immature Schwann cells (E16)
onwards, whereas the protein expression was low in P4 Schwann cells and increased afterwards. As myelination starts in the first postnatal weeks, this concurrence might suggest that myelin-formation is accompanied by expression of Cx32.

5.4 Cellular localization of connexins in vitro

In addition to the membrane localization of Cx43, Cx29 and Cx32, the cellular localization also revealed a general cytoplasmic expression at different stages of the Schwann cell lineage. As expounded by Laird (1996) and by Beyer and Berthoud (2000), this cytoplasmic localization of connexins may represent newly synthesized and/or degraded connexin proteins because of their short life cycle. Moreover, evidence exists that the phosphorylation state of Cx43 might also influence the protein’s subcellular localization (de Feijter et al., 1996; Nagy et al., 1997).

Epidermal growth factor (EGF) has been demonstrated to induce an increase in serine phosphorylation of Cx43, and elicits a rapid but transient reduction of gap junctional communication (Lau et al., 1992). Insulin like growth factor-1 (IGF-1) and FGF-2 have also been demonstrated to be involved in the regulation of Cx43 phosphorylation (Reuss et al., 1998; Lin et al., 2003). The observation of the cytoplasmic distribution of connexins at different Schwann cell developmental stages might provide additional evidence, since in our in vitro assay, all cultures were exposed to ß-NRG (which also has an EGF-like domain), FGF-2,
and/or IGF-1 (only in NCC cultures). Furthermore, in some cultures, a presumptive Golgi localization could also be observed, indicating the assembly of gap junction proteins in the Golgi apparatus or in the trans-Golgi network (Diez et al., 1999; Martin et al., 2001; reviewed by Saez et al., 2003). However, Golgi localization of these connexins needs to be confirmed by marker proteins.

5.5 Expression of other connexins in neural crest cells

Transcripts of Cx26, Cx30, and Cx45 were also detected in NCC, whereas after NCC differentiation into the defined Schwann cell lineage, mRNAs of these connexins were no longer detectable. These connexins have previously been described in embryos at the preimplantation stage (Davies et al., 1996; Houghton et al., 2002; Bloor et al., 2004), and are expressed in various adult tissues, for instance in astrocytes. As NCCs are a group of multipotent stem cells, which give rise to various derivatives, it is not surprising that these connexins were found in these non-committed cells. They seem, however, not to be related to Schwann cell development. In addition, Cx47, which is expressed in oligodendrocytes in the CNS (Menichella et al., 2003; Odermatt et al., 2003), was not expressed in Schwann cell lineage even at mRNA level. Although the transcript of Cx36 has been reported to be expressed dynamically in the developing CNS, as well as in sympathetic and spinal ganglia at E12.5 (Gulisano et al., 2000), which also develop from trunk NCC, we were
unable to detect consistent Cx36 mRNA expression in NCC. Even in the presence of BMP-2, which promotes neuronal differentiation in culture, Cx36 expression was not transcribed. Although expression of Cx36 is suggested to be related to neurogenesis in the CNS (Gulisano et al., 2000; Rozental et al., 2000), our data indicate that expression of Cx36 in the PNS might commence after neurogenesis has established, and probably requires extracellular signal(s) other than BMP-2.

5.6 Localization of connexin29 in embryonic stages of Schwann cell development

The onset of Cx29 expression paralleled NCC differentiation into Schwann cell precursors in vitro. By taking advantage of the LacZCx29 transgenic mouse model, the spatial expression of Cx29 during embryonic Schwann cell development was investigated in detail. Interestingly, Cx29 expression showed distinct patterns between dorsal and ventral roots of spinal nerves at embryonic stages. Connexin29 expression was observed from E12 onwards in ventral roots as well as in peripheral nerves, whereas expression of Cx29 in dorsal roots and within the DRG commenced at E16. This unique temporal-spatial pattern of Cx29 expression raised the issue of synchronization of Schwann cell development in both derivatives of NCC.

