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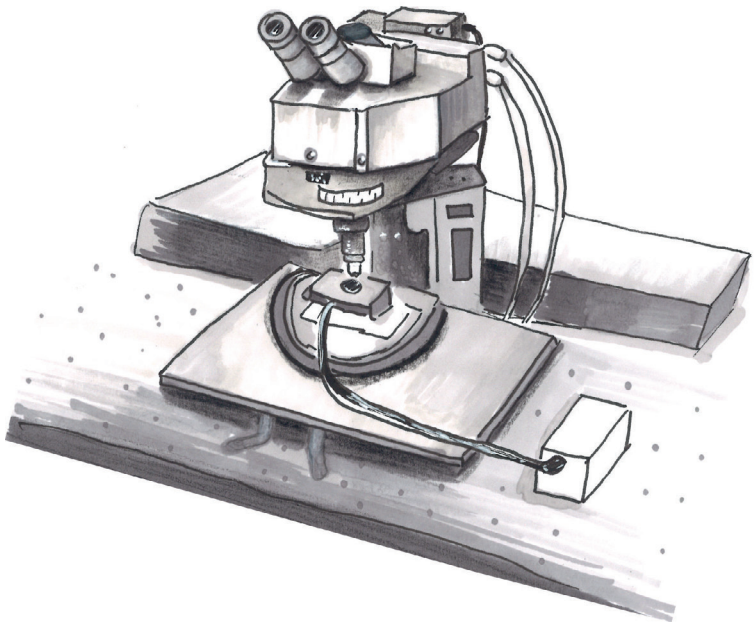
In Vivo Imaging of the Early Embryonic Cortex in Rodents



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In vivo imaging of the early embryonic cortex in rodents

Mikhail Yuryev

ACADEMIC DISSERTATION

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Contents

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III. Yuryev, M.*, Andriichuk, L.*, M. Leiwe, V. Jokinen and C. Rivera. "*In vivo* two-photon imaging of the embryonic cortex reveals spontaneous NMDA-sensitive calcium activity." Manuscript. *These authors contributed equally to this work.

These articles have been reprinted with the permissions of their copyright holders. In addition, some unpublished material is presented.
Author's contribution to the original publications:

I. Planning and performing in vivo experiments, design of the imaging chamber, data analysis, writing the manuscript.
II. Planning and performing in vivo experiments and ex vivo microscopy (including sample preparations), data analysis, writing the manuscript.
III. Planning and performing in vivo experiments, data analysis (except Figure 2), writing the manuscript.

Abbreviations

2D	two-dimensional
3D	three-dimensional
ACSF	artificial cerebro-spinal fluid
ADP	adenosine diphosphate
AM	acetoxymethyl
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N′ ,N′ -tetraacetic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
Ca2+	calcium ion
CaMK	calcium-calmodulin-dependent protein kinase
CCD	charge-coupled device
CNO	clozapine-N-oxide
CP	cortical plate
CREB	cyclic adenosine monophosphate response element-binding protein
CRISPR	clustered regularly interspaced short palindromic repeats
CSF	cerebro-spinal fluid
DREADD	designer receptor exclusively activated by designer drugs
E	embryonic day
FDA	food and drug administration
FITC	fluorescein isothiocyanate
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GECI	genetically encoded calcium indicator
GFP	green fluorescent protein
GPCR	G-protein coupled receptor

GTP	guanosine triphosphate
HSF/SF	hepatocyte growth factor/scatter factor
IP_3	inositol triphosphate
IZ	intermediate zone
IZ/SVZ	intermediate/subventricular zone
KCC	potassium-chloride cotransporter
LED	light-emitting diode
LFP	local field potential
LGE	lateral ganglionic eminence
MAPK	mitogen-activated protein kinase
MGE	medial ganglionic eminence
mGluR	metabotropic glutamate receptor
MRI	magnetic-resonance imaging
MZ	marginal zone
NCx	neocortex
NKCC	sodium-potassium-chloride cotransporter
NMDA	N-methyl-D-aspartate
NP	nanoparticle
OCT	optical coherence tomography
ORMOSIL	organically modified silica
P	postnatal day
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PFA	paraformaldehyde
PI_3	phosphoinositide-3
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC-b	phospholipase C β
PSi	porous silicon

ROI	region of interest
ROS	reactive oxygen species
STF-1	somatostatin transactivating factor 1
Str	striatum
SVZ	subventricular zone
TRITC	tetramethylrhodamine isothiocyanate
TRP	transient receptor potential
VGAT	vesicular GABA transporter
VZ	ventricular zone

Abstract

Embryonic brain development is a highly dynamic period in human life. Any disturbances at this stage can cause life-long negative consequences, such as developmentally related diseases, including autism, schizophrenia, and bipolar spectrum disorders. During development the mother-embryo interface plays a crucial role in supplementing the growing organism with oxygen and nutrients, regulating chemical cues, and protecting it from infections and potentially hazardous compounds. However, most of the studies of embryonic brain development have utilized *ex vivo* systems such as slices and neuronal cultures. Despite the great value of the data obtained from *ex vivo* studies, they usually completely ignore the significance of the mother-embryo interaction. Thus, there is desperate need for novel preclinical models of embryonic development.

In this thesis, we developed a novel technique for *in vivo* two-photon imaging in mouse embryos connected to the mother via umbilical cord. We developed the special chamber with polymer membrane allowing to keep embryos separately in the heated physiological solution while the umbilical connection to the anesthetized mother is preserved. We developed the protocol for stimulation of calcium activity using high-power laser radiation and studied the propagation of the resulting calcium waves in the mouse embryonic cortex *in vivo* under ketamine/xylazine anesthesia. We confirm the enhancing effect of caffeine on the evoked activity and the suppressing effect of the adenosine triphosphate (ATP) -receptor blockade, known from previous *ex vivo* studies. We analyzed the patterns of wave propagation and show the non-uniform spreading, which suggests the presence of differing connectivity patterns in the cortex already during the early stage of development.

Further, we studied spontaneous calcium activity and cellular motility in the mouse embryonic cortex *in vivo* under light isoflurane anesthesia. We demonstrate two various patterns of ongoing activity: sporadic activation of single cells and correlated activity in the form of calcium waves. We show that blockade of N-methyl-D-

aspartate (NMDA) receptors with ketamine inhibits the calcium activity *in vivo*, corresponding with the arrest of cellular motility.

In the last part of the thesis, we studied the dynamics of the externally introduced substance to the mouse embryonic brains *in vivo*. We used porous silicon nanoparticles, which are a promising drug delivery platform, as they can be loaded with poorly water-soluble drugs. We show that the nanoparticles can breach the placental barrier and accumulate in the brains of the embryo. To study the dynamics of nanoparticles when already in the cortex, we injected the embryonic brains intraventricularly. Nanoparticles, including ones 3-4 μm in size, were distributed in 80% of the cortex already 4 hours following the injection, thus demonstrating high motility in the brain tissue of embryos. We confirmed the motility of nanoparticles in real time using the *in vivo* two-photon imaging of embryos connected to the mother under ketamine/xylazine anesthesia. The results emphasize the susceptibility of the embryonic cortex at the early stage of development to external particles, which should be taken into account in nanomedicine development.

In summary, the developed *in vivo* imaging technique allowed functional studies in the embryonic cortex in real time. This will allow preclinical pharmacological investigations of the compounds while maintaining the physiological mother-embryo interface.

1. Introduction

At tissue and cellular levels, the developing brain differs fundamentally from the adult brain. The processes of cell proliferation, differentiation and migration are tightly intermixed and together orchestrate brain growth. The malfunction of any of these processes might cause developmentally related diseases, such as schizophrenia, mental retardation and neurodegenerative disorders, in the later stages of life.

The cerebral cortex is the part of the brain responsible for various behavior-related functions, in particular, cognitive. It actively forms during the midgestation stage of pregnancy in humans, a time during which the embryo is most vulnerable to non-physiological cues (Rakic, 2009).

The cerebral cortex forms in an inside-out manner, with the newly born excitatory neurons migrating towards the cortical plate from the ventricular zone (Rakic, 1988). Inhibitory neurons, in turn, are born in the distant region of the brain in the ganglionic eminences, and then undergo long-distance migration to reach the final destination in the cortical layers (Marin et al., 2010). This process allows neurogenesis to happen in distant areas under the influence of different external cues, while already differentiated populations of various cell types mix in the target cortical layers.

To ensure proper brain development, various intra- and extracellular cues regulate neuronal differentiation, migration, and maturation. Intracellular cues comprise timely expression of proteins, such as transcription factors (Kwan et al., 2012), while extracellular cues include motogenic and repellent factors, such as semaphorins and neurotransmitters (Marin, 2013). Among the intracellular factors involved in cellular development of the growing cortex, one can emphasize calcium (Berridge, 1995a) among the earliest second messengers that emerged during evolution. In the developing brain, calcium signaling regulates various processes, including protein expression, cell division, and migration. At the same time, calcium as a charged ion is involved in electrical processes specifically important for the neurons. Proper calcium homeostasis is crucial for the cell, as calcium ion (Ca^{2+}) can be involved in new cell generation and in cell death through apoptosis (Berridge, 2012;

Clapham, 2007). The calcium also translates extracellular cues into the intracellular signaling, thus allowing newly born neurons to interact with each other through gap junctions and synaptic contacts.

Until recently, most of the studies of the calcium signaling in the developing cortex were performed in *in vitro* and *ex vivo* models (Crepel et al., 2007; Weissman et al., 2004). However, these models lack the full connectivity of the real developing brain, where cells receive natural oxygenation, nutrition, and hormonal supplementation through the mother. Moreover, the whole developing brain is affected by cues from the maternal organism via the mother-embryo interface. The development of *in vivo* methods would allow direct observations of newly born neurons in the developing cortex, e.g. migration and calcium signaling under physiological conditions.

For studies of the brain formation and cortical development rodents represent valuable model due to large similarities in brain development in mechanistic as well as in morphological aspects (Figure 1).

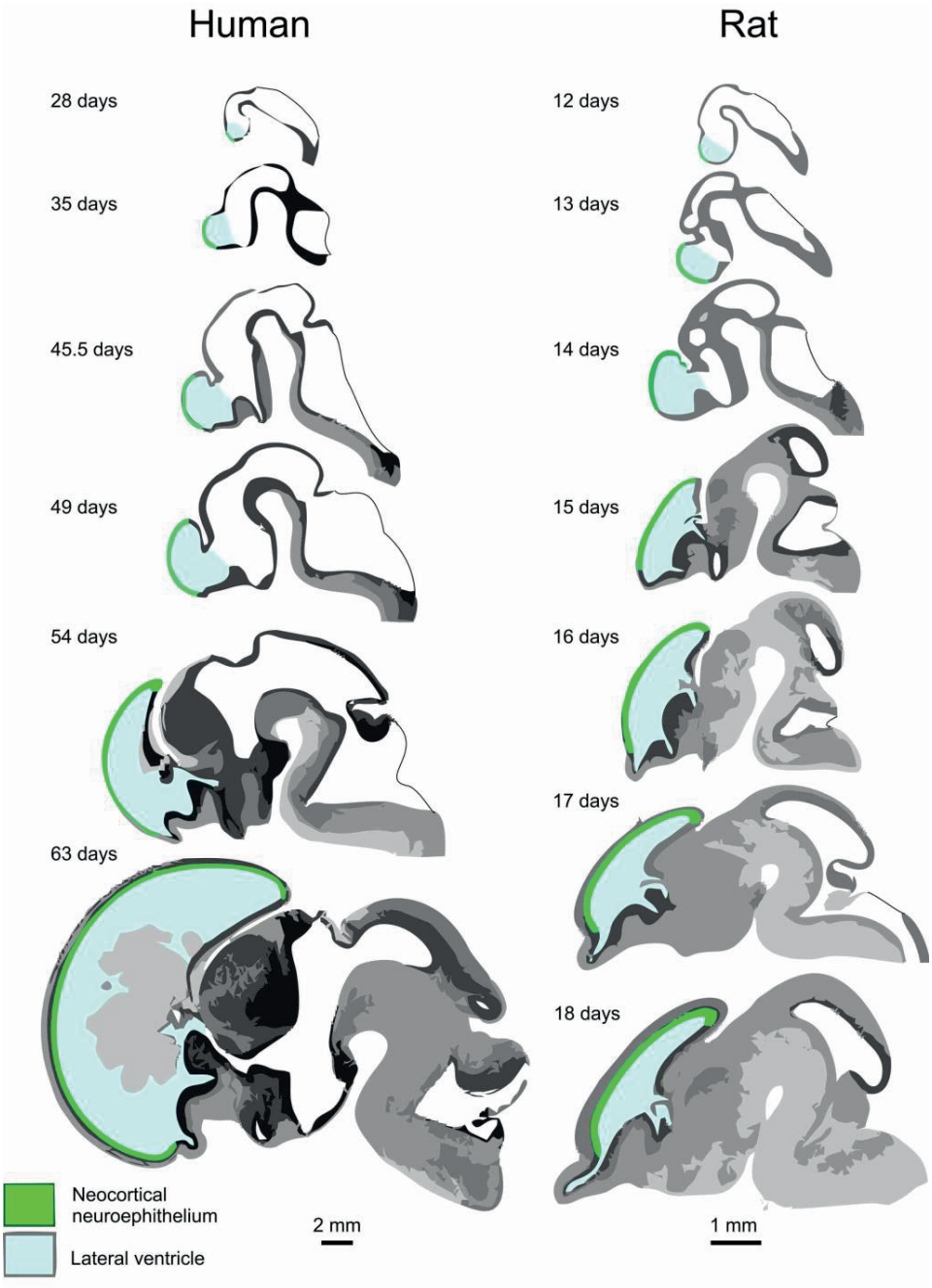

Figure 1. Comparison of the human and rat developing brains at various stages. Modified from neurondevelopment.org, The Laboratory of Developmental Neurobiology, Inc.

2. Review of the literature

2.1. Neurogenesis in the cortex

Most of the cortical cells stem from the ventricular zone (VZ), which is the epithelial layer of progenitor cells (Rakic, 1988). This process occurs extensively during the second trimester of pregnancy in humans. A major population of VZ is radial glia (Rakic, 2009). Radial glial cells have long apical processes extending throughout the cortical thickness to the marginal zone (MZ) (Figure 2A). Most of the excitatory neurons in the cortex stem from the radial glia, which divides in two ways: symmetrical division, giving birth to new radial glial cells, and asymmetrical division, producing a neuron and a radial glial cell. Another form of neurogenesis is through production of intermediate progenitor cells, which divide in the subventricular zone (SVZ) and then migrate along the radial glial fiber towards the cortical plate (Noctor et al., 2001). This type of neurogenesis is present more in humans than in rodents (Kriegstein, 2005), which reflecting the evolutionary development of the cortex (Kriegstein et al., 2006). The cortex of the human brain also differs from that of rodents by the abundance of outer radial glia lacking the apical process (Pollen et al., 2015). Radial glia serves as a source of neurons until the later gestation period when they differentiate to astrocytes (Kwan et al., 2012).

Most of the inhibitory GABAergic interneurons are produced in the ganglionic eminences and then tangentially migrate towards the target cortical layers (Figure 2B) (Marin, 2013). The GABAergic interneurons are generated in three populations: the majority stems from the medial ganglionic eminence (MGE), another population comes from the lateral ganglionic eminence (LGE), and the late-born interneurons migrate from the caudal ganglionic eminence.

Neurogenesis is tightly regulated by various genetic factors. The neuronal progenitors and radial glia express the factor Sox2, which is downregulated during cell maturation (Graham et al., 2003). The differentiation of the cells is regulated by

various factors such as Cux2, Fgf8, Emx2, and Pax6 (Nieto et al., 2004) (Bishop et al., 2000; Crossley et al., 1996; Englund et al., 2005).

