

The aim of this project is to test hypotheses about the molecular mechanisms of differentiation and development of the cerebellar Purkinje cells on a model of a laboratory opossum which is a suitable for developmental studies, including studies of brain development.

The gray short-tailed opossum is an omnivorous, nocturnal marsupial from the family of Didelphidae. The opossum pups (3-14 pups in a litter) are born after 13.5 days of gestation at a very immature stage. Newborn pups weigh approximately 100 mg and attach to the mother teats for 4 weeks. At birth their nervous system is at a developmental stage comparable to the early embryonic stage in eutherians: E11 in the mouse or 6 weeks of pregnancy in humans. Studies in the opossum have shown that the general pattern of brain structure development and the sequence of structures' formation are similar to those in eutherians. However, the main difference between marsupials and eutherians is that in opossums, the development of the majority of the brain regions occurs after birth. The opossum pups are a model of choice for ex utero studies of the mechanisms of early stages of mammalian development and of the influence of external factors on development at these stages.

The cerebellar cortex of mammals, including opossum, is composed of three layers: the molecular, Purkinje and granular layer. The molecular layer contains axons of interneurons, Purkinje cell dendrites and glial cells fibers. The second layer is formed by cell bodies of Purkinje cells, aligned in a single row. Gamma-aminobutyric acid (GABA) is the neurotransmitter in the Purkinje cells. The deepest third layer of the cerebellar cortex consists of a huge number of very small granule cells, that are glutamatergic, stellate cells (GABAergic interneurons), Golgi cells, Lugaro and Bruch interneurons with different morphology and connections. Development of the mammalian cerebellum is complex and continues for a long time.

The main hypothesis to be tested is the assumption, that the GABA-ergic phenotype of these neurons is already determined at the stage of dividing progenitor cells, therefore it might not be changed by environmental influences acting on newly generated neuroblasts, while morphology of neurons developing from those neuroblasts, e.g. the size and shape of their perikarya, size and morphology of the dendritic trees, local or external targets of their axons, are not determined by (or not only by) the early genetic program, but predominantly by interaction of the migrating neuroblasts and young, developing neurons with other cells of the structure in which the neuroblasts settle. Therefore they depend at least partially on the interactions of these cells with their environment.

The second innovative element of the project is to construct the first transgenic laboratory opossum, expressing the green fluorescent protein (GFP). It could be later useful in many other experiments performed on this species.

We plan to create the transgenic opossum encoding GFP under the control of Sox2 promoter. First, we will isolate fertilized oocytes from females. Then embryos will be injected with the DNA construct (Sox2-eGFP construct) and those that will divide into 2-cells' stage during the overnight culture, are going to be transferred into pseudopregnant females.

In order to determine the fate of newly-generated cells in the cerebellum, animals at various developmental time points (from newborn to postnatal day 60) will receive subcutaneous injections of bromodeoxyuridine (BrdU). BrdU is incorporated into DNA during the S-phase of cell cycle. The long survival period after BrdU injections (3 months) will allow to determine the final location and phenotype of the labeled cells implanted into brain at specific developmental time points. Using the antibody against BrdU we will analyze BrdU-labeled cells of the known time of generation.

During the development of the mammalian cerebellum, neurons are generated by progenitor cells of the roof of the IV ventricle and in its rhombic lip. Then they migrate and reach their final destinations. The expression pattern of genes known to be involved in cerebellar development of mice, such as *shh* (sonic hedgehog), *Lhx1*, *Lhx5* i *Ptf1a* (pancreas transcription factor 1a) will be investigated with a highly sensitive in situ hybridization method. Primers will be designed, and the polymerase chain reaction (PCR) will be performed. Obtained fragment of DNA will be cloned into a plasmid. After sequence verification, RNA polymerase will be used to synthesize RNA complementary to DNA substrate. Prime kit will be used for random primed labeling of RNA probes.

The factors shaping general architecture of Purkinje cells' dendritic trees are still unknown. For this purpose, in the in vitro experiments we will test the effects of selected genes that are going to be downregulated on the fate of cerebral progenitor cells. Next, in vivo experiments will be performed, based on the results of in vitro cultures using shRNA or the adenoviral vector to study selected genes involved in formation of the Purkinje cells. We will use the electroporation technique to transfer foreign DNA into the opossum brain.

Finally, transplantation experiments will be performed to test our main hypothesis. Cells from the transgenic GFP opossum will be used for cellular transplantation experiments. The progenitor cells of the newborn donor opossums expressing GFP will be isolated from the roof of the IV ventricle. They will be injected into the roof of the IV ventricle or to the subventricular zone of the lateral ventricle of the opossum brain. Three weeks after transplantation the phenotype of cells expressing GFP will be determined.

As a result of these experiments we should gain knowledge on the timing and place of expression of some genes participating in morphogenesis of the cerebellar Purkinje cells and on the role of epigenetic factors in shaping the cerebellum. These data should also provide a better understanding of mechanisms involved in the mammalian evolution. Results of the investigations may contribute to elaboration of prevention methods of cerebellar developmental disorders in prematurely born children and children suffering cerebellar damage due to complicated delivery or later accidents.