Previous studies have reported that a subgroup of late migratory NCC named boundary cap (BC) cells is present at the boundary between the
PNS and the spinal cord (Niederlander and Lumsden, 1996). Investigations using Egr2 (also known as Krox20, the expression of which is initially restricted to BC cells) knock-in mouse have demonstrated that these BC cells also migrate out of the entry or exit points where they become arrested. Later in development, they differentiate into sensory neurons and glial cells within the DRG, and to Schwann cell precursors of dorsal roots. However, some Schwann cell precursors in the ventral root also differentiate from BC cells. Boundary cap cell migration is initiated around E11.5, and, by E12.5, their derivatives (presumptive Schwann cell precursors) can be observed ensheathing axons in the dorsal root (Maro et al., 2004). This time course is consistent with that of Schwann cell development. However, there is no direct evidence whether BC cells are already committed to the Schwann cell lineage or still maintain their NCC properties at that time. Therefore, the issue of asymmetric differentiation of Schwann cells in dorsal and ventral roots of spinal nerves needs to be investigated in more detail. Studies on quail using the pan-glial marker 7B3 for NCC-derived glial precursors have revealed that 7B3 antibodies label Schwann cell precursors in dorsal and ventral roots of spinal nerves at the same stage (Henion et al., 2000; J.A. Weston; personal communication), indicating that, at least in the avian, Schwann cells in dorsal and ventral roots seem to develop synchronously.
Expression of the Schwann cell marker protein S100 was examined at the transition stage from Schwann cell precursors to immature Schwann cells at E16, when Cx29 expression in the dorsal root also commences. Expression of S100 was detected in immature Schwann cells in the ventral roots as well as in sensory neurons and their axons within the DRG at E16. In the dorsal root, S100 immunoreactivity was likely to be localized in the nerve fibers rather than in the presumptive immature Schwann cells. This suggests that immature Schwann cells in the dorsal root might still not have differentiated at E16 and that the Schwann cell lineage in the dorsal root probably converts to immature Schwann cells later than those in the ventral root of the spinal nerve in the mouse (at E16).

As previously discussed (5.2), various marker proteins are expressed during Schwann cell development. However, there is no distinct protein, which is exclusively expressed either by NCC or by Schwann cell precursors in the mouse system. Therefore, to distinguish unambiguously between these two stages of the Schwann cell lineage, particularly important for cells of the dorsal root between E12 and E16, a combination of marker proteins will have to be investigated.

During early postnatal stages, the immunosignal for Cx29 as well as β-galactosidase activity were distributed homogenously along the peripheral nerves, which is different to the distribution in adult
myelinated nerve fibers, where Cx29 localizes to the innermost aspects of Schmidt-Lanterman incisures and to juxtaparanodes (Altevogt et al., 2002; Li et al., 2002). Myelin formation commences in postnatal Schwann cells, with the first cells starting myelination in the first postnatal week. Therefore, the process of myelination might be accompanied by the site restriction of Cx29 in Schmidt-Lanterman incisures and juxtaparanodes.

## 5.7 Localization of connexin29 in the nervous system

Previous studies have demonstrated that Cx29 is uniquely distributed in the nervous system, i.e. in oligodendrocytes of the CNS and Schwann cells of the PNS in adult mice (Sohl et al., 2001; Altevogt et al., 2002). We investigated expression of Cx29 during embryonic development, and observed that at E9.5 the expression of LacZCx29 was restricted to the roof of the developing hindbrain. This might be due to Cx29 expression in migrating cranial NCC committed to become glial cells of some cranial ganglia (V, VII, VIII, IX, X, etc). An alternative explanation is that LacZCx29 expression in these regions is located in a part of the ectodermal placodes which give rise to additional parts of the cranial nervous system. Later on, LacZCx29 expression was distributed in various cranial ganglia as well as along the nerves which emerge from both of the origins, i.e. cranial NCC and ectodermal placode (Noden, 1991; Graham and Begbie, 2000; Baker and Bronner-Fraser, 2001). At E16, expression of Cx29 was detected in close approximation to some
trunk organs, like esophagus and trachea, also indicating its occurrence in the PNS. Taken together, the LacZCx29 expression pattern at E9.5, E12 and E16, strongly suggests that Cx29 expression is restricted to the nervous system during embryonic development.