2.2. Migration of cortical neurons

2.2.1. Radial migration

Radial migration occurs in an inside-out manner, with newer cells migrating towards upper layers of the cortex (Rakic, 1988). Thus, the first neurons occupy eventually the deepest layers starting from the preplate region and the last ones to appear are situated in the superficial cortical layers. According to the current view, neurons stemming from the same radial glial cell occupy common space in the forming cortex, organizing in clusters known as cortical columns (Rakic, 2009).

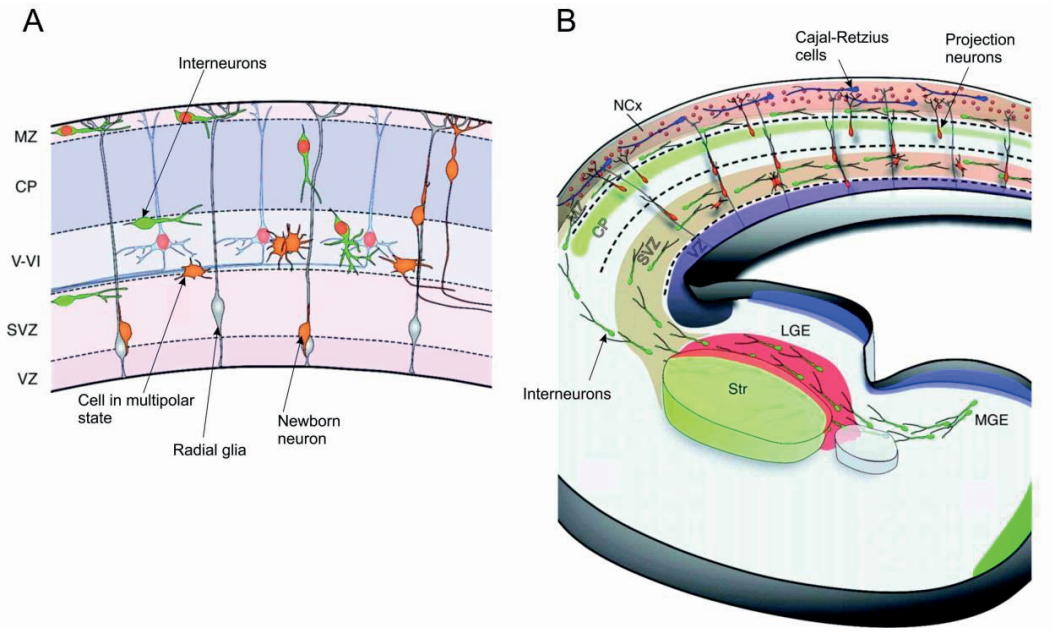

Figure 2. Neurogenesis in the embryonic cortex. **A.** Scheme of the forming layers of the embryonic cortex. **B**. Scheme of migrating neurons arriving to the cortical layers. MZ, marginal zone; CP, cortical plate; V-VI – cortical layers V-VI; SVZ, subventricular zone; VZ, ventricular zone; NCx, neocortex; Str, striatum; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Modified from (Marin et al., 2010) with permission.

From the VZ, the newly born neurons migrate through the intermediate zone (IZ), following the radial glia fiber (Rakic, 1972). Alternatively, the cells that already have their apical process in the marginal zone, radially translocate their somas (Nadarajah and Parnavelas, 2002). The radial migration strongly depends on the correct interactions with radial glia. The radial glia fibers, which extend throughout the thickness of the growing cortex, are used for guidance of the migration of newly born neurons (Rakic, 1988). The interactions between the neurons and radial glial fibers are conveyed by cell-adhesion molecules, residing on the membrane. Astrotactin, neuregulin, and integrins are also responsible for the radial glial fiber interactions with the neurons (Adams et al., 2002; Anton et al., 1997; Edmondson et al., 1988; Fishell and Hatten, 1991). Electron microscopy (EM) studies have shown that the migrating cells produce tight connections with the radial glia, and connexin channels are also involved in the process (Elias et al., 2007).

Radial migration is also regulated by various extracellular cues such as semaphorins, netrins, slits, and Reelin. For example, the reeler mice, with mutations in Reelin (D'Arcangelo et al., 1995), have defects in cortex layering, and show inverted lamination (Caviness, 1982). The Reelin molecule is released by Cajal-Retzius cells, which reside in the marginal zone (D'Arcangelo et al., 1999). Mice with mutations in gene Dab1 (Howell et al., 1999a; Howell et al., 1999b) show a similar phenotype as Reeler mice because addition of phosphoryl group to Dab1 protein affects microtubule dynamics (Beffert et al., 2004). Reelin signaling also plays a part in the phosphorylation processes, which are involved in the regulation of microtubule dynamics, affecting motion of the cell (Marin et al., 2010).

2.2.2. Tangential migration

The cortical inhibitory interneurons are born in ganglionic eminences distant from the regions where excitatory neurons are born. This allows initial cellular differentiation under the control of non-interfering environmental cues (Marin,

2013). When generated, the interneurons migrate tangentially along the cortical layers towards the target regions. The first population of the GABAergic cells are Cajal-Rezius cells in the upper layers of the cortex, and they are believed to mature already by the time when the major population of MGE- and LGE-produced GABAergic neurons are tangentially migrating along the cortical regions (Marin et al., 2010).

The direction of tangential migration is regulated by chemotaxis. Several motogenic factors are involved in the regulation of tangential migration. These factors ensure that the cells do not engage in radial migration. Brain-derived neurotrophic factor (BDNF) and neurotrophin 4 have been shown to stimulate neurons to migrate to the marginal zone of the cortex (Brunstrom et al., 1997). PI3-kinase signaling (Polleux et al., 2002) and the ERK pathway (Segarra et al., 2006) are involved in the mechanism of the growth factor-mediated neuronal migration. Hepatocyte growth factor/scatter factor (HSF/SF) also acts as a mitogen (Powell et al., 2001). Somatostatin transactivating factor-1 (STF-1) attracts the tangentially migrating interneurons to IZ/SVZ via CXCR4 receptors, expressed in migrating interneurons. Ambient neurotransmitters, such as gamma-aminobutyric acid (GABA), regulate tangential migration as well (Luhmann et al., 2015).
Migrating neurons utilize axons of the other neurons as extracellular substrate (Metin and Godement, 1996). The neurons can also interact with extracellular matrix of other cells during migration (Bespalov et al., 2011).

The molecules involved in axon guidance can also affect neuronal tangential migration. The interneuronal migration in the MZ and SVZ is controlled by chemokines, which cause the neurons to stay in the area (Li 2009, Lopez-Bendito 2008). Semaphorins, slits, and netrins, involved in radial migration regulation, are also able to regulate tangential migration. The semaphorins Sema3A and Sema3F repel neurons from the LGE and from migration to the striatum (Marin et al., 2001).

Interestingly, the migration modes are not always constant. Thus, newly generated interneurons first migrate radially, then tangentially to the region of

residence through IZ/SVZ, then when positioned in MZ they express a 'random walk'-type behavior and finally descend radially to the cortical layers (Hatanaka et al., 2016). In the cerebellum, in turn, the excitatory neurons, granule cells, also demonstrate a switch in migration mode; initially, they are born in the rhombic lip and migrate tangentially, later migrating radially to the final positions (Fink et al., 2006; Komuro and Yacubova, 2003). Changing of migration modes is advantageous for generation of various neuronal populations. Thus, types of neurons can be defined already where they are born, in distant areas, which chemical environments do not interfere with each other. Later, after they are differentiated, they can migrate to the target brain tissues and intermix there.

2.2.3. Migration mechanisms

Neuronal migration depends on the polarization of the cellular morphology (Marin et al., 2010). Polarization is maintained by a leading process that defines the direction of the motion. Leading processes of migrating neurons share similarities with growth cones of axons in the roles of sensing the external environment. The leading process detects the chemotactic cues that guide the migration. Usually, the process of cellular motion works in three steps: the leading process extension, the nucleus translocation towards the leading process, and the removal of the back process of the cell.

Cellular motion is based on polymerization and depolymerization of actin fibers that push the cellular membrane. Small GTPases such as Rac regulate the protrusion formation, while Cdc42 is involved in the determination of migration direction (Lauffenburger and Horwitz, 1996). Rho is another small GTPase responsible for the contraction of actomyosin, which leads to overall cell movement forwards (Hall, 1998; Marin et al., 2010).

A serine/threonine cyclin-dependent kinase CDK5 is involved in the extension of the leading edge of the cells, via interaction with actin regulators (Dhavan and Tsai, 2001; Kawauchi et al., 2006; Nikolic et al., 1998). The Golgi apparatus is involved in the centrosomal translocation, which precedes the somatic

displacement. *In vivo* imaging in mice has revealed involvement of the Golgi complex in defining branching direction of the migrating interneurons (Yanagida et al., 2012).

Cellular motility is also regulated by Ca2+ (Tsai et al., 2015). Calcium affects myosin light chain kinase (MLCK) and myosin (Kasturi et al., 1993), which are involved in the motion of the cell forward. Calcium regulates actin dynamics as well, via protein kinase C activation (Larsson, 2006) and via calmodulin-dependent kinase pathways (Braun and Schulman, 1995). Intracellular calcium can also influence adhesion, which affects the overall motion of the cell (Su et al., 2006).

2.2.4. Electrical activity effects on migration

Calcium activity correlates with neuronal migration *in vitro* (Komuro and Rakic, 1996). Experiments in brain slices have shown calcium activity correlation with the nuclear translocation of the radial glia (Liu et al., 2010b), which are involved in ATP release. Also, calcium bursts correlate with periods of motion of the soma of radial glia (Rash et al., 2016). The migration of interneurons also depends on calcium activity. Potassium-chloride cotransporter KCC2 expression, which leads to the GABA signaling shift from depolarizing to hyperpolarizing, decreases the calcium oscillation frequency, thus reducing the migration of interneurons (Bortone and Polleux, 2009). Also, reduction of GABA-mediated depolarization via blocking of sodium-potassium cotransporter NKCC1 with bumetanide slows down the migration of vesicular GABA transporter (VGAT) interneurons *in vivo* (Inada et al., 2011). This is in line with measurements of reduced interneuron migration rate in glutamic acid decarboxylase GAD67-GFP mice, which have a lower level of GABA than VGAT-GFP mice, because one allele responsible for GABA production is exchanged for GFP. In the same study, chelation of Ca2+ with BAPTA-AM, which blocks Ca2+ oscillation, virtually stopped the neurons (Inada et al., 2011).

In neuroblast cultures, calcium channels are involved in migration of the neural progenitors. During differentiation, the low-threshold voltage-dependent calcium channels are expressed in the progenitor cells at the early stage (Louhivuori

et al., 2013). Blocking of these channels significantly reduced the migration of the progenitors, also affecting neurite formation.

In the developing cerebellum, N-type calcium channels participate in the migration process (Komuro and Rakic, 1992). These could be activated by the various neurotransmitter receptors that are also involved in neuronal migration. In cerebellar slice preparation, addition of antagonist of glutamate NMDA receptor caused a significant reduction in migration distance (Komuro and Rakic, 1993). Magnesium and glycine also affected neuronal migration. In contrast, the GABA-receptors antagonist did not significantly affect the migration. Chelating intracellular calcium also reduced migration (Komuro and Rakic, 1993). Furthermore, in the cerebellum, somatostatin causes neurons to either migrate or stop depending on the frequency of calcium oscillations (Yacubova and Komuro, 2002). Glutamate receptors mGluR5 block causes an increase in the motility of neuronal progenitors (Jansson et al., 2012). AMPA/kainate receptors block, in turn, causes a reduction in cells motility. Another neurotransmitter, ATP, regulates migration of intermediate neuronal progenitors (Liu et al., 2008).

As the neuronal migration mechanisms share several principles with axon guidance and axons growth is dependent on neuronal activity, it could be speculated that local electrical activity would also affect neuronal migration. As an example of the emerging long-distance connectivity between different parts of the brain, thalamocortical axon guidance depends on the activity in the subplate region (Hoerder-Suabedissen and Molnar, 2015). Aberrations in the subplate formation affect the activity patterns and also the proper organization of cortical structures such as the barrel cortex in rats (Tolner et al., 2012). Various mutations in the subplate, such as in p35, Sox5 and Tbr1, lead to improper cortical layering, suggesting incorrect positioning during the migration phase. The classical example of aberrant cortical layering due to incorrect migration is Reeler mice (Hoerder-Suabedissen and Molnar, 2015).

2.3. Calcium signaling in the developing cortex

2.3.1. Principles of calcium signaling

The ability of calcium to regulate so many intracellular processes relies on the very low intracellular calcium concentration, which is 20 000 times lower then the extracellular concentration. One of the main signaling features of calcium is its binding to carboxylate oxygen in amino acids, which causes conformational changes in the proteins and alters the local electrostatic fields (Clapham, 1995).

The low intracellular concentration of calcium is mainly kept by active extrusion machinery. The most crucial component, calcium ATPase, uses energy of ATP dissociation to adenosine diphosphate (ADP) to pump calcium outside the cell (Figure 3). Sodium calcium exchanger uses the membrane gradient of the sodium ion, letting sodium inside the cell in exchange for extruded calcium. Part of the intracellular calcium can be stored in cellular compartments such as the endoplasmic reticulum and mitochondria. Calcium ATPase also uses energy of ATP dissociation to ADP to pump calcium into the endoplasmic reticulum. Also, Ca^{2+} can penetrate NMDA receptors and AMPA receptors, which are involved in the fast calcium dynamic during electrical signaling.

Calcium is involved in signaling processes of various speeds. The fast calcium signaling (on the timescale of tens of ms) is initiated by voltage-gated Ca^{2+}-selective channels, e.g. following an action potential. The change in the potential difference on the membrane forces the charged aminoacid residues in the channel to induce change in the conformation and opening of the pore (Clapham, 2007). This type of fast calcium signaling allows calcium transients to be used as an indicator of electrical activity in cells.

The slower calcium signaling (on the timescale of hundreds of ms) independent of membrane potential changes, is via G-coupled receptors (GPCR). It could be caused by cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol triphosphate (IP_3), which causes the release from the intracellular stores in

endoplasmic reticulum (Berridge and Irvine, 1989). This type of signaling is important in undifferentiated cells which do not have synaptic connections (such as neuronal progenitors and e.g. tumor cells). An alternative way for intracellular signaling is via activation of transient receptor potential (TRP) channels and tyrosine kinase (Trk) receptors (Clapham, 1995). Ca2+ is also released upon activation of ryanodine receptors. The common ryanodine receptors activator is caffeine (Friel and Tsien, 1992). Inside the cell, calcium regulates variety of processes such as gene expression and kinase activity.

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2.3.2. Patterns of calcium signaling in the developing brain

In mammals, calcium is involved in the intracellular processes from fertilization (Gilkey et al., 1978). When spermatozoa reach the egg, calcium wave propagate throughout the cell, signaling the start of life. Further, the calcium oscillations are ongoing throughout brain development; e.g. highly dividing cells such as neuronal progenitors show calcium oscillations.

In the early embryonic cortex, calcium oscillations in *ex vivo* slices have been observed in the mode of single-cell transients, coupled synchronous calcium spikes, and calcium waves (Owens and Kriegstein, 1998). Calcium waves at these early stages during massive cell proliferation were mainly observed in the ventricular zone of the cortex. Calcium waves propagate in radial glia through consequential release of ATP-activating P2Y1 receptors, which are G-protein-coupled receptors. This leads to cleavage of PIP2 into IP3, which releases Ca2+ from intracellular calcium stores (Weissman et al., 2004). Blocking of ATP receptors with suramin (Dunn and Blakeley, 1988) has been shown to affect the cell cycle of proliferating radial glial cells. The waves observed in those experiments were also gap junction-dependent. However, as the authors manipulated the gap junctions by decreasing the calcium level, the physiological meaning of the radial glia waves remains unclear.

