*Final report*

# Neuronal migration, polarization and differentiation in the superficial spinal dorsal horn during embryogenesis

## 1. Introduction

Cellular migration is a well-known biological process during tissue- and organogenesis that is even more essential for development of the nervous system. Neuronal migration is required for proper neuronal connections by providing the pre- and postsynaptic elements to be in the right time and right place. With this process the spinal dorsal horn (SDH) become a highly organized laminated structure since different laminas are specialized for receiving functionally different sensory inputs from the periphery and processing and relaying toward to other parts of the central nervous system (Willis and Coggeshall 2004). The superficial laminae of Rexed (I-III) are specialized mainly for relaying nociceptive information. How neurons were arranged into laminas in this area was our primary question in this proposal. The neurons here are quite unique since they are deriving from a common progenitor group (Wildner et al. 2006). Despite their common origin of the inhibitory (dILA) and excitatory (dILB) neurons populating the superficial laminae they acquire a large morpho-functional heterogeneity (Réthelyi and Szentágothai 1969; Todd 2010) during embryonic development. These cells develop into either projection or propriospinal and interneurons resulted by numerous recently unknown probably interacting factors. However, based on transcription factor profile of the dILA and dILB neurons, this heterogeneity cannot be classified. These two groups differ from each other initially with the expression of the transcription factor Ptf1a exclusively (Gross et al. 2002; Glasgow et al. 2005; Wildner et al. 2006). Due to serial and interactive expressions of other transcription factors (Lbx1, Pax2, Tlx3, Lmx1b, Drg11, Bhlhb5) dILA (Ptf1a+) neurons become inhibitory while dILB (Ptf1a-) cells differentiate into excitatory neurons (Cheng et al. 2004; 2005; Mizuguchi et al. 2006; Hori et al. 2008; Ross et al. 2010).

Since these heterogeneous neuron populations are quite homogenous in intrinsic factors we placed our focus more on the extrinsic factors in our proposal. A number of in vivo and in vitro studies have proved the importance of integrins, N-CAM and ECM molecules in neuronal migration (reviewed by Venstrom and Reichardt 1993; Barros et al. 2011). Hyaluronan and chondroitin-sulfate proteoglycans have central roles in the neuronal ECM organization. Semiquantitative analysis of hyaluronan revealed that it accumulates in the highest relative concentration at the border of the ventricular and intermedier zones, where progenitor cells terminate their last division and daughter cells start migrating toward to their destinations in the developing spinal cord (Mészár et al. 2008). Semaforins are also important molecules for morphological differentiation and expressions of some elements of the signalling pathways mediated by semaforins are described in the spinal cord. Their attractant or repellent effects on the axons are largely depending on the composition of the ECM around the neurons, particularly on the presence of chondroitin- and heparane-sulfates.

Taken together, answering the following questions was initiated and motivated our work:

1) How do these differentiating cells migrate into the superficial spinal dorsal horn?
2) When and how are these neurons differentiate further? What types of neurons are they differentiating into?
3) What are the molecular background behind these changes?

Our primary hypotheses were that dILA and dILB neurons migrate into the superficial dorsal horn by radial and possibly non-radial manner by passing through or around the early born SDH neurons settle in the deep dorsal horn. We also hypothesized that differentiation of dILA and dILB neurons depend on the microenvironment where they terminated their migration.

## *2. Results*

### *2.1. Establishment of migratory and morpho-genetic maps on dILA (inhibitory) and dILB (excitatory) neurons*

#### 2.1.1. Plasmid vectors used for experiments

We tested several expression vectors with different transcription regulatory sequences (CMV, Beta-actin) and fluorescent reporters (GFP, YFP, tdTomato, mCherry). The most stable and reliable was the ones with CAG promoter that contain an enhancer from cytomegalovirus (CMV) and a promoter from chicken beta actin and globin and a chimeric intronic sequence. For labelling dILA neurons we followed two strategies: 1) we used Ptf1a-cre vector in a combination with cre sensitive pCALN5-mCherry or pCALN5-GFP providing a sporadic labelling (red or green fluorescence) of dILA neurons, or 2) we used Ptf1a-cre transgenic mice crossbred with tdTomato reporter strain (Ai69) for labelling all Ptf1a neurons. For migration assays we used pCAG-GFP transcription vector. pCAG-GFP, pCALN5-mCherry, pCALN5-GFP vectors was a kind gift of our collaborator Professor Fujio Murakami (University of Osaka). The Ptf1a-cre vector was a kind gift of our collaborator Dr Dalit Sela-Donenfeld (The Hebrew University of Jerusalem).