5.8 Connexin31 expression in peripheral sensory and spinal motor neurons

Studies by Jungbluth et al. (2002) demonstrated that Cx31 was expressed at the entry/exit points of all mixed sensory/motor nerves and at the entry points of pure sensory nerves into the spinal cord in the mouse from E11.5 onwards, and this expression was regulated by Krox20. In addition, expression of Cx31 was not present in BC cells at the exit points of pure motor nerves. It was therefore evaluated whether Cx31 might be the connexin expressed in dorsal roots of spinal nerves between E12 and E16, and possibly compensate for Cx29. We found that Cx31 was expressed by sensory neurons within the DRG, which are also derivatives of BC cells, and by motor neurons in the spinal cord. However, expression of Cx31 was absent from glial cells in the dorsal root. Previous studies have shown that axonal signals determine the survival, proliferation and differentiation of Schwann cell precursors (Jessen et al., 1994; Dong et al., 1995 and 1999; Ciutat et al., 1996; reviewed by Jessen and Mirsky, 2002). In turn, signals from Schwann cell precursors are also required for the survival of neuronal cells during development (Riethmacher et al., 1997;
Jessen and Mirsky, 1999). The distinct patterns of Cx29 and Cx31 expression in the PNS suggest a differential role of both connexins in coordinating or transporting signals between neuronal and glial cells during development. However, both Cx29 and Cx31 are rather atypical connexins in the sense that Cx29 gap junctions have not yet been shown to form functional channels at all, and Cx31 expressing cells only form functional channels with themselves (Elfgang et al., 1995), pointing to a potential role of these two connexins in development independent of gap junction communication.

5.9 Sonic hedgehog regulates connexin29 expression in vitro

Recent studies provide evidence that gap junction expression is regulated by extracellular signaling molecules such as neurotransmitters, growth factors and cytokines (Patel and Kos, 2005; Vine and Bertram, 2005). Given the differences of Cx29 expression between dorsal and ventral roots of spinal nerves, one could assume that extracellular signal(s) which regulate dorsal-ventral patterning, or ventral localized diffusible signal(s) released from nearby tissues, might be involved in regulation of Cx29 expression during Schwann cell development. We therefore focused on a possible role of sonic hedgehog (Shh) as a regulating factor.

Sonic hedgehog is secreted from the notochord and floor plate (Echelard et al., 1993; Marti et al., 1995; Ericson et al., 1997) and accounts for induction of differentiation of ventrally located cells in a concentration-
dependent manner (Roelink et al., 1995; Liem et al., 2000). A steroidal alkaloid, cyclopamine (CP), has been demonstrated to act as an effective antagonist of the Shh pathway in vitro (Incardona et al., 1999; Taipale et al., 2000; Chen et al., 2002). Moreover, studies have shown that the functions of Shh are not restricted to ventral patterning during embryogenesis; Shh is also involved in NCC survival, proliferation, differentiation and migration in avian and zebrafish (Ahlgren et al., 1999 and 2002; Testaz et al., 2001; Fu et al., 2004; Wada et al., 2005). In our in vitro assay, a significant amount of cell death was observed in the presence of higher concentrations of Shh and CP. Moreover, in the presence of CP, NCC migration was distinguishably reduced. These observations indicate that Shh signaling might play a role in mammalian NCC migration and/or survival. However, cell death of NCC might also result from the cytotoxic effect of Shh and CP which has been revealed in a recent study (Mimeault et al., 2005).

Most importantly, Shh was shown to be involved in the regulation of Cx29 expression during Schwann cell development in vitro. Sonic hedgehog promoted the onset of Cx29 expression in cultured NCC (E9.5+2) one day before that of controls. Studies in chick have also shown that Shh is required for the initial expression of the oligodendrocyte precursor marker protein O4+ in the metencephalon and spinal cord. Moreover, increase of oligodendrocyte precursors in response
to Shh is dose-dependent (Davies and Miller, 2001). Furthermore, inhibition of the hedgehog pathway by CP led to the loss of DRG neurons, and its ablation in zebrafish was also shown to be dose-dependent (Ungos et al., 2003). In our studies Cx29 was also identified as a target of Shh. However, inhibiting the Shh pathway in vitro using CP did not prevent Cx29 expression in general, Cx29 expression was observed after three days in vitro, at E9.5+3, though its expression was at lower level than in CP-free cultures. These data indicate that Shh signaling seems to be involved in regulating the timing of Cx29 expression, but not its general expression.

Given the fact that Shh signals in vivo originate ventrally, and that its effect is dose-dependent in vitro, these data also provide an explanation for the asynchronous dorsal-ventral expression pattern of Cx29. Schwann cell precursors associated with axons from ventral horn of the spinal cord are in close proximity to the source of Shh, and thereby, receive higher concentrations than precursor cells in the dorsal root, which are more distant. This topological situation may be one of the reasons for the early onset of Cx29 expression in ventral roots (at E12) as compared to the Schwann cell precursors in dorsal roots (at E16).