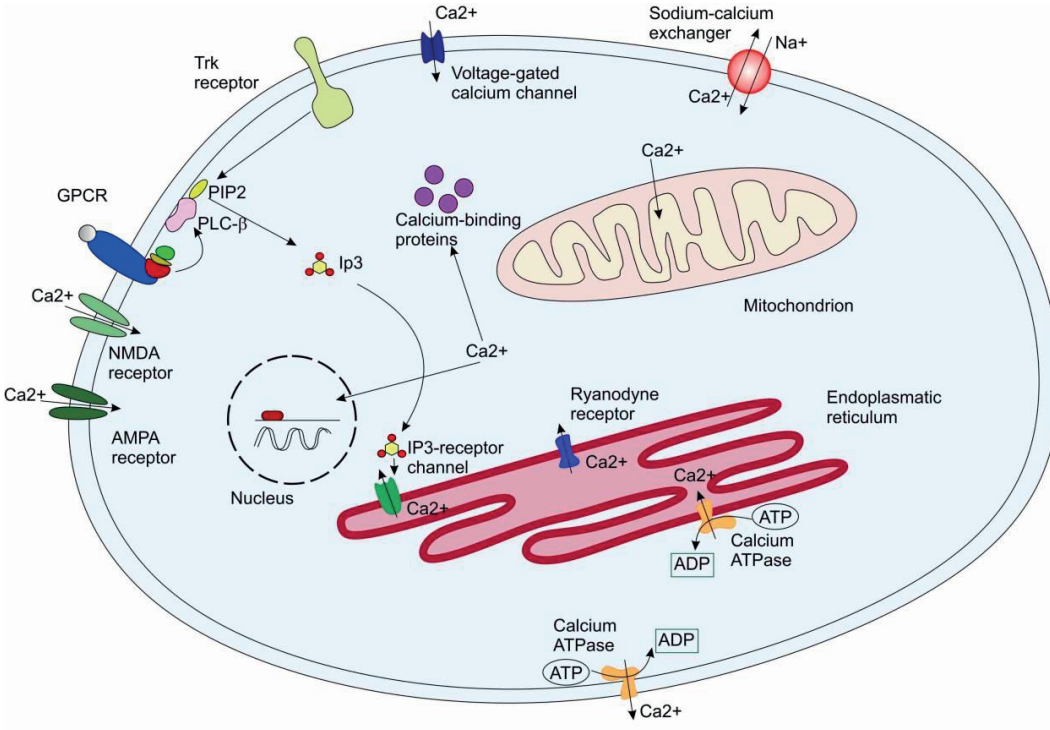

Figure 3. Principles of calcium signaling in the cell. GPCR, G-protein coupled receptor; PLC-β, phospholipase C beta; PIP2, Phosphatidylinositol 4,5-bisphosphate; IP3, inositol triphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Trk, tyrosine kinase; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

Single calcium oscillations in the embryonic brains during active cell proliferation follow the small-world network organization already at developmental stage E9 (9th embryonic day of development) in mice (Malmersjo et al., 2013). Small-world network is characterized by small number of steps for one cell to communicate with the other, even though they are not connected directly. This suggests the existence of early network patterns already prior to the appearance of coordinated activity, which occurs later in ensembles of differentiated neurons (Crepel et al., 2007). The networks at these early stages are connected via gap junctions. Gap junctions are present in various types of cells, such as cardiomyocytes, pancreatic

islets, and astrocytes, as well as in tumor cells. In developing brains, gap junctions are the first contacts, appearing before synaptic connections (Montoro and Yuste, 2004; Yuste et al., 1992).

Gap junctions consist of connexin channels, making pores in the membranes that allow ions, and small molecules like ATP, to propagate from one cell to another. The initial approach to the study of gap junctions used this property, i.e. after injecting a dye with relatively high molecular weight in one cell, it propagated to connected cells. Calcium ions readily pass the gap junctions, providing robust intercellular communication. Importantly, gap junction permeability can be attenuated by various factors, e.g. intracellular calcium concentration (Weissman et al., 2004), thus fine-tuning the communication parameters.

2.3.3. Changes in calcium signaling over the course of development

The early calcium oscillations in dividing cells are initially present in the epithelial layers of progenitor cells (Malmersjo et al., 2013). During further developmental stages (E13-E15 in mice), calcium activity is still mainly observed in the ventricular zone in radial glia, thus in the highly dividing cells (Owens and Kriegstein, 1998). At that stage calcium oscillations are connected to the cell cycle, which was shown in *ex vivo* experiments (Weissman et al., 2004) as well as in mathematical calculations (Barrack et al., 2014). Mathematical modeling has revealed that calcium oscillations follows the cyclins concentrations changes, which could, in turn, affect the cell cycle (Barrack et al., 2015). Thus, in the dividing cells calcium works as an intercellular messenger to regulate the cell cycle. Moreover, calcium activity can communicate the signals from the dividing epithelial progenitors to the differentiated migrated cells via radial glial fibers, as they pass through the thickness of the newly forming cortex (Rash et al., 2016).

As the number of differentiated neurons in the brain expands at stages E14-E17, spontaneous activity becomes present in the maturing neuronal population and

could be observed as sporadic and correlated calcium activity (Conhaim et al., 2010) (Ferrari et al., 2012; Gust et al., 2003). The calcium activity observed includes short-duration calcium spikes (sharp rises and fast drops of the amplitude) and long-duration calcium plateaus (sharp rises, but long keeping of the same amplitude before drop).

During the early postnatal development of the cortex in mice, some calcium oscillations appear in clusters, or 'patches' (Yuste et al., 1992), connected via gap junctions. These clusters are believed to share the same predecessor (Rakic, 1988) which produces the cells later forming the same cortical column. Later, during synapse formation, correlated activity begins to appear and is observed not only in the ventricular zone, but also in the hippocampus, caused by giant depolarizing potentials through GABA-mediated excitation (Crepel et al., 2007). At these stages, the mechanism of the calcium activity is also changing, from G-coupled protein-mediated intracellular calcium release (Berridge, 1995b) to activity-dependent activity.

The duration of the calcium signal decreases over development. As gap junctions decrease, the intercellular signal propagation is mediated mainly through synaptic connections. However, input of gap junctions in the postnatal stage of development is still significant (Montoro and Yuste, 2004). At the postnatal stage, not only neurons, but also the astrocytes get involved in the signal mediation and regulation of the network calcium activity (Thrane et al., 2012).

2.3.4. Disturbances in calcium signaling in the developing brain

As calcium signaling is involved in a vast variety of processes in the cell, an aberrant calcium signal can cause malfunctions in the brain (Berridge, 2012). Calcium signaling is involved in various aspects of schizophrenia, leading to problems for the person understanding what is real (Berridge, 2012). In GABAergic cells, Ca2+ controls cAMP

response element-binding protein (CREB) phosphorylation, which, among other things, affects the production of GABA. In schizophrenia, GABA production is insufficient, leading to malfunctioning of the inhibitory system, one of the hallmarks of schizophrenia. The changes could be observed as impairmed gamma-rhythm generation, which is reflected in frequency band 25-100 Hz in electroencephalographic recordings, and related to the attentive focus (Lee et al., 2003; Meyer-Lindenberg, 2010; Uhlhaas and Singer, 2010). Ca2+ signaling can also be affected in bipolar disorder, which causes oscillations in the moods and shifts from depression to the elevation. One of the mutations (in gene B-cell lymphoma 2, Bcl-2) associated with risk of bipolar disorder results in increased level of Ca2+ in cells and also stronger IP3-induced release of Ca2+ from stores (Machado-Vieira et al., 2011).
Calcium signaling changes are also involved in autism spectrum disorders. Mutations in Long QT (LQT) genes, which affect ion pumping channels, inevitably change the calcium signaling (Gargus, 2009). Integrating various existing data on the involvement of calcium signaling in autism spectrum disorders, the signaling pathway analysis combined with statistical detection of mutations, suggested general involvement of the PKC-MAPK cascade in the disease outcome (Wen et al., 2016).
The most frequent inherited autism form is Fragile X syndrome. *In vitro* studies in neuronal progenitors derived from Fragile X syndrome patients using induced pluripotent stem cells have shown that the calcium responses mediated by the mGluR5 receptor differ from the controls (Achuta et al., 2016). In the Fragile X mouse model, the calcium responses to depolarization through potassium were increased (Achuta et al., 2014).

2.4. Anesthesia effects on the developing brain

Evidence indicates neurocognitive deficits following long neonatal exposure to repetitive anesthesia. The effects of short-term anesthesia are less clear. There is a growing body of knowledge from animal experiments on animals regarding the

adverse anesthesia effects; however, often the data from human studies do not show significant effects of short-term anesthesia.

2.4.1. Apoptotic effects

Anesthesia, e.g. by ketamine and isoflurane, has been shown to cause apoptosis in embryonic brains of primates and mice (Brambrink et al., 2012a; Brambrink et al., 2012b). As apoptosis appears in the normally developing embryonic brain as part of the brain formation process, there is still uncertainty whether anesthesia speeds up the apoptosis of cells already destined to death or drives new cells into apoptosis (Brambrink et al., 2012c).

NMDA receptor blockade has shown an increase in apoptosis in the developing rat brain (Ikonomidou et al., 1999). The NMDA receptor blocker MK801 caused a concentration-dependent neuronal degeneration via apoptosis at stages E17-P14 of development. Most of the neurons degenerated in the course of 48 hours following the agent administration, with degeneration increasing over 24 hours. The effect of MK801 blocker increased up to developmental stage P7 (7^{th} postnatal day of development), and then decreased, with no significant apoptotic effects at P21.

NMDA receptors blocking ketamine anesthesia have also been shown to produce apoptosis in the brains of rhesus macaque embryos, which development closely resembles one of the human (Brambrink et al., 2012b). The apoptosis rate in the brain of fetuses, whose mothers had received ketamine anesthesia was higher than in neonates who received anesthesia at a comparable level. The cerebellum was especially affected in the fetuses exposed to ketamine, with internal granule cells undergoing apoptosis.

Various anesthethics can affect different areas of the brain. For example, the primary visual cortex in neonates exposed to isoflurane has shown a much higher rate of apoptosis than with ketamine. Isoflurane induced apoptosis in neuronal layers II and V of visual cortex (Brambrink et al., 2012a; Brambrink et al., 2010). In mice, the cell survival and apoptosis upon exposure to isoflurane has also been tested for dependence on cell age, controlled by bromodeoxyuridine injections (Hofacer et al.,

2013). In hippocampus, the dentate granule cells were the most affected by the isoflurane anesthesia at the age of P21, although at the age of P7 the effect was not significant, in contrast to the whole brains (Ikonomidou et al., 1999). At P49, the neuroapoptosis was strongly reduced, albeit still significant, in dentate gyrus, relative to control conditions.

Experiments in mice have also shown specific vulnerability of GABAergic neurons to anesthesia. Exposure of cortical slices from early postnatal mouse brains to NMDA blockers induced autophagy in GABAergic neurons (Roux et al., 2015). Inhibition of autophagy reduced the increase in apoptosis caused by the NMDA blockade by MK-801. Enhancement of the autophagy, on the other hand, caused an increase in the apoptotic rate.

In vitro, ketamine conveys its toxic effects on the differentiating cells via suppression of calcium activity (Sinner et al., 2011). Application of ketamine and MK801 for 24 hours led to decrease in calcium-calmodulin-dependent protein kinase CaMKII expression. It also resulted in the lower synapsin expression. Importantly, at the differentiating stage, even concentration of ketamine 3 μM, which is low in comparison to anesthetic doses, turned out to be significantly toxic. Ketamine also affects the development of dendrites (Vutskits et al., 2006). Thus, overall, ketamine affects the process of synaptogenesis.

2.4.2. Effects of anesthesia on network activity

Different types of anesthesia produce different effects on the network activity and these effects vary depending on the developmental stage of the organism. In adults, the effects of anesthesia are well studied, e.g. effects on waves, which are detected by electroencephalography. The cellular mechanisms of anesthesia are also known in vivo (Thrane et al., 2012). However, the effects of anesthesia on activity in immature brains are still largely unknown.

Isoflurane was shown to suppress the early gamma and spindle-burst oscillations in early postnatal rats (Sitdikova et al., 2014). Isoflurane affects mainly

GABA, glutamate and glycine receptors. Due to these multiple actions, frequency of the spontaneous bursts decreases with an isoflurane concentration for light anesthesia above 0.5%, with blockade of spontaneous bursts at a concentration of 1.5%, which is sufficient for deep anesthesia in adults. Multi-unit activity is also suppressed. Interestingly, the suppression of spontaneous alpha/beta oscillations, as well as spontaneous gamma oscillations, is less profound in the later stages of development and not significant at the age P16-P69. Evoked responses from whisker stimulations have revealed similar findings, with a decreasing effect over the course of the rat's development. This implies that the immature brain network is more susceptible to the effects of isoflurane anesthesia, then adult brain. Ketamine-midazolam anesthesia also shuts down the cortical activity in early postnatal rats (Lebedeva et al., 2016). At the stage P5, 40 mg/kg of ketamine and 9 mg/kg of midazolam almost completely suppressed spontaneous activity in layer IV of the somatosensory cortex. Ketamine is non-competitive antagonist of the NMDA receptors, while it also modulates nicotinic acetylcholine receptors. Midazolam, in turn, affects GABA receptors activation. Thus, the precise molecular mechanisms of the effects are still to be elucidated.

In line with the anesthesia effects, NMDA receptor disturbance by alcohol also suppressed the spontaneous activity in P1-P2 neonatal rats (Lebedeva et al., 2015). At P5, ethanol suppressed the burst activity in the cortex. The developmental profile of the activity inhibition corresponded to the apoptosis rate, with apoptosis reaching its peak during the first postnatal week, when the alcohol-inhibiting action on the network was the most profound. Alcohol caused concentration-dependent apoptosis in the cortex, with a significant apoptosis increase at exposure to concentrations higher than 1 g/kg tissue. Also, the multiunit activity suppression by alcohol was activity dependent, with a significant reduction occurring already at 1 g/kg tissue concentration. Movements were reduced under alcohol exposure as well. This is important for development of treatments for fetal alcohol syndrome, which develops upon maternal exposure to alcohol.

As the early postnatal rat is a model of human brain development during the embryonic stage *in utero*, these activity inhibition effects are important for explaining the toxic effects of anesthesia in the developing brain in contrast to the relatively safe action in the adult, developed brain. A possible explanation for the different outcomes is strong dependency of the immature neurons on activity for survival (Mennerick and Zorumski, 2000).

2.5. Methods for visualization of the developing brain

2.5.1. Imaging methods for the embryonic brain *in vivo*

Probably the biggest body of knowledge about the mechanisms of brain development in the current literature comes from traditional histological investigations. Despite the significant improvements in quality and functionality of histological techniques, such as use of antibodies, for long time they were mere modifications of the techniques developed in the 1900s by Ramon y Cajal and others. Extensive basic research was done by Hans Spemann and others using traditional microscopy.

The breakthrough for *in vivo* imaging of humans came from the 1941 invention of ultrasound imaging techniques by Karl Theo Dussik and Friedreich Dussik. The ultrasound imaging became popular in the 1960s. This remained the most popular medical imaging method for following human embryo development. An improvement in resolution could be achieved by using magnetic resonance imaging (MRI) (Roberts et al., 2014) (Figure 4A). MRI can be used to detect malformations of the brain such as double cortex or mysencefalia. Further improvements became possible with invention of *in vivo* optical imaging methods in animals.