#### 2.1.2. Neurons populating the superficial spinal dorsal horn born in short time interval

We examined how dILA and dILB neurons migrate into and populate the superficial SDH and arranged into laminar distribution. First, we had to determine the proper time intervals for birthday of dILA and dILB neurons. During animal surgery of time mated pregnant mice, we transfected GFP expression vector (pCAG-GFP) of the spinal cord of mice embryos by in utero electroporation at embryonic days ranging from 11 to 13 (E11-13). This labelled the progenitor cells found in the ventricular zone surrounding the central canal but the GFP expression remained stable in those of cells that became postmitotic at the time of the electroporation. We let the treated embryos to be born and reach the young adult ages and then we sacrificed mice for histological processing of their spinal cords. We examined the laminar distribution, morphology and neurochemical properties of GFP positive neurons. Combining of these experiments with BrdU incorporation assays we found the lamina I-III neurons born in a very short time interval (approx. six hours) in mice. Our finding was specifying considerably the data published by others.

### 2.1.3. dILA and dILB neurons were migrating with the same manner to reach and build the superficial laminae

For migratory route assays, we labelled the newly born dILA and dILB neurons with GFP vector by in utero electroporation at E12. We made organotypic spinal cord slice cultures from the GFP positive embryos after 1-2 days of their survival and maintained in microscope stage incubator for 1-3 days. Confocal time-lapse images were taken from the slices in 15-20 min intervals for at least 6 hours on each in vitro days and we examined the migration of the GFP labelled neurons into the superficial SDH and their lamination process. We obtained the first appearance of the superficial laminae as early as E14 but the GFP labelled neurons were still migrating intensively at this age. The labelled neurons were migrating radially first until they reached the lamina of their destination then they turn their direction and moved medio-laterally by intralaminar displacement. During this period the morphological differentiation, axonal and dendritic growth and branching started before they the termination of migration. Since organotypic slice cultures have limitations about mimicking in vivo conditions and examining possible rostro-caudal migrations in the superficial laminas, we aimed to perform a long term (2-photon) time lapse imaging in a living whole embryo. For holding exo-utero embryos stable, we designed a stage adapter that physically separated the maternal respiratory movement from the embryo. Our stage prototype was suitable for holding the imaged embryo quasi stable at least four hours. We have obtained however a stereotypic motion which was impossible to mask entirely by image alignment. We hope that these motion artefacts could be corrected more efficiently by further development of the stage adapter.
Two master theses and a conference abstracts based on these data. We also intend to publish our results in peer-reviewed journal (presubmission phase).

### 2.1.4. Morpho-functional description of dILA and dILB neurons

Using transgenic GAD65 and GAD67 mice lines, we described that the differentiation process of the inhibitory dILA neurons were not finished after birth. Immuno-histochemical double labelling on serial samples of mice embryos from different embryonic ages showed that these neurons appear first in the superficial laminae at around E14 and the relative numbers of cells showed the GABA-ergic phenotype was highest at around E16. From this time, we found a considerable decrease in the numbers of inhibitory neurons that stabilized after the first postnatal week (P7).
We also described that a paired domain transcription factor Pax2 expression that is required for GABA-ergic differentiation of the dILA neuron remain active during the whole life of the inhibitory cells. Individual intracellular labelled neurons were co-immunolabelled for Pax2 and vesicular GABA transporter VGAT allowed us to prove that Pax2 expression is restricted exclusively in the adult dILA neurons. Therefore, we described Pax2 as a stable and reliable inhibitory neuron marker in the SDH that labels the cell nuclei.
We described our results in two conference abstracts and submitted one publication to the journal Brain Structure and Function. Currently we are working on the modification of the paper that was requested by the referees. We also intend to publish our results on the Pax2 in the Journal of Neuroscience Methods. This latter is in presubmission phase.

## *2.2. Observation of axon initiation in dILA and dILB neurons*

### 2.2.1. Plasmid vectors used in these studies

Our original aim was to construct an expression vector that transfected into neurons drive tdTomato expression (pCMV-Brainbow-1.0H) in the soma and GFP exclusively in newly formed axons as early phase as axon initiation. For the latter we obtained a vector (pBa-Kif560-GFP) that express a truncated for of the kinesin 5C tagged with GFP. We turned down the idea to build a bicistronic vector coding these two markers (tdTomato in the soma and Kif5C-GFP in the axon) as we used the two markers in separated since using co-transfection allowed us to manipulate the ratio of the vector concentration. It turned out that the Kif560-GFP vector had a considerable cytotoxic effects in vivo. Since this system was needed further development we examine axon initiation and differentiation of dILA and dILB neurons by using pCAG-GAP-GFP vector a membrane bound form of the GFP. This allowed us to follow the rapid and transient changes in the cell surface during axonal and dendritic growth.