It has been suggested that oligodendrocytes in spinal cord, share a common origin with motor neurons (Pringle et al., 1996; reviewed by Richardson et al., 2000; Nobel et al., 2004) and both sensory neurons
within the DRG and Schwann cells generate from NCC. The regulating effect of Shh on Cx29 expression indicates that Shh may be an instructive signal for Schwann cell lineage differentiation. However, according to our observation that NCC did not remarkably change their phenotype to that of Schwann cell precursors after cultured for 2 days in the presence of Shh (while Cx29 expression was promoted), it seems unlikely that Shh is a direct signal for gliogenesis. Alternatively, Shh might influence differentiation of NCC into cells of the Schwann cell lineage indirectly by regulation of Cx29 expression. However, Cx29 deficient mice do not show dominant deficit in Schwann cell lineage progression or in the morphology of adult nerve fibers (our own unpublished observation). This suggests that other connexins might be present during Schwann cell development for functional compensation.

5.10 Connexin43 is involved in neural crest migration

Neural crest cells have been observed to migrate in clusters or sheets (Bancroft and Bellairs, 1976; Davis and Trinkaus, 1981; Kasemeier-Kulesa et al., 2005). This behavior provides a chance for extensive cell-cell contact. Assembly of functional gap junction requires appropriate cell adhesion which is mediated by Ca$^{2+}$-dependent molecules, the cadherins (Musli et al., 1990; Jongen et al., 1991). Previous studies have shown that connexin hemichannels (connexons) are inserted into the plasma membrane at regions of cadherin/catenin-mediated cell adhesion
(Fujimoto et al., 1997). Consistent with this finding is the observation that Cx43 is colocalized with N-cadherin in NIH3T3 cells (Wei et al., 2005). Members of the cadherin family are widely expressed in NCC. In chick, N-cadherin and cadherin-6B are expressed in the most dorsal region, where NCCs are generated, and cadherin-7 is expressed in migrating NCC (Nakagawa and Takeichi, 1995). In mammals, expression of cadherin-19, cadherin-20 and N-cadherin has also been detected (Xu et al., 2001; Moore et al., 2004; Takahashi and Osumi, 2005). These studies suggest a propensity of NCC to form gap junctions. The fraction of hemichannels in the plasma membranes is low compared to that of gap junctional plaques as could be demonstrated by our immunocytochemical double labeling approach (Chapter 4.3.2). This is likely to be one reason for the inability of hemichannel blockers to inhibit migration. Likewise, hemichannels may be not involved in migration at all and this relationship is more attributable to functional gap junction channels in the sense of coordination and organization of directed movement of cohorts of cells. This argument, however, seems less likely since both inhibitors (mimetic peptides and ELII-site directed antibodies) were proven able to block intercellular communication (Hofer and Dermietzel, 1998; Chapter 4.3.1). A more reasonable answer to the observed inability of hemichannel blockers to inhibit migration could be the steady state conditions and the accessibility of unpaired connexons in the plasma membrane of
Gap junctions are dynamic structures that turn over rapidly (Bruzzone et al., 1996). In cultured cells, studies have determined gap junctional half-lives in the range of one to three hours (Laird et al., 1991; Darrow et al., 1995). During the life cycle of a gap junction plaque, new hemi-channels are continuously recruited to the plasma membrane and endocytosed (Dermietzel et al., 2003). The kinetics of this turnover is not known yet, but a feasible explanation of the lack of inhibition is that the proteinaceous inhibitors are rapidly degraded to an extent that is not sufficient to achieve blocking thresholds.

This explanation is in agreement with our observation that silencing the Cx43 gene using siRNA or disruption of gap junction intercellular coupling using oleamide affected NCC migration significantly. The difference between the blocking and silencing effect can be explained by their selectivity.

Oleamide, previously proven to be a potent inhibitor of gap junctional communication (Guan et al., 1997; Boger et al., 1998) shows low selectivity in terms of connexin preference. As mRNA expression of additional connexins was detected in NCC, these might also be a target for oleamide, and explain the more robust effect of this inhibitor.