2.5.2. Optical imaging in embryos

The advantage of optical imaging is that it is possible to combine with non-invasive manipulation using optogenetic and uncaging approaches. Moreover, one can use the intrinsic properties of the neurons to release neurotransmitters such as ATP by direct photostimulation of the cell, thus activating the cellular network.

At the very early stages of development the processes on going inside the embryo can be observed with conventional wide-field microscopy. Hans Spemann has performed experiments with live embryos using optical microscopes as early as

in the late 19th century. These experiments led to development of such concepts as Spemann organizer principles and a variety of other basic biological development theories.

Nowadays, wide-field fluorescence microscopy is commonly used for the purposes of studying early developing embryos, which can be cultured in an externally oxygenated medium. The use of fluorescent tags for specific proteins is very convenient for studying, for instance, cell lineages (Mavrakis et al., 2008). Confocal microscopy is also widely employed because the more the embryo grows, the more profound the need for optical sectioning for identification of cell layers and separate cells. The modern spinning disc confocal imaging allows detailed imaging at high speed (Lam et al., 2014), which is highly desirable for studies of calcium signaling (Hou et al., 2014). The latest achievements in the development of fluorescent microscopy are super-resolution techniques, which allow imaging beyond the diffraction limit of the light. Such methods have already been applied for the imaging of live Drosophila embryos, allowing examination of protein dynamics *in situ* at a precision unimaginable earlier (Truong Quang and Lenne, 2015).

Another developing method is optical coherence tomography (Figure 4B). It can be used for non-invasive imaging of embryos and to follow the development of limbs, brain, heart, and other organs over several days (Larina et al., 2009; Larina et al., 2012). For example, alcohol effects on embryo development were tracked in the same animal for several days (Sudheendran et al., 2013). The resolution thus far is below subcellular, but higher than with the other non-invasive methods. Optical imaging methods are especially convenient for use in studies of zebrafish embryo development, as the embryo is completely transparent at the beginning of development. The short-term imaging of cultured murine embryos is possible at the stage E9.5 with wide-field fluorescent microscopy (Malmersjo et al., 2013) provided that there is sufficient oxygenation of the medium.

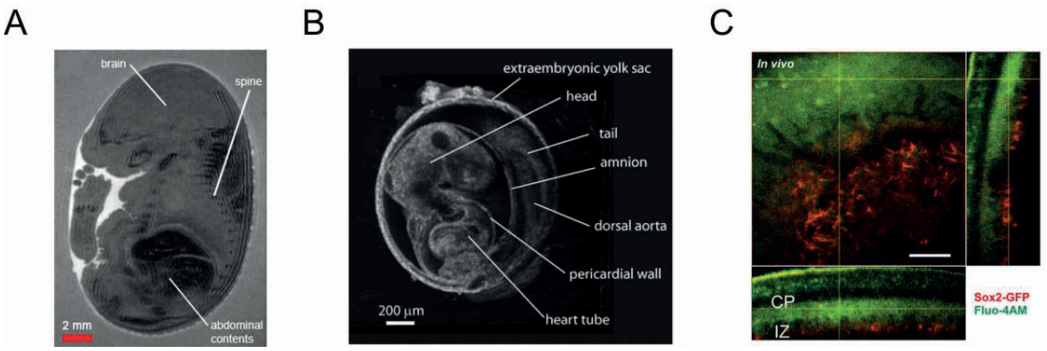

Figure 4. Comparison of methods for imaging embryos. **A**. Embryo image obtained with magnetic resonance imaging (MRI). Scale bar 2 mm. Modified from (Roberts et al., 2014). **B**. Embryo image obtained with optical coherent tomography (OCT). Scale bar 200 µm. Modified from (Larina et al., 2009). **C**. Embryonic cortex image obtained with two-photon microscopy. Scale bar 100 µm. Modified from (Yuryev et al., 2016).

2.5.3. Two-photon microscopy of embryos

One of the methods coming into routine use in the basic studies of brain development is two-photon microscopy (Denk et al., 1990; Helmchen and Denk, 2005). The two-photon imaging method is based on non-linear two-photon absorbance (Göppert-Mayer, 1931). In the one-photon excitation methods (e.g. conventional confocal microscopy), the probability for the fluorescent molecule to adsorb the photon for excitation is proportional to the intensity of illumination. In contrast, for two-photon excitation the adsorption probability has squared dependence on the intensity. This leads to the effect that simultaneous adsorption of two photons for excitation of the molecule appears only in the focal point of the objective (Figure 5C), where the intensity is maximal, and virtually no molecules are excited outside of the focus (Helmchen and Denk, 2005). Moreover, the excitation can appear only with extremely high intensities that could not be achieved with continuous emission lasers and become possible only with femtosecond lasers, in which the very short femtosecond pulses of extremely high intensities are followed by long periods without emission. In such a case, the power of light intensity in the focal point reaches the level of gigawatts.

Since the illuminating light is in the infrared spectra (Figure 5A), the energy of single photons is not enough for excitation of the conventional fluorophores and so not adsorbed (Figure 5B and C). Importantly, the infrared light is much less scattered by biological tissues than the visible spectra light. Taken together, the parameters of two-photon microscopy achieve significant improvements for the imaging of biological tissues, enabling visualization *in vivo* with the resolution of confocal microscopy (Helmchen and Denk, 2005).

As it allows subcellular resolution and ultimately can provide imaging of histological quality, *in vivo* two-photon imaging became very popular in adult rodents and zebrafish embryos. More recently, the method has been adapted for imaging of mouse embryos *in vivo*, connected to the mother (Figure 4C). The first attempts to stabilize embryo sufficiently for cellular imaging without disrupting the connections with the mother were made already in 2003 for interneuronal migration studies (Ang et al., 2003). Later, the technique was used to show the random behavior of the interneurons during migration (Yokota et al., 2007) and to elucidate the Golgi-mediated dynamics in interneurons (Yanagida et al., 2012). The *in vivo* two-photon imaging of embryos separated from the mother and maintained in an oxygenated medium were performed as well (Franco et al., 2012; Rash et al., 2016). In that case, stability sufficient for calcium imaging could be achieved.

The problem with immobilization of embryos is the soft tissue structure and high vulnerability. The umbilical cord connection of mice is very easy to damage and greatly affects the condition of the embryo. The common immobilization technique (Ang et al., 2003; Higuchi et al., 2016; Yanagida et al., 2012) is embedding the embryo in low-melting agarose to stabilize the motion. However, muscular movements of the embryo even inside the tissue might disturb image acquisition. To circumvent this problem, in Yanagida et al. (2012), for instance, the embryos were paralyzed. Such an approach might not be optimal for the studies of electrical activity in embryos, as peripheral activity can stimulate activity in the brain (Khazipov et al., 2004), and so complete paralysis of the body strongly interferes with such activity patterns.

Nevertheless, the resolution in such imaging is at the level of conventional histology, and thus allows investigation of subcellular compartments (Yanagida et al., 2012). At the early stage of embryonic development, in turn, the low phototoxicity of two-photon microscopy allows continuous imaging over several days in the culture (Squirrell et al., 1999) .

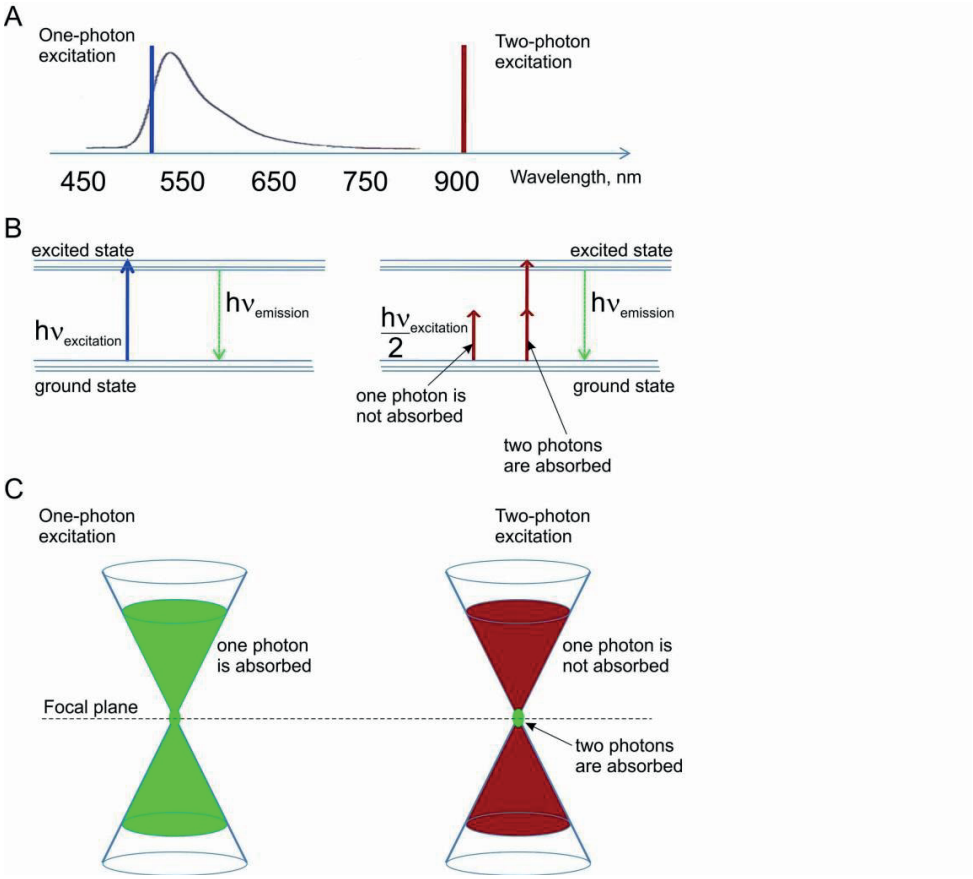

Figure 5. Principles of one- and two-photon imaging. **A.** Scheme of excitation lengths differences for one-photon and two-photon excitation. **B.** Representative Jablonski diagrams for one-photon (left) and two-photon (right) excitation and following fluorescence emission. **C.** Representation of the fluorescence around the focal point for one-photon excitation (left) and two-photon excitation (right).

2.5.4. Calcium imaging *in vivo*

In differentiated neurons, calcium oscillations are directly connected to the spiking of the neurons. Appearance of highly sensitive fluorescent calcium indicators like Fura-2 (Grynkiewicz et al., 1985) with fast kinetics made it possible to indirectly measure the electrical activity in large populations of cells with cellular specificity, which was not feasible before. Initially, calcium imaging was performed using fast charge-coupled device (CCD) cameras under wide-field illumination. Introduction of the confocal microscopes in the early 1990s facilitated calcium imaging in single cells.

Previously, the main intercellular electric signaling in neurons was measured by electrical recordings in several cells or using local field potential (LFP) and the results were extrapolated to the neuronal population. With calcium imaging, one could actually detect the borders of the event to measure the actual size of active zone. For example, using calcium imaging the function of gap junctions (Yuste et al., 1992) was studied, where the correlation of patches of calcium-active cells with filling by the small molecular weight dye was shown.

Using calcium imaging, one could study the large population of neurons and find the cells involved in the network regulation, or hubs (Bonifazi et al., 2009). The first calcium indicators were not fast enough to resolve the single spike, but could detect the spike trains. Depending on the calcium affinity for binding of the dye it is possible either to detect high calcium oscillations or low ones, to achieve high speed of kinetics for measurements of separate signals (Oregon Green-1), or to get higher sensitivity for the detection at the expense of speed (e.g. Fluo-4). The bulk loading of acetoxymethyl ester (AM) form of dyes enabled calcium imaging in large populations of cells *in vivo*, in the cortex of mice (Stosiek et al., 2003). Using optical fibers, it was possible to study the calcium activity of large cell populations *in vivo* in the early postnatal stage. Thus, the early network oscillations have been shown, which before had only been studied using local field potentials (Garaschuk et al., 2000).

Recently, methods for two-photon calcium imaging in early postnatal brains were developed (Kirmse et al., 2015). The population calcium activity imaging was

employed to study the effect of neurotransmitter GABA on the network. As the effects of GABA on the developing brain network have mainly been studied *in vitro*, the *in vivo* effects are still a subject of debate (Ben-Ari et al., 2012; Zilberter, 2016). In this regard, further development of calcium imaging methods in early postnatal mice might resolve the question of GABA action.

Also, it become possible to measure calcium activity in cells using green fluorescent protein fused with calmodulin (GCaMP) (Nakai et al., 2001). Introduction of genetically encoded calcium sensors made it possible to visualize calcium specifically in the desired population of cells. Expression of GCaMP using adeno-associated virus enabled the study of calcium activity in hippocampal cells *in vivo* (Dombeck et al., 2010).

The latest generation of genetically encoded calcium indicators (GECI) is able to resolve single spikes in trains (Chen et al., 2013) and also allows imaging in the spectra shifted towards infra-red (Zhao et al., 2011). Since the scattering of the red-shifted light in brain tissue is lower than the blue and green light, it is possible to detect the infra-red emission from the molecules deeper in the tissue. Thus, further development of infra-red calcium sensors will increase penetration of imaging and allow visualization of calcium activity in deeper brain structures. Using GECI, it become possible to measure the activity of specific cell populations in the behavioral context. In mice, for example, the calcium activity of cells populations is studied when the awake mouse is placed in virtual reality (Dombeck et al., 2007) or in a physical 'home cage' (Kislin et al., 2014).

Despite these advantages for calcium imaging *in vivo* in adult and early postnatal rodents, the imaging of activity in the brain at the embryonic stage remains to be developed. There may be a lack of interest in studies of electrical activity in the cortex at the early embryonic stage since only a few works have addressed the patterns of calcium activity in slices. The activity has been extensively studied in the midbrain (Hughes et al., 2009) in slices of mouse embryo brains. Propagating waves of activity

have been shown in the midbrain at that stage (Momose-Sato et al., 2007). Spontaneous activity also has been observed in the spinal cord (Spitzer, 2006).

Interestingly, much attention has been paid to studies of activity in early postnatal mice, which corresponds to the cortical development in humans at the third trimester (Khazipov and Luhmann, 2006; Vanhatalo et al., 2005). Meanwhile, the human embryo is much more susceptible to environmental factors upon maternal exposure through the mother-embryo interface, possibly leading to brain malfunctions. The placental connection, which is an essential filter in such a case is absent in the postnatal mouse model of brain development and does not reflect the real exposure scenario. Moreover, at these early stages of development (first and second trimesters in humans, corresponding to embryonic stages in mice) the basic intercellular signaling mechanisms are still largely unknown.

2.5.5. Optical stimulation methods

Once observations of physiological phenomena in the brain are possible, manipulation of the processes will follow. This will allow direct testing of hypotheses regarding the principles of how some cellular mechanisms work. The most popular stimulations utilized in brain imaging could be divided into three groups: 1) genetically controlled (optogenetic approaches), 2) chemically controlled (e.g. uncaging), and 3) spatially controlled (e.g. local lesion). Spatially controlled methods, such as focal laser radiation or wide-field fluorescence illumination, are widely used to study the consequences of trauma on neurons, e.g. in the peripheral nervous system by laser axotomy (Yuryev and Khiroug, 2012; Yuryev et al., 2014), axotomy in the brain (Allegra Mascaro et al., 2014)(Figure 6A), and stroke models (Kislin et al., 2016) (Figure 6B). There, the high-intensity radiation is spatially localized in a desired region under the assumption that the other regions are less affected. Chemically controlled optical manipulations use, for example, caged compounds – active molecules, inactivated by anchoring with a photo-active domain which could be removed under certain illumination (Figure 6C), thus restoring the activity of the

molecule (Ellis-Davies, 2007). Such approaches found applications in the studies of neurotransmitters action since they enabled the local release of neurotransmitters (Kantevari et al., 2010; Khirug et al., 2008; Matsuzaki et al., 2004). In contrast to the local microinjection system, the optical localization achieved is at the level of single microns, thus one can control single spines on the dendrite. Another advantage is the possibility of less invasive action and less effects on cell volume regulation than with the microinjection system, which is important e.g. for studies of GABA action since chloride regulation is easily affected by any traumatic disturbances (Kaila et al., 2014). A disadvantage of the uncaging approach for use in the studies of E13-E15 in this thesis was the necessity of using two different light sources, which on top of the complications of the *in vivo* two-photon imaging in embryos makes the system highly unreliable.