### 2.2.2. The dILA and dILB neurons polarize after termination of migration

We labelled E12 embryos by in utero electroporation using expression vector for membrane bound GFP and we made organotypic slice culture after one day of the survived embryos. Using long term two photon time-lapse imaging, we found that the polarization of the GFP labelled neurons occurred when they arrived in the superficial SDH but before the termination of their migration. Most of the cells showed a multipolar migration and the axon initiated from a de novo process as a branch of an existing leading or trailing process. This observation could be strengthened by what was reported on these neurons about their morphology in adult mice (Szucs et al. 2010; 2013).
We published a master thesis and a conference abstract based on these results.

### 2.2.3. Expression patterns of extracellular macromolecules and semaforins around dILA and dILB neurons during differentiation of spinal dorsal horn

Histochemical assays to hyaluronan on serial samples of mice embryos showed positive signal around the migrating neurons. In contrast, the non-migrating neurons were immunopositive for chondroitin-sulfates instead. After this observation, we carried out functional studies on ex vivo organoid culture since in vivo manipulation of the extracellular matrix molecules was not solved with a lack of useful expression vector. For minimizing the effects of intrinsic factors we made organotypic slice cultures of E13 embryos previously transfected by Ptf1a-cre and pCALN5-GFP vector combinations and kept in culture medium supplemented with or without ECM digesting enzymes (hyaluronidase and chondroitinases). Images were taken form the whole culture with 2-photon microscope after the second in vitro day. Neurolucida software was used for 3D reconstruction and annotation of GFP positive neurons and numerical data (surface, area orientation, process, sholl analysis) was analyzed further. Disruption of any component of the ECM were increased the branching and growth of the axons and dendrites of the late-born Ptf1a positive neurons. The dendritic branching was facilitated most by using chondroitinaseABC while hyaluronidase was promoting longer dendritic growth. Our data indicated the morphogenic effects of hyaluronic acid and chondroitin-sulfate proteoglycans in differentiating neurons in the SDH.

These results were published in conference abstracts MSc theses. We aimed to publish these results in peer-reviewed after increasing the numbers of the experiments.
We found the results of this experiments quite interesting therefore we stepped further than it was described as the original aim of the proposal in order to increase the impact of the publication based on this experiment. We designed such an electroporable expression vector that coding the chondroitinase AC optimalized for mouse codon usage. We then added an artificial sequence to the coding sequence for driving extracellular secretion of the enzyme (Leahy et al. 2000). This chimera gene was then cloned into a bicistronic P2A GFP vector that was also containing a cre inducible transcription. We tested the vector in cell culture with chondoitinase activity assay that proved our vector functionality. We aimed to use this vector in in utero electroporation and also to generate a conventional transgenic mouse line later.

### 2.3.2 Semaforin signaling may play a role in differentiation of dILA és dILB neurons

ECM molecules acting by interaction with morphogen molecules such as semaforins (Dick et al. 2013; Vo et al. 2013). We showed by immunohistochemistry and in situ hybridization that dILA and dILB cells are expressing the sema3 receptors neuropilin 1 and plexin A2 at the time of their differentiation. These receptors were immunopositive on the soma and processes as well and we assumed importance in dendritic and axonal growth. This requires further experiments to answer this assumption. We designed loss of function study based on dominant negative technology. The experiments are in progress. We published a master thesis from the results so far.

## *3. Utilization and application of the results*

Result of our basic research is beneficial mainly for education as our newest findings are presented on MSc and BSc lectures and practical courses. Our methods are following the trend of the modern experimental embryology thus these methods we implemented in the graduate program of biologists. The most of our data are useful for biomaterial science (i.e. material and cell interface, ECM as scaffold) and for studies focusing on neuronal regeneration and stem cell research. Our experimental model for neuronal migration assay could be a useful test for examining teratogen factors and also help us to understand more some congenital migratory and neurodegenerative diseases.

## *4. Personnel*

The following students graduated and finished studies in the research group as well:

Mikler, Boldizsár – graduation / finishing date: 31. August 2014.

Tóth, Fruzsina – graduation / finishing date: 31. August 2014.

The following students was joined to the group:

Sulik, Máté – from 01 October 2014.

Horák, Emma – from 01 December 2014.

Other non-researcher colleges: our college Török Éva was retired at 1 August 2015. Her administrative work in the project was taken over by Lékóné Fecskovics Szilvia

### *5. Problems*

There was no serious problem

## *6. Reference list*


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