The less robust effect of Cx43 siRNA on NCC migration can be explained by the restriction of its actual effect to the last 24 hrs of the
culture period, together with the fact that Cx43 expression was endogenously downregulated during this time period. For the effect of Cx36 siRNA, it is not clear, whether the inhibition on NCC migration (though insignificant compared to the controls) resulted from a cross-hybridization to Cx43 RNA which could lead to partial silencing, or was due to specific inhibition of Cx36 RNA, given that mRNA of Cx36 was detected in NCC, though was inconsistent in all samples.
6. Summary

First, our data demonstrated that connexins were sequentially expressed during Schwann cell development. This expression pattern suggested different roles of these connexins in the Schwann cell lineage.

Second, we revealed that the onset of Cx29 expression coincided with the differentiation of NCC to Schwann cell precursors \textit{in vitro}. This finding indicated that Cx29 is a novel marker for the defined Schwann cell lineage.

Third, we observed an asymmetric spatial pattern of Cx29 expression between dorsal and ventral roots of spinal nerves, showing that Cx29 expression commenced in the ventral root (at E12) earlier than in the dorsal root (at E16), which might suggest that Schwann cell differentiation in dorsal and ventral roots of spinal nerves might progress asynchronously.

Fourth, Cx29 expression seemed to be regulated by extracellular cues like Shh, which promoted and accelerated Cx29 expression by 24 hrs in cultured NCC \textit{in vitro}.

Fifth, Cx43 with its predominant expression in NCC was involved in the regulation of trunk NCC migration, in a similar pattern that has been demonstrated in cardiac NCC (Huang et al., 1998).
7. Reference


Ek-Vitorin JF, Calero G, Morley GE, Coombs W, Taffet SM. 1996. PH regulation of connexin43:

21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:
494-98.

Elfgang C, Eckert R, Lichtenberg-Frate H, Butterweck A, Traub O, Klein RA, Hulser DF,
Willecke K. 1995. Specific permeability and selective formation of gap junction channels in

and the specification of cell fate in the ventral neural tube. Cold Spring Harbor Symp Quant


1997. Heart and neural tube defects in transgenic mice overexpressing the Cx43 gap junction

1994. Mutations in the connexin 32 gene in X-linked dominant Charcot-Marie-Tooth disease
(CMTX1) Hum Mol Genet. 3(1):29-34.

during development, in neurological mutants and in the peripheral nervous system. Brain Res.

Falls. DL. 2003. Neuregulins and the neuromuscular system: 10 years of answers and questions. J
Neurocytol. 32: 619-49. Review.

Fraser SE, Bronner-Fraser M. 1991. Migrating neural crest cells in the trunk of the avian embryo are

cells transfected with connexins 43 or 32. Microsc Res Tech. 52(3):289-300.


Le N, Nagarajan R, Wang JY, Araki T, Schmidt RE, Milbrandt J. 2005: Analysis of congenital hypomyelinating Egr2Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell


Musil LS, Goodenough DA. 1991. Biochemical analysis of connexin43 intracellular transport,


A study with freeze-etch technique. Prog Brain Res. 46:1-384.


Curriculum Vitae

Jing Li

Jing.Li@rub.de

Dept. of Neuroanatomy and Molecular Brain Research
Ruhr University Bochum
Universitätstrasse 150, D-44801
Bochum, Germany
Telephone: 49-(0) 234-32-25004
Fax: 49-(0) 234-32-14655

Education/Employment

Sep. 2002-present Doctoral student, International Graduate School of Neurosciences (IGSN), Ruhr University, Bochum, Germany

Oct. 2001-Aug. 2002 Study for Master equivalent degree, IGSN & Department of Neuroanatomy and Molecular Brain Research Medical Faculty, Ruhr University, Bochum, Germany

Sep. 2000-Jun. 2001 German
Zhejiang University, Hangzhou, P.R. China

Sir Run Run Shaw Hospital, Hangzhou

Jun. 1998-Jun. 1999 Internship in People’s Hospital, Beijing, P.R. China

Sep. 1994-Jul. 1999 Bachelor of Medicine
Beijing Medical University (now Beijing University), Beijing
Fellowship

Research fellowship from the International Graduate School of Neuroscience, “From molecules to cognition” program, Ruhr University, Bochum, Germany.

Publication


Language

Chinese: mother language
English: fluent
German: good