Optogenetic methods of neuronal stimulation include expression of light-activated channels (Klapoetke et al., 2014; Miesenbock, 2011). For example, transmembrane protein channelrhodopsin changes conformation upon blue light illumination and causes extracellular sodium ions to pass through the membrane into the cell, leading to depolarization (Figure 6D). In the mature neurons, this leads to spike activation. Another protein – halorhodopsin – is a chloride channel, which can be activated by yellow light. In intact mature neurons, halorhodopsin activation causes chloride influx into the cell, leading to hyperpolarization, and inhibits the activity. Overall, the combination of the light-activated channels is a powerful tool to manipulate the activity in the brain networks using light illumination. A special advantage of such an approach is the possibility of controlling the activity of cell lineages selectively. One can, for example, activate or deactivate large or small subpopulations of cells provided the suitable genetic markers are available. Similar to this, drug-mediated activity control systems are being developed. For example, the DREADD system could be used to either activate or shut down the activity of the cells by supplying the specific drug – clozapine-N-oxide (CNO), which does not significantly affect the other organism (Zhu and Roth, 2014). The main disadvantage

of these methods explaining why they were not used in the current thesis, is the necessity of the expression of the genetic construct. For the early developmental stages, such as E13-E15, the cell-population control of the construct expression is very challenging, since the viral injection approaches need 2-3 days for the expression, and the faster *in utero* electroporation approach is extremely challenging at the stage E11-E12, which should be used to target the E13-E15 studies.

In the current thesis, the 'crude' photostimulation has been used by activating the cells with high laser illumination (Figure 6E). Such an approach has been developed in the cell cultures (Smith, 2001) and later extended to slice cultures of the brain (Liu et al., 2010b). The photoactivation of a single cell can force it to release ATP, which activates neighboring cells, thus enabling the study of ATP-mediated calcium waves propagation with high spatial precision. The technique utilized the fact that the ATP receptors are highly present in immature cells at the embryonic stage of development (Weissman et al., 2004).

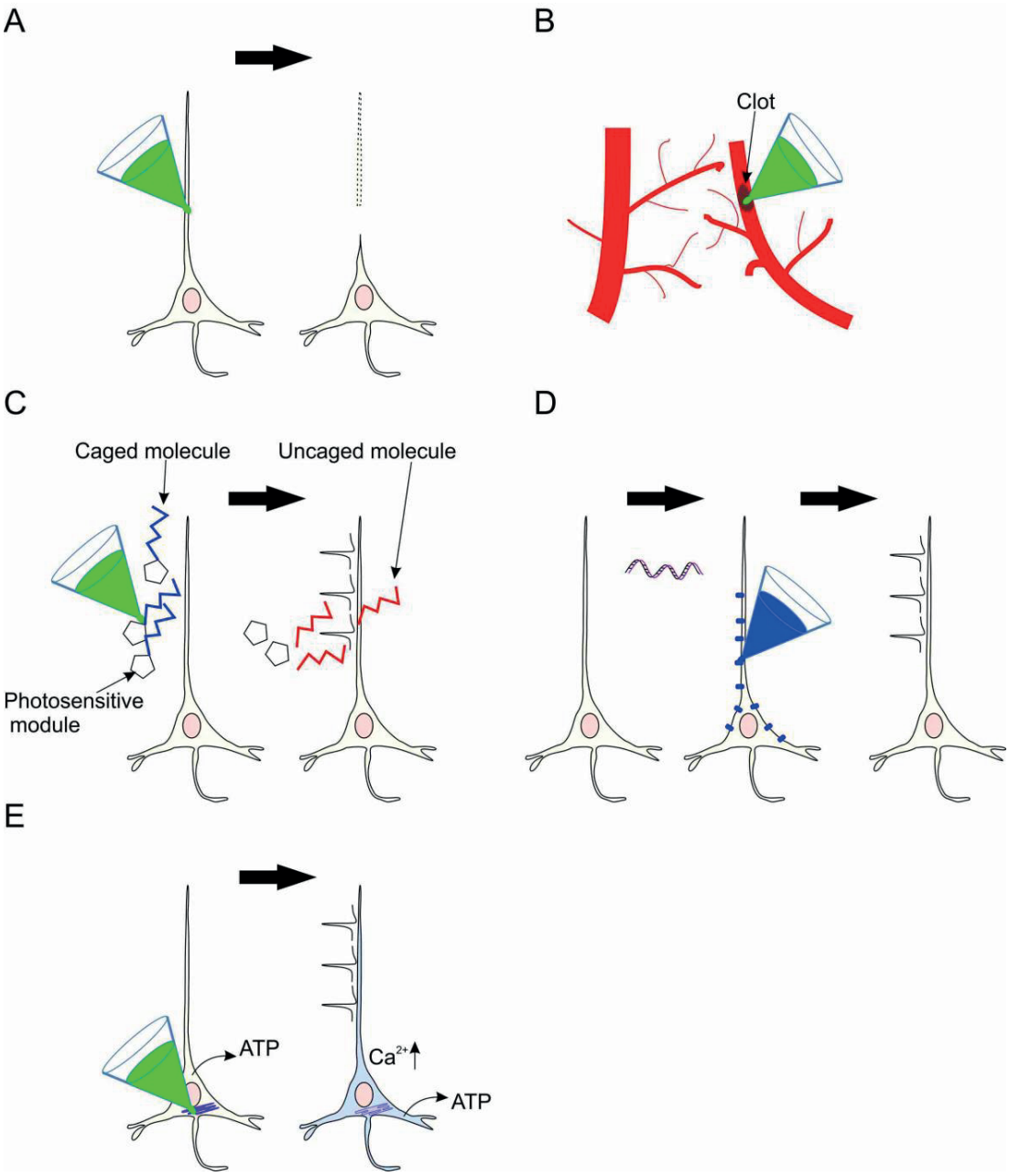

Figure 6. Optical stimulation methods. **A.** Focal laser axotomy. **B**. Focal laser microthrombosis. **C.** Uncaging. **D.** Optogenetical stimulation. **E.** High illumination optical stimulation.

2.5.6. Image processing aspects

In vivo imaging usually involves a variety of artifacts connected to tissue instability, blood vessel pulsations, and heartbeat of the animal. For embryonic imaging, it is an especially relevant problem due to the high dynamics in the tissue, the soft cranium, and the heartbeat not only of the embryo, but also of the mother. To get useful information from the rough imaging data in embryonic imaging, post-processing and image correction techniques are highly desirable. One should pay particular attention to the image processing, as it can affect the quantification of the data. Essentially, the image is a numerical matrix of intensities. The common mathematical routines applied to the images deal with 2-dimensional (2D) matrices, in this case, 2D one-channel images.

The common way to stabilize the image stacks obtained in the course of time-lapse measurements is to register images based on comparing sequential image frames (Figure 7A). One of the most widely used methods for this is the pyramid-based stack registration method (Thevenaz et al., 1998) which can be openly accessed as a plugin to the commonly used open-source program ImageJ (Wayne Rasband, NIH) for image analysis. The computational efficiency of the method allows fast registration of the image stacks (e.g. in the order of several minutes for 1000 frames of 512x512 resolution). However, sometimes the method does not work well for constant image drift.

The more computational-consuming method is the descriptor-based registration method (Preibisch et al., 2010). The method defines certain landmarks (descriptors) in the image, and aligns the images of the stack according to these landmarks. The method is powerful for the time-lapse image correction, however, it requires higher computational capabilities, and the processing time increases markedly with the increase in resolution of the image. For analyzing the long-term imaging data, sometimes the stack intensity normalization can be beneficial in the case of dye bleaching. Thus, the brightness of each image is adjusted to the reference frame brightness – e.g. the first frame.

Another important processing of the images is application of the proper filtering method. Probably the most common filtering technique is Gaussian filtering (Lee, 1983), which convolutes the image matrix with the specific window introduced by the researcher. Gaussian filtering is based on the assumption that the noise in most systems (such as electric noise in microscope detectors) has a Gaussian distribution of intensities (Figure 7B). Thus, upon introduction of Gaussian filtering the normal Gaussian noise is strongly suppressed in the image. Another possible filtering method is Kernel filtering (Takeda et al., 2007), in case one wants to enhance the contrast at the edges, e.g. to detect the borders of the cell. Such filters can work in 2D image, and so be applied to the rough non-stabilized image.

The type of filtering that can be especially beneficial for long time-lapse recordings is statistical-based filtering, which includes not only noise distribution in space (2D image in the case), but also in time. One of the most common filters of such types is Kalman filter (Matthies, 1989) (the plug-in for ImageJ developed by Christopher Philip Mauer). The filter builds a model of the image changes in time according to the statistical noise distribution and re-builds the filtered image with the noise suppressed according to that model (Figure 6D). However, for *in vivo* imaging data analysis one should first stabilize the motion artifacts of the images, because in case of the sudden changes in the neighboring image frames (e.g. due to heart beat) these motions would not fit the model based on the noise, and rather just blur the resulting image.

When the image is stabilized, normalized, and properly filtered, the next part of the analysis is object detection. Regardless of whether the object of the study is migrating cells or calcium signals, one needs to impose image segmentation routine to detect the objects (Figure 7C), in the present case, cells. Image segmentation techniques usually utilize the further parameters for object separation: the intensity change at the border of the object, the size of the object, and the shape. In case of calcium imaging the cell loaded with dye upon activation has a higher intensity than the background. The size is defined by the researcher, by measuring clearly

identifiable separate cells. The shape could assume that the cell soma is approximately circular and does not have long protrusions, so the object borders would be set at a certain distance from the object center.

The image segmentation can be optimized for specific experimental parameters. For instance, in (Aronov, 2003) the cell detection for calcium imaging data analysis utilized the property of Fura-2 dye, for which fluorescence is high at the basal calcium level, and drops upon the calcium rise. In contrast, the same segmentation does not work directly for Fluo-4 dye, which is bright only when the calcium is increased (Smedler et al., 2014).

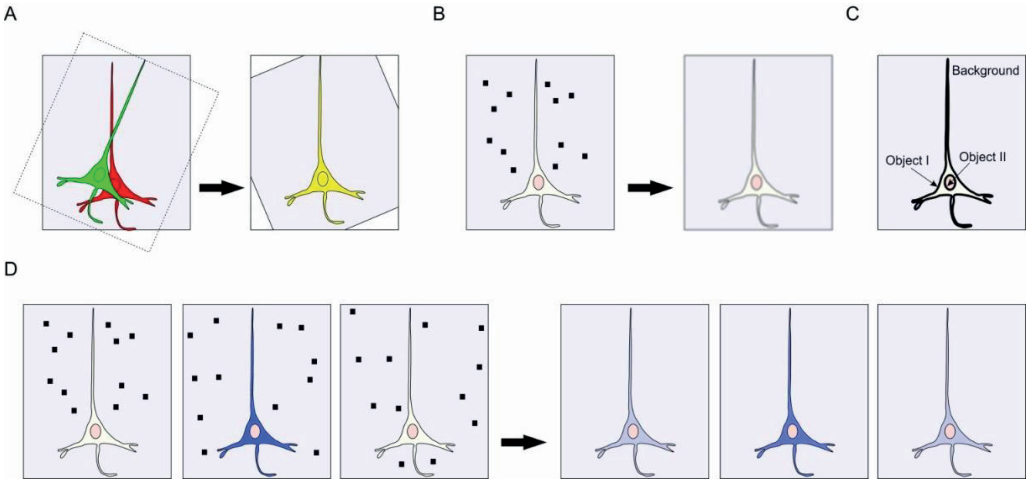

Figure 7. Image processing methods. **A.** Registration (motion correction). **B.** Gaussian blur. **C.** Image segmentation into objects. **D**. Kalman filtering.

2.5.7. Data analysis

The common data analysis paradigm in calcium signaling investigation is building of spatial raster plot of event. Raster plot designates only the time of appearance of the event, which, in turn, is defined as the rise or fall of the signal intensity over a defined threshold. Often, the rise above threshold is considered as exceeding 3 standard deviations. In this case, the complex massive of data of events intensities is simplified to the yes/no definition. This approach works, for example,

for detection of neuronal spikes, which appear in an all-or-none fashion when the depolarization overcomes the spiking threshold.

With raster plot, one can study the cluster activity of the cells and define the subgroups. For example, in (Cossart et al., 2003) it was possible to identify the whole network up-states by analysis of raster plots. In (Bonifazi et al., 2009), the events analysis revealed coordination of the network by single cells defined as hubs. It is worth noting that the intensity of the events did not play a role in the mathematics of the network analysis, as long as the signal-to-noise ratio was sufficient for reliable event detection.

A similar approach is valid for identification of the small-world networks, self-organized compartments of the network repetitively appearing in the brain systems (Feldt et al., 2011). An important part of such an analysis is proper modeling of the scrambled data to define the statistical significance of the measure (Smedler et al., 2014). Usually, scrambled data comprise randomly distributed data points of the same raster plot. One should take care to define correctly the percentiles of the probabilities for the distribution to appear randomly, as the standard statistical tests are not necessarily applicable in such a case. Usually, 95% certainty of the non-random appearing difference is considered significant (e.g. in Student's t-test).

In addition to the network events measurements, the absolute value of calcium transients can carry useful information. For calcium imaging with fluorescent indicators, one can measure the amplitude of the responses in single cells, or even bulk fluorescence change in the overall field of view, thus estimating the whole network calcium change. Although the details of the information are lost in this case, it reduces the artifacts related to the incorrect cell borders detection and incorrect clustering. It is also a computationally simpler task than the cell-by-cell signal detection. In line with this bulk approach, it is possible to introduce a coordinate system (Huang et al., 2004) to the image and divide it into equal-sized regions of interests, and then analyze the calcium transients in each region of interest (ROI), and e.g. build a raster plot. The information on the cellular interaction would be lost,

but the overall signal propagation and connectivity will remain, and, importantly, this data would be easier to measure than the cell-to-cell detection. Such an approach does not apply to the motion systems, though, and for the migration studies the cell detection might still be preferable as the moving cell undergoes calcium transients (Rash et al., 2016).

For cellular migration studies the common analysis types are directionality and speed of the cells (Higuchi et al., 2016; Inada et al., 2011). By single-cell detection, one can track its position in the image frame, and, thus, calculate the speeds at a given point in time and space. Here, one should be careful about 3-dimensional (3D) data interpretation since, as has been mentioned before, most of the image processing routines work better for 2D images, e.g. maximum projection of 3D stack. However, the vertical component of the speed of the cells would be lost in that case, and 2D-measurements of the cell motility might incorrectly estimate actual 3D behavior.

2.6. Drug delivery to the developing brain

As the number of developmental-related diseases in developed countries is increasing (Boyle et al., 2011), the question of the treatment of such disorders is becoming more and more acute. The possibilities of genetic editing in human embryos are already being considered in China (Liang et al., 2015). In Western Europe, experiments with the human genome in embryos are not readily permitted, however, the results of developments of techniques like CRISPR9 in other countries might affect the policies (Cyranoski and Reardon, 2015). Thus, the prevention of developmental-related diseases from the full effect is being actively considered. One option could be the treatment of diseases before the phenotype is fully developed, e.g. supplements with medication already *in utero*. Thus, under known pathological conditions by results of external diagnostics, it might be possible in the future to treat the embryo at the early development, thus recovering normal development, which would lead to the birth and growth of a healthy child. It is becoming evident that various disorders, such as autism and schizophrenia, stem from changes that have started already at the cortical growth stage.

However, controlled delivery of drugs to the embryos remains a great challenge. Nature has developed complex systems and mechanisms of placental barrier to protect the embryo from external potentially hazardous materials. Also, the immune system of the mother adapts to the development of the embryo inside the body so that it is not attacked as an alien object. The placental penetration of any given drug is usually lower than the concentration of the drug that remains in the body of the mother. Thus, if one can develop a 'universal' carrier that could be loaded with any drug and will efficiently penetrate the placental barrier, one can use this encapsulation/delivery system to treat the embryos at the early developmental stage.

2.6.1. Types of drug formulations

The small-molecule drugs, which are commonly used in clinical practice, are not necessarily the most efficient carriers. Indeed, the pharmacokinetics of the small

molecule has often a 'saw' shape, with sharp rises and slopes, thus causing the strong variations in drug presence in the blood or the targeted tissue. The drug is eliminated through the reticuloendothelial system organs, such as the liver and spleen, and through the kidneys to end up in urine. For drug delivery to embryos, these pharmacokinetic properties could be even more skewed since most of the drug can be taken up by the mother, and even if the concentration in the embryo is comparable to maternal exposure, the adverse effect on mother might still be strong due to the large integral amounts of the untargeted drug used. Moreover, the penetration of the placental barrier is another hindrance that might affect the drug pharmacokinetics, so to reach therapeutic concentrations in the embryo the mother might need to be exposed to the toxic drug concentrations for a long period.

Possible manipulations of the drug in adults could be addition of targeting modules such as antibodies, aptamers or targeting peptides, addition of carbohydrates to affect hydrophobicity and interaction with immune cells, and addition of inactivating fragments that could be hydrolysed e.g. upon reaching lyzosomes, thus reducing the toxic effect of the drug before it reached it destination (Tiwari et al., 2012). The approach that is currently actively being developed is encapsulating the drug in vesicles (Torchilin, 2010). Nanoparticles are a promising vesicle-resembling carrier. By attenuating the properties of the nanoparticles, one can achieve higher targeting of the drug, reduction of adverse effects, and external manipulation of local drug actions, e.g. with magnetic fields (Brusentsov, 2012). Thus, nanoparticles (Figure 8A) could be a promising candidate for a carrier, which with the necessary modification would be able to penetrate the placental barrier and target the given organ of the embryos, thus allowing targeted therapy at this early stage.

2.6.2. Barriers to penetration of the embryonic brain

To end up in the embryo, the drug or the carrier must first cross the placental barrier (Muoth et al., 2016) (Figure 8 B). To target specifically the embryonic brain,

the carrier should not be immediately eliminated by the embryo's reticuloendothelial system (Nie, 2010), and must be able to cross the blood-brain barrier (Wohlfart et al., 2012). In contrast to a common misconception, the blood-brain barrier in embryos is already formed and active (Saunders et al., 2012). However, the mechanisms of its functioning differ from the adult brain. The important feature of the barrier system in embryos is separation of the cerebro-spinal fluid from the blood, formed by the epithelial cells in the choroid plexus (Saunders et al., 2012). As cerebro-spinal fluid (CSF) is omnipresent in the embryonic brain, another barrier is strap junctions of neuroependyma, which separate CSF from the extracellular fluid inside the brain tissue. Electron microscopy studies indicate that some substances can enter the embryonic brain via active transport mechanisms. The protein concentration in embryonic CSF is higher than in adults, because in embryos CSF turnover is slower.

The choroid plexus is responsible for active transport of proteins from the blood to the CSF. Transcellular transport has been first demonstrated with albumin (Dziegielewska et al., 1980). The transport system was shown to selectively transport albumin of the corresponding species. Amino acid transport was demonstrated with the radiolabeling of ^{32}P, revealing that uptake declines with age. In 1971, Oldendorf showed that transport of amino acids to the developing brain was faster than to the adult brain (Oldendorf, 1971).

The opposite process in developing brains is efflux of entering compounds. The epithelial cells in the choroid plexus of developing brains express the efflux transport proteins such as P-glycoprotein, multidrug-resistance-associated proteins, and breast cancer resistance proteins (Ek et al., 2010). Thus, the mechanisms of the extrusion of the compounds from the developing brain cells have similarities to cancer cell drug resistance.

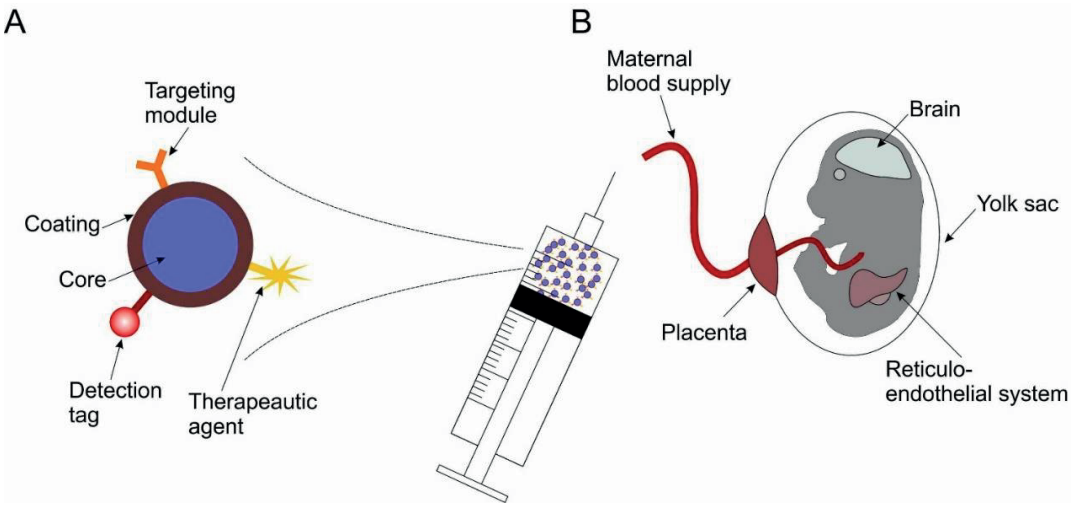

Figure 8. Nanoparticles and barriers to reaching the embryonic brain. **A.** Scheme of the nanoparticles with combined therapeutic and diagnostic capabilities. **B.** Mother-embryo interface.

2.7. Nanoparticles as a drug delivery system

2.7.1. Types of nanoparticles

A nanoparticle is defined as a structure of size below 100 nm in at least one of its dimensions. The first type of known artificially synthesized nano-products – colloidal gold – was invented by Faraday more than a hundred years ago (Daniel and Astruc, 2004). Since then, much progress has been made and nanoparticles currently find applications in various fields such as medicine, cosmetics, and food supplements. Nanoparticles comprise a variety of types: liposomes, magnetic nanoparticles, titanium oxide particles, quantum dots, porous silica, and porous silicon.

Nanoparticles received substantial attention in the field of biomedicine due to the possibility of combining various functions in one particle. The concept of a 'magic bullet' was proposed by Paul Ehrlich (Strebhardt and Ullrich, 2008). The modification of a particle with antibodies, targeting peptides, or aptamers allows delivery of the particle to the specific area needing treatment (e.g. tumor tissue) while avoiding healthy tissues, thus reducing side effects (Cho et al., 2008). Various types

of particles allow either loading of a drug in the pores or in the particle core (liposomes, porous silica, porous silicone) or covalent bonding of the drug with the particle coating. The particle core itself can carry the properties useful for active treatment, e.g. magnetic nanoparticles could generate heat introduced into the alternating magnetic field, or gold nanoparticles can produce the thermal energy under light radiation. The same effects such as heat generation could be used to modulate the local effects of drugs, e.g. by locally increasing the efficiency of chemotherapy (Brusentsov, 2012). At the same time, the particles could be used for detection of unhealthy tissues like tumors (magnetic nanoparticles, gold nanoparticles). Thus, the nanoparticles could combine complex machinery from various modules (Nikitin et al., 2010), which can even produce the calculated outputs based on the environment and integrating two or more parameters such as blood sugar level and level of insulin at the same time (Nikitin et al., 2014).

Nanoparticles bear an important property for chemistry – they have a high surface-to-volume ratio. That is why reactions on the nanoparticle surface can happen much faster than on a flat surface. At the same time, nanoparticles can have crystal-like properties, which the free molecules they consist of do not have. Thus, for example, iron oxide nanoparticles represent magnetic properties, although molecular iron oxide does not (Pankhurst, 2003).

Right now, probably the most widely presented example of a nanomedicine is Doxyl, a liposome-encapsulated doxorubicine in which the nanoparticle formulation significantly reduces heart toxicity (Torchilin, 2010). Liposomes have a bilipid membrane that allows loading of a drug inside the particle. Another widely known type of nanoparticles is titanium oxide, used in cosmetics such as sunscreen (Shi et al., 2013).

A range of magnetic nanoparticles is used as contrast agents for magnetic resonance imaging (Pankhurst, 2009). They are used also for detection of metastasis for tumor resection procedures (Ahmed et al., 2015). Recently, iron oxide magnetic nanoparticles were approved by U.S. Food and Drug Administration (FDA) for

treatment of anemia, as they slowly degrade, with iron from the particles being transformed into iron-containing proteins such as ferritin (Rosner and Auerbach, 2011).

2.7.2. Size and surface chemistry effects of nanoparticles

Size, shape and surface properties are crucial characteristics for nanoparticles, affecting their interaction with the organism in case of use *in vivo* (Blanco et al., 2015). The particle coating affects the "visibility" of nanoparticles for macrophages and other immune cells. For example, hydrophilic coating with polymers such as polyethylenglycol (PEG), hinders the accessibility of the nanoparticle by macrophages recognition sites, thus reducing the absorbance of the nanoparticles (Petros and DeSimone, 2010a). The charge also affects the particles behavior *in vivo*. Dendrimers are less accessible for the macrophages to absorb the particle.

It is important to bear in mind that any theranostic (simultaneous therapeutic and diagnostic) improvements of the particles, such as modification with targeting antibodies, loading of the drugs to particles, and adding tracer to the particle, might change the surface properties of the particle, its charge, or its shape, and thus, will inevitably affect the dynamics of the particle in the organism *in vivo*.

The pharmacokinetics of nanoparticles in the organism can work as an advantage. Thus, probably the most efficient use of magnetic nanoparticles so far has been found in the contrast of liver (Pankhurst, 2009) since organs of the reticulo-endothelial system (liver and spleen) readily adsorb any particles above 50 nm, thus working as a filtering system for blood. As well, liposome-based Doxyl was initially developed for targeting the liver for the same reasons of high accumulation in the liver (Torchilin, 2010). Nanoparticles with a size of less than 5 nm are eliminated from the organism by renal excretion, and thus, cannot be used for such purposes.

For particles with a spherical shape, the larger diameter generally leads to a shorter circulation time in the blood-stream due to fast elimination by Kupffer cells of the liver in case of general administration. On the other hand, sometimes the larger size might have beneficial effects. For example, nanoparticles with a size of 100 nm can selectively penetrate tumors due to enhanced permeability and a retention effect caused by large pores formed during non-systematic tumor formation, as the cells are not properly aligned, unlike in healthy tissue (Petros and DeSimone, 2010b). Thus, by manipulating particle size targeting effects can be achieved.

2.7.3. Effects of nanoparticles on neurons

Use of nanoparticles in brain has been of high interest for glioma treatment. The reason is non-uniform spread of glioma in the brain, which makes the resection of the tumor tissue highly challenging and entails a high risk of recurrence. Nanoparticles have some advantages to circumvent the problem. For example, magnetic nanoparticles injected in glioma tissue are used for hyperthermia treatment (heating the tissue to cause apoptosis) using an external alternating magnetic field (Pourgholi et al., 2016). The particles can be held in place by using a local magnet, thereby avoiding diffusion.

Various metal oxide particles have been tested for their effects on neuronal cell lines *in vitro*. In PC12 cells, iron oxide nanoparticles with anionic coating got internalized into cells, and caused concentration-dependent reduction of the filaments sprouting upon exposure to the neural growth factor (NGF) (Pisanic et al., 2007). The nanoparticles caused concentration-dependent reduction in the formation of intercellular contacts as well. The lower response to NGF also manifested in a decrease in growth-associated protein-43 (GAP-43), involved in axonal growth.

Other metal oxide particles have also shown adverse effects in PC12 cells. TiO_2 nanoparticles exposure increased generation of reactive oxygen species (ROS) in cells

(Liu et al., 2010a), which is a common toxic effect, reducing cell viability. Mn nanoparticles and Mn2+ (acetate) nanoparticles resulted in depletion of dopamine from PC12 cells at concentrations causing low ROS production (Hussain et al., 2006). Another effect that nanostructures can impose upon cells is a decrease in overall DNA content, which was observed in spinal cord cells of chicken embryos after exposure to single-walled carbon nanotubes (Belyanskaya et al., 2009).

Metal oxide particles can affect sodium currents in hippocampal neurons. Measured with patch-clamp, ZnO nanoparticles increased sodium currents, while silver nanoparticles caused a reduction in sodium currents (Liu et al., 2009; Zhao et al., 2009). Nanoparticles can also disturb calcium signaling of the cells. For example, residual oil fly ash can activate TRPV1 receptors (Oortgiesen et al., 2000). Often the calcium increase corresponds to toxic effects and ROS generation (Lovisolo et al., 2014). Meanwhile, silver nanoparticles can produce calcium increases in neurons and astrocytes independently of the ROS (Haase et al., 2012). SiO_2 nanoparticles induce calcium changes depending on their size and concentration (Ariano et al., 2011). Nanoparticles of 50 nm caused calcium transients at concentrations of up to 15 μg/ml, with long increase in calcium at concentration of 73 μg/ml or higher, however, nanoparticles of 200 nm induced transient responses even at high doses.

Some types of nanoparticles have been considered for targeting neuronal cells. For example, organically modified silica (ORMOSIL) particles have been shown to penetrate neuronal cells (Barandeh et al., 2012) and be trafficked inside the cell without causing significant toxic effects (Bharali et al., 2005). Porous silica oxide nanoparticles have been tested *in vitro* as a possible delivery system of lipophilic agents to neurons (Rosenholm et al., 2009). As lipophilic drugs are very challenging to deliver in free form through the blood, the use of such a carrier would be highly advantageous. Nanoparticle formulation can also be used for neuroprotective action. For example, auto-catalytic ceria nanoparticles can impose ROS scavenging effects and increase survival of damaged spinal cord neurons (Das et al., 2007).

2.8. Effects of nanoparticles on the developing brain

2.8.1. Nanoparticles in embryos

As the presence of nanoparticles in the environment increases, the question arises of how they might affect highly vulnerable fetuses. Nanoparticles are being used in hygiene products, such as shower gels, in food additives, and in cosmetics, in addition to the aforementioned biomedical applications. Even exhaustive gas from cars can contain nanoparticles (Oortgiesen et al., 2000). Thus, exposure of pregnant mothers to nanoparticles is highly probable. However, the effects of nanoparticles on embryos have been poorly studied to date. There are relatively few studies addressing the effects of nanoparticles on developing embryos. Some studies have been performed in invertebrates like zebrafish, as it is a useful model for following embryonic development due to tissue transparency (Asharani et al., 2011). However, these models lack the complex interaction of the mother and embryo through the placenta, which is the natural interface for human embryo development, as the mother being directly exposed to the nanoparticles will transfer the nanoparticles to the embryo through the placenta.

It has been shown in pregnant mice (Yamashita et al., 2011) that silica (70 nm size) and titanium (35 nm size) dioxide nanoparticle exposure affects embryonic development. After intravenous injection, the particles penetrate through the placenta and can reach embryonic organs such as the liver and brain. Following the exposure, the embryos were underdeveloped relative to the control groups. The authors did not observe such strong effects after injections of larger particles (fullerene of 300 nm and silica particles of 1000 nm). Moreover, the harmful effects could be significantly reduced by particle modification with carboxyl and amine groups. These results emphasize the complexity of interaction of the developing organism with the particles depending on the surface properties and the size.

In another study (Huang et al., 2015), penetration of polystyrene particles through the placental barrier has been shown. The particles were observed in various

organs of the fetus: brain, lungs, and liver. The authors found that the placenta's retention of nanoparticles depends on their size; generally, the larger the particles size, the lower the retention rate (in the range of 40-500 nm). However, the retention of nanoparticles of 20 nm has been significantly lower than of nanoparticles of 40 nm, at the level of nanoparticles of 500 nm. This raises the question of the retention mechanism in the placenta, which could depend on diffusion, vesicular transport, or other processes such as phagocytosis or endocytosis. However, one should be careful when analyzing these parameters under systemic administration to the mouse, as most of the particles are rapidly taken up by the organs of the reticulo-endothelial system (liver and spleen) and by the lungs, and the time for interaction with the organism is reasonably short.

It should be noted that the majority of studies on the nanoparticles in embryos have focused on either proving the toxicity of the particles to the fetus, or the opposite, proving their harmlessness, which would support use of the particles. There are relatively few studies focusing on the basic principles of nanoparticles behavior in the embryos, especially in the brains. Meanwhile, the toxicity of a given type of nanoparticles can change tremendously by simply changing the coating type (Yamashita et al., 2011), and the same might not apply to other particles. Thus, the general mechanisms of nanoparticles behavior in the embryonic brain need to be elucidated in order to produce a targeted drug design and not a stochastic serendipity approach.

2.9. Porous silicon

Porous silicon (PSi) nanoparticles are a promising type of nanoparticles for drug delivery (Santos et al., 2011). In essence, porous silicon is a crystalline Si structure with empty pores introduced (Santos et al., 2014). The porous structure of the material defines its extremely high internal surface area, achieving 700 m^2/g. The pore volume of the material reaches more than 0.9 cm^3/g. These parameters allow the material to absorb relatively large amounts of drugs, 5-40 wt. %.

An important feature, of porous silicon nanoparticles, is a top-down approach to synthesis, which can be easily scaled up for manufacturing purposes. Usually, porous silicon is synthesized by anodization in the hydrofluoric acid-ethanolic electrolytes of the monocrystalline Si wafer, thus producing the pores in the course of etching (Santos et al., 2014). The changes in the etching parameters lead to control of pore type. To produce the nanoparticles, a thin layer of porous silicon is usually used, which is broken into smaller bits. Further milling and sonication are used to reduce the size of the particles. Finally, the particles are segregated by size using membrane filters and centrifugation.

After the particle production, the surface of the PSi should be modified to reduce the reactivity, thus stabilizing the particles. The stabilization of the surface could be achieved by thermal oxidation or thermal carbonization, hydrosilylation or silanization (Makila et al., 2012). The resulting surface could be further modified to fine-tune the properties of the nanoparticles (Bimbo et al., 2011b). Multifunctionalization, with the further possibility of various attachments to the particles, could also be employed.

The major advantage of the porous silicon system is the amorphous state of the drug loaded inside the particles, thus drugs poorly soluble in water can be incorporated in the porous silicon nanoparticles which would act as a carrier (Santos et al., 2013). This application has been shown with drugs against influenza (Bimbo et al., 2013) and cancer (Santos et al., 2011). Importantly, the silicon element (the main component of porous silicon systems) is not toxic for the organism. Silicon dioxides have been approved by the FDA already in 1979 as compounds for food ingredients and orally and topically applied drugs. The porous silicon is, in turn, biodegradable and does not produce significant toxic effects upon injection into an organism (Bimbo et al., 2010). Over the course of several days, the porous silicon nanoparticles degrade in physiological solutions to silicic acid, which is eliminated from the organism in urine. *In vitro* tests have shown that the PSi microparticles produced higher cytotoxicity than the nanoparticles (Bimbo et al., 2011a).

To load the drugs, the porous silicon should be exposed to the drug solution (Santos et al., 2013). The drug could be covalently bonded to the particles, or spontaneously adsorbed. The drug loading could be performed in hydrophobic solution in which the hydrophobic drug is easily soluble, and then the solution can be changed to a physiological water-based buffer, the drug being retained in the pores. The PSi nanoparticles could be optimized to target various tissues, such as cancer cells or heart cells (Ferreira et al., 2015). However, most of the previous works on PSi focused on cancers (Santos et al., 2011). The question of porous silicon delivery to neuronal cells has not been extensively studied yet.

3. Aims of the study

Despite great advancements in the understanding of the mechanisms of neurogenesis and cortical formation achieved from *ex vivo* models and cell cultures, the *in vivo* role of these mechanisms remains unclear. The *in vivo* studies of these mechanisms have suffered from a lack of robust tools for investigations. We aimed to develop a method for *in vivo* imaging of mouse embryos with which the activity and cellular dynamics of the developing cortex could be examined.

Aims of the study were as follows:

1. To develop a method for two-photon imaging of mouse embryos connected to the mother.
2. To investigate the properties of evoked calcium activity in the embryonic cortex in vivo.
3. To evaluate the effects of spontaneous calcium activity on cellular migration in embryos *in vivo*.
4. To characterize the motility of externally introduced nanoparticles in the embryonic cortex.

4. Materials and methods

The materials and methods used in the studies are described in detail in the original publications.

4.1. Animals

All experiments involving animal subjects were approved by the National Animal Experiment Board, Finland.

In the experiments, inbred c57bl/6JOla mice and outbred ICR mice were utilized. The following transgenic lines were employed: Sox2-GFP reporter mouse (D'Amour and Gage, 2003) and GAD67-GFP mouse (Tamamaki et al., 2001).

4.2. *In vivo* imaging

In vivo imaging procedure is described in detail in Studies I and II.

Pregnant mice were anesthetized with ketamine/xylazine 80/10 mg/kg or isoflurane 4% for induction and 1-1.3% for maintenance, and placed at the heating pad operating at 37°C. The abdomen was opened with single incision and the uterine horn exposed.

In Studies I and II, the embryos were injected intraventricularly with calcium dye Fluo4-AM (0.4mM) or nanoparticles solution, dissolved in artificial cerebro-spinal fluid (ACSF, in mM: 125 NaCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 5 KCl, 20 D-glucose, 10 HEPES) with addition of 13 mM of FastGreen for injection guidance. Injections were performed with Picosprizer II until the filling of thee ventricles, through a glass pipette (Drummond Scientific) with a pulled tip of length 12 mm and tip diameter 50 um. The uterine horn was rinsed with 37°C ACSF every 5 min to avoid dehydration and hypothermia. The embryos were placed back in the abdomen of the mother and the cavity was sutured.

Imaging was performed 30 min after the surgery in Study I and 20 min after the surgery in Study II. The abdomen was opened again and the uterine horn exposed. The custom-made heating chamber was placed above the uterine horn. The 10 cm culture dish with a 2 cm hole in the center covered by a polydimethylsiloxane

membrane was placed in the chamber, and an incision 15-20 mm long in one direction and 10 mm in the perpendicular direction was made in the center of the membrane. The uterine horn was rinsed with ACSF and carefully exposed through the incision in the membrane. The membrane was sealed with acrylamide glue (Henkel) and further with 4% agarose (Bioline). The dish was then filled with ACSF.

The uterine horn was cut and the embryo in the yolk sac exposed. The yolk sac was carefully cut from the side of the embryo tail to avoid damage to the head and taken out, keeping the vessels connected to the uterine horn intact. The embryo was positioned in either a custom-made metal holder or next to a screw-nut with Blu-Tack (Bostik), the ACSF was temporarily removed from the plate, and the embryo was glued to the holder or screw-nut with acrylamide glue. Agarose was applied around the body to further immobilize the embryo. The dish was then filled again with ACSF.

In Study III, the embryos were injected with the calcium dye Fluo4-AM, but after immobilization. The two-photon imaging was performed with the Olympus FV-1000MPE system through the 25X water-immersion objective XLPLAN (Olympus). In Study II, z-stacks with a thickness of 170 μm were acquired with step 10 μm, and in Study III z-stacks with step 5 μm were acquired.

In Study I, one focal plane was imaged in time-lapse series, at a resolution of 512x512 and an acquisition rate of 1.2 fps. Before stimulation, the baseline measurements of 5 frames were acquired. For stimulations, bleaching tool in the Olympus software package was used. Irradiation at maximum power was produced for 3 s at a wavelength of 800 nm with the same femtosecond laser as for the imaging, in a circular area of diameter 20-30 μm. Fluorescence was collected using a filter with a bandwidth of 515-560 nm. The embryo's condition was monitored by blood flow, which could be observed as moving erythrocytes shadows in the autofluorescence signal.

4.3. Image processing

The *in vivo* microscopy imaging data were processed using ImageJ (Rayband, NIH) plugins.

In Study II, the maximum z-projections were calculated from the initial time-lapse series, and the calculated stacks were corrected with StackReg plugin in ImageJ or with descriptor-based registration (Preibisch et al., 2010). The stitching of the wide-field fluorescent images from histological investigations was performed with Mosaic plugin in ImageJ.

In Study I, image stacks were stabilized with StackReg plugin. Immunohistochemistry images before and after the staining were overlapped using UnwarpJ plugin. Polar transform of the images was calculated with the plugin Polar Transform (written by Edwin Donnely and Frederic Mothe) in ImageJ, with the stimulation point as a transform center. The transformed image was divided into 50 um x 50 um regions of interest. For each region of interest, the intensity profile over time was calculated, and the event moment was designated as half-maximal intensity. With the event times, the raster plot was built. The active cell was defined as a 5% fluorescence increase over the basal fluorescence. The number of active cells was normalized to the dye-loaded area in the image. Wave propagation speed was measured as the half-maximal wave propagation distance divided by the time the wave reached half of the maximum spread distance.

4.4. Nanoparticle preparation

To detect APS-TCPSi NPs in the tissues, the NPs were labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich) in a ratio 1:10 (PSi:FITC) (w/w) in ethanol:(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH7.8 (4:1) solution. The mixture was stirred at room temperature for 30 min. Particles were precipitated with a centrifuge and washed with ethanol and further with MilliQ-water. Following the washing, tetramethylrhodamine isothiocyanate (TRITC, Sigma-Aldrich) was loaded to the NPs in a ratio 10:1 (PSi:TRITC) (w/w). The particles were stirred at room temperature for 2 h, precipitated with a centrifuge, and washed with MilliQ-water twice. After the labeling procedures, the NPs were dissolved in ACSF to reach a concentration of 0.75 mg/ml.

4.5. Nanoparticle injections

For intravenous injections, pregnant mice were anesthetized with ketamine/xylazine 80/10 mg/kg and placed on a heating pad operating at 37°C. A stock particles solution of concentration 0.75 mg/ml was diluted four times in ACSF of injection facilitation and supplied with 13 mM of Fast Green dye for injection guidance. The particles were injected into the tail vein with G27 needles. Mice were injected with 0.63 μg of particles/g tissue. After the procedure, 200 μl of phosphate-buffered saline (PBS) was administered subcutaneously to avoid dehydration.

For *in utero* injections, pregnant mice were anesthetized with ketamine/xylazine 80/10 mg/kg and placed on a heating pad operating at 37°C. The abdomen was open with a single incision and the uterine horn exposed.

Injections were performed with Picosprizer II until the filling of thee ventricles, through a glass pipette (Drummond Scientific) with a pulled tip of length 12 mm and tip diameter 50 μm. The uterine horn was rinsed with 37°C ACSF every 5 min to avoid dehydration and hypothermia. The embryos were placed back in the abdomen of the mother, and the cavity was filled with warm PBS at 37°C and sutured. The next day the mice were supplemented with ketoprofen 0.015 mg/kg and buprenorphine (Vetergesic) 0.013 mg/kg.

After 4 h, 2 days, or 3 days, the mice were euthanized with CO_2, the embryos harvested and euthanized by decapitation, and the brains put in 4% paraformaldehyde in PBS overnight.

4.6. Histology and immunohistochemistry

In Study I, some embryos' brains were put in overnight fixation in 4% paraformaldehyde (PFA). The PFA solution was changed to 30% sucrose in PBS on the following day. Two days later, the brains were frozen in TissueTek (Sakura), and coronal slices of 30 and 50 μm thickness were prepared by cutting at -20°C. The sections were placed on Superfrost Plus microscope slides (Thermo Scientific). The

immunostainings were performed according to the manufacturer's guidelines. The slices were labeled with primary Tuj1 specific monoclonal mouse antibodies (1:500) to distinguish differentiated neurons and further labeled with secondary antibody conjugated with Alexa-568 (1:800, Molecular Probes). The sections were mounted with ProLong (Life Technologies) and stored at 4°C. In Study II, after fixation, brains slices of thickness 200 μm were prepared using vibratome.

For immunostanings, 7-μm-thick paraffin sections were prepared. The sections were stained with rabbit cleaved caspase-3 antibody (1:300) in 2% bovine serum albumin (BSA). For detection, the slices were further labeled with secondary antibody conjugated with Alexa-405 (1:200) dissolved in 2% BSA in PBS.

Wide-field fluorescence imaging was performed with an Imager.M1 Colibri system (Zeiss) with LED illumination.

The imaging was performed with 4X, 20X, and 40X objectives.

4.7. Statistical analysis

The non-parametric Mann-Whitney U-test was used to define statistical significance in control measures. In Study III, Student's t-test was used for data that had passed the normality test. Statistical significance was set at $p<0.05$.

5. Results and discussion

5.1. Study I

5.1.1. Development of method for *in vivo* calcium imaging in embryos connected to the mother

The task of *in vivo* measurements of intracellular calcium in mouse embryos is highly challenging. Two-photon calcium imaging is a popular method for the measuring intracellular calcium in adult rodents because of the possibility of deep tissue imaging with high resolution (Helmchen and Denk, 2005). Such qualities are the result of intrinsic optical sectioning and low light scattering in tissues due to the use of infra-red light. However, two-photon imaging for live mouse embryos connected to the mother is problematic. Although *in vivo* imaging of cellular migration was performed already in 2003 by Ang et al. (Ang et al., 2003), intracellular calcium imaging requires higher stabilization. In previous works focusing on interneurons migration (Ang et al., 2003; Yanagida et al., 2012; Yokota et al., 2007), stabilization of the embryonic head has been achieved by submergence of the whole embryo in a low-melting agarose. However, the stability of the tissue in such an approach might not be sufficient for intracellular measurements due to intrinsic tissue movements from heartbeat and vessels pulsations. In Study I, we developed an embryo stabilization approach by gluing the embryonic head to the metal screw-nut with consequent submergence of the embryo into agarose (Figure 1, Study I). Such a system allowed high local stability in the area of imaging with the embryo's whole body immobilized. At developmental stages E13-E15, the imaging was possible directly through the skin as the skull was not yet fully formed. At stages E13-E14, the imaging of the whole cortical thickness was possible, while from E15 onwards the forming skull and rapidly expanding cortex are already preventing imaging of the full depth of the cortex. However, at stages E17-E18, it is possible to remove the skin (Yanagida et al.,

2012), which greatly facilitates the imaging, as it scatters light probably more strongly than the unformed skull.

Another difference in the imaging system that allowed high local stabilization of the embryonic brain is utilization of the separate chamber for the embryos (Pierfelice and Gaiano, 2010). Thus, motion artifacts from the mother's breathing are not attributed to the embryos. The chamber is heated up (Caetano et al., 2012), which maintains the physiological ambient temperature of the embryos. In future studies, this configuration may also allow bath applications to test different solutions or drugs.

To monitor calcium in the cells, we microinjected the acetoxymethyl form of Fluo4 calcium indicator into the brains ventricle. Interestingly, only a subpopulation of cells was selectively loaded with the dye (Figure 1B, Study I). Using Sox2-GFP reporter mice, we observed *in vivo* that the loaded cells were almost entirely Sox2-negative (Figure 1D, Study I), so only differentiated cells took up the Fluo4-AM-dye. We confirmed this by staining the cryosections with neuronal marker Tuj1, showing that the majority of Fluo4-AM-dye-loaded cells were Tuj1-positive (Figure 1E, Study I).

For the future investigations, the use of expression of genetically-encoded calcium indicators like GCAMPs in transgenic mice or introduced by electroporation would be highly advantageous, since it would be possible to measure calcium specifically in the desired cell population and might increase signal-to-noise ratio due to lack of fluorescence elsewhere. However, expression of the indicators at such early stages as E13-E14 is a considerable challenge. It should be noted that the condition of the embryo could be monitored by blood flow, which can be observed as shadows of erythrocytes in the vicinity of vessels walls (Figure 1C, Study 1). Further, the introduction of the blood vessel markers would improve the vessel imaging. However, the common dye-injection approach such as fluorescently labeled dextran should be thoroughly optimized for embryos, since the large molecules do not readily cross the placental barrier.

5.1.2. Evoked calcium waves measurements

To evoke the calcium wave, the same laser as used in imaging was focused on a small round area (diameter 20 μm) and high-power illumination was delivered for 3 s. With this stimulation, the energy exceeded by about 1000-fold the normal imaging mode delivered to the cell. This caused calcium elevations in the cells in the vicinity of the stimulated zone, with formation of the consequential wave.

In slices, selective stimulation of the single cell is possible using a high-illumination technique (Liu et al., 2010b). *In vivo*, due to small tissue vibration, the stimulation volume is significantly increased and can have a much broader effect producing the simultaneous stimulation of 3-4 cells with an equal illumination periods (3 s). The stabilization quality of the tissue is thus crucially important for production of a highly localized efficient stimulation point. It is worth mentioning that in addition to achieving higher stability of the live sample there is an increasing risk of damaging the embryo; thus, the final stabilization level could reasonably depend on the measured parameters and an acceptable range of precision. For development of measurements of faster processes (such as electrical excitation-mediated synaptic connectivity studies), a higher level of stabilization might be required.

In our set-up, a 25x objective was used for illumination, achieving an imaging area of 500 μm x 500 μm. Around one-third of the cells in the imaged area were activated upon a single stimulation. This gave us the necessary dynamic range to detect changes in the waves since in the case of increased cell number the wave was still observed in the field of the objective. It thus allowed us to investigate the wave propagation parameters (Figure 2, Study I).

Although local high-power illumination is a crude technique that inevitably produces side effects, its simplicity makes it a powerful tool to analyze the properties of the network, as it does not require expression of additional proteins or introduction of the puffs that would release the drugs non-selectively in the tissue or separate illumination source, as is needed for uncaging experiments (Smith, 2001). Also, at the

age of E13-E14 the stimulation could be produced throughout the cortical thickness, thus enabling the interaction between different newly forming layers of neurons to be assessed.

The configuration of our set-up with the uterine horn separated from the mother in the dish with a water-based buffer has an advantage for the calcium waves studies due to the possibilities of changing of the imaged embryos inside the same chamber. Thus, from the same mother it was possible to measure the calcium waves from 3-4 different embryos with 3-4 stimulation in each, thus reaching a high statistical power of measurements. For fine cellular processes, however, the use of physiological optogenetic approaches in the future might yield higher statistical power.

5.1.3. Stimulated calcium wave properties

The calcium waves produced in slices by Liu et al. were mediated by ATP (Liu et al., 2010b). To probe whether ATP is involved in the calcium wave propagation *in vivo*, we injected the embryonic brains with suramin (Dunn and Blakeley, 1988) to non-selectively block P2 receptors. Indeed, the stimulated wave propagation was significantly reduced.

We measured various parameters of the evoked waves: number of engaged cells, speed and distance of the wave propagation, and overall fluorescence increase. After injection of suramin, all of the measured parameters were significantly decreased, indicating the involvement of P2 receptors in calcium wave propagation (Figure 3, Study I). Thus, based on the previous data on *in vitro* stimulations (Liu et al., 2010b; Smith, 2001; Weissman et al., 2004) ATP is the likely mediator of the propagating calcium wave *in vivo*.

To investigate the calcium waves propagation under the conditions of facilitated intracellular calcium release, we injected the embryonic brains with caffeine, which, besides antagonizing adenosine receptors, also activates ryanodine receptors (Friel and Tsien, 1992). The calcium waves were boosted, with significantly higher number of cells involved in the cascade (Figure 3E, Study I) and an overall

fluorescence increase (Figure 3G, Study I). The speed of the wave propagation was slightly increased, albeit, not significantly (Figure 3F, Study I).

The measurements of the evoked calcium signal can be advantageous to spontaneous calcium activity due to the controllable reproducibility (Weissman et al., 2004). The possibility of the pharmacological manipulation of the calcium waves with the evoked calcium signal as a read-out makes the method on one hand a promising *in vivo* testing platform for various pharmacological candidates. On the other hand, the same *in vivo* measurements could be conducted to assess the acute toxicity of various chemicals to which pregnant mothers might be exposed. Currently, most toxicology investigations of effects on embryos are conducted mainly *ex vivo* with histology, without direct measurements of the effect of the agent in the brain. The method presented will allow evaluation of the effects of the chemicals on processes in real time. The mouse model of embryonic development in this case is more relevant to the human development than the currently used zebrafish model, as the mouse model has placental mother-embryo interface. Another advantage of the mouse model over other rodents systems is the abundancy of transgenic mice available.

5.1.4. Calcium wave directionality analysis

To determine whether the calcium waves propagate uniformly through the cortex, we employed the polar transformation approach (Huang et al., 2004). The images were transformed from Decartes coordinates (X, Y) to polar coordinates (r, phi), with the reference zero point in the center of the stimulus area, where r is the radial coordinate (distance to the stimulus point) and phi is the angle varying from -180° to +180° . After this transformation, we could represent the wave propagation image stacks in the traditional rectangle view, with the wave propagating from the left to right (Figure 4A, B, Study I). The advantage of such an approach is that we were able to divide resulting images into equal-sized-square regions of interest, and follow the calcium signal in these regions. In contrast, for the same type of processing in the original image one needs to arrange non-even regions of interest, and this makes

analysis especially challenging if the stimulus is not precisely in the center of the image. After the polar transformation, we could apply the same analysis to the waves independently of where they were evoked in the initial image.

We divided the polar-transformed images into 'blocks' of 50 μm along the radial coordinate (distance to the stimulus point). The binning could be adjusted depending on the image resolution, and, importantly, the stability of the specimen. In the case of 50 μm binning, the small motion artifacts were averaged out by measurements of calcium signals from several neighboring cells.

By measuring the half-maximal appearances of the calcium signals, we built the raster plot of the wave propagation (Figure 4 C-E, Study I). It turned out that some populations of cells were activated faster than others, which could be detected in the blocks of the same distance to the stimulus. Thus, in most of the cases one could observe the leading (fast) wave front, and the following slow wave front. The non-uniformity of the wave speed was maintained also upon facilitation of intracellular calcium release with caffeine (Figure 4 E, Study I). After injection of suramin, the waves were suppressed, although the activation times were still non-uniform for the cells close to the stimulus point (Figure 4 D, Study I).

The non-uniform speed of the wave propagation indicates the presence of a subpopulation of connected cells already at an early stage of development (E14). The non-uniform spread of the calcium waves has also been observed in slices upon mechanical stimulation (Weissman et al., 2004).

The *in vivo* measurements of evoked calcium waves thus be a means to study the changes in neuronal connectivity during early cortical development and also a platform to test pharmacological agents affecting neuronal connectivity. For the evoked responses, polar transformation with the chosen binning of the region of interest size could be an alternative to the fine cell-to-cell measurements in a case where the motion artifacts are challenging to get rid of completely (as in *in vivo* mouse embryos imaging). For tasks where the cell-to-cell connectivity studies are not

necessary, polar transformation could be a simplified way to estimate the non-uniformity of the signal spreading.

5.2. Study II

5.2.1. PSi nanoparticles penetrate the embryonic brain after maternal intravenous injection

The effects of nanoparticles ln embryos are often studied in invertebrate models like Drosophila or Zebrafish, which lack the placental barrier. Alhough various studies have addressed the effects of nanoparticles on adult brains, the effects on embryonic brains are still unclear. To investigate the dynamics of nanoparticles in embryonic brain, we have chosen porous silicon (PSi) nanoparticles, which are currently under extensive investigations as a promising drug-carrier candidate for low -water-soluble drugs (Santos et al., 2011). To test whether PSi can penetrate the placental barrier and reach the embryonic brain, we injected pregnant mother mice with PSi intravenously. We chose a low toxic concentration of nanoparticles, estimated from the previous *in vitro* studies (Makila et al., 2012). Upon injection of this amount, some nanoparticles were observed in embryonic brains 2 days later (Figure 1b, Study II). Interestingly, we observed the particles in the distant parts of the brain tissue (Supplementary Figure 2, Study II). These results indicate the possibility of penetration of various parts of the embryonic cortex after maternal exposure to PSi nanoparticles.

To investigate the dynamics of nanoparticles when already in embryonic brains, we employed the model of direct intraventricular injection to the embryonic brains *in utero* (Figure 1c, Study II). Two days after the injection, the nanoparticles were detected in various parts of the embryonic cortex (Figure 1d, Study II). Thus, we decided to use the *in utero* nanoparticle injection model throughout the study, as we were able to better control the amount of nanoparticles in the embryonic brains.

In future studies, quantitative measurements of placental penetration by nanoparticles would be highly beneficial (Huang et al., 2015). Another important issue to investigate is the nanoparticles size and coating effects on placental barrier penetration. Also, the mechanisms of the placental barrier are still somewhat obscure and need to be elucidated for smart-drug design. Some studies have shown penetration of metallic oxide particles through the placental barrier and to various organs of embryos (Yamashita et al., 2011). However, the dynamics of nanoparticles inside the brain, which is one of the most vulnerable embryonic organs, has not been thoroughly investigated. Here, we focused on the dynamics of nanoparticles already in the brain.

5.2.2. Distribution of PSi nanoparticles in the embryonic brains over time

We investigated the distribution of PSi nanoparticles in embryonic brains over time. We collected the brains 4 hours, 2 days, or 3 days after intraventricular injection. Already 4 hours following the injection the particles were observed in various parts of the cortex (Figure 2a, Study II). The particles were observed at a distance of up to 480 μm from the ventricle, while the cortical thickness at the region is 600 um. Thus, already 4 hours following the injection PSi particles were able to penetrate up to 80% of the cortical depth.

Particles of various sizes could be observed at different depths in the cortex. We analyzed the distribution of the particles at the different distances from the injection site 4 hours after the injection according to the size of the particles (Figure 3c, Study II). Interestingly, the largest particles did not accumulate in the injection area, but rather propagated through the cortex around 200 μm, thus, 30-35 % of the cortical depth. We observed three separate pools of particles: large particles (4 μm in diameter) close to the injection area, with apparently slow motility; another pool of

large particles (3-6 μm) at 200 um distance; and small particles, which propagated larger distances. Thus, smaller particles appear to more easily diffuse to deeper tissues.

The strong penetration of the cortical tissues by large particles (3-6 μm) is rather surprising. One explanation could be the active cell division and constantly ongoing somatic translocation, causing non-stable quality of the tissue of this region (ventricular zone), thus allowing particles to diffuse through the zone (Kwan et al., 2012). Another explanation may lie in active engagement of the particles to the motion together with cellular motility. Also, at these early embryonic stages the brain tissues do not yet possess extracellular matrix, which could hinder tissue penetration at later stages (Lau et al., 2013).

Two and three days later, only large particle agglomerates could be observed. Thus, the smaller particles are either eliminated from the brains of embryos by this point or are accumulated into the agglomerates. Agglomerate formation could be the consequence of active motility in the tissue, while the elimination of the particles could either happen following degradation to free silicic acid or elimination from the brain and then from other organs of embryos, probably via the reticular-endothelial system (Bimbo et al., 2010). *In vitro* dissociation test of the particles in PBS showed dissolution of around 30% of the particles after 72 hours. *In vivo* conditions of the embryonic cerebro-spinal fluid could facilitate the degradation of PSi particles. The elimination of the particles is highly important for consideration of biomedical applications in the embryos as well as in pregnant mothers.

The particles measured in the study were relatively large, and smaller nanoparticles might have been missed. Spatial resolution can be improved by using higher magnification confocal imaging, enabling single nanoparticle detection. However, the focus of the study of particle spread in the present work is rather opposite: even very large particles (3-6 μm) are able to penetrate the deep tissue of embryonic brains. This shows that embryonic brains are 'transparent' to the

nanoparticles, which should be taken into account in biomedical nanoparticle applications.

5.2.3. Motility of PSi particles

To investigate the motility of nanoparticles in embryonic brains in real time, we adapted the *in vivo* imaging method from Study I. We used the same imaging system with a separate chamber for the uterine horn, and the same fixation procedure for the embryos (Figure 3a, Study II). We injected the embryos intraventricularly with PSi nanoparticles, as in the other experiments of this study. To detect nanoparticle motility, which is a significantly slower process than calcium signaling in time, we tried to image as large volumes as possible to capture the simultaneous behavior of different particles. On the imaged z-stacks on the time scale of 50 min, we observed the active motility of several small PSi particles (Figure 3b, Study II). The larger agglomerates, however, mainly stayed still during the course of the imaging experiment.

The speed of the small particle motion measured with *in vivo* imaging was 1.7±0.2 μm/min. This speed correlates with the speed of propagation of the smaller particles estimated from *ex vivo* analysis. In slices, the smaller particles were observed up to a distance of 480 um from the injection zone after 4 hours, thus reaching an average speed of 2 μm/min. These motion speeds are comparable with the speed of migrating interneurons (Inada et al., 2011). Since the observed *in vivo* particles moved in saltatory manner, but with preferred directionality, one can suggest that the particles were engaged in the cellular motility processes.

Further experiments with transgenic models for visualization of the various cell populations might shed light on the particle-to-cell interactions *in vivo*. This would be important for optimizing drug delivery in rodent disease models. As *in vivo* imaging methods for the placenta are already developed (Zenclussen et al., 2012), the presented *in vivo* embryo imaging system can be adapted to study the mechanisms of placental barrier penetration in real time. Also, the developed *in vivo* imaging system

in embryos can be a useful tool to access the particles behavior *in vivo*, as well as particle-particle interactions which are important for the development of multifunctional nano-systems (Nikitin et al., 2014). Different particles can be imaged simultaneously provided that they have separate fluorescent markers.

The resolution of the two-photon microscope is at the level of other optical microscopes, up to 200 nm, so it is impossible to distinguish single nanoparticles below that limit. However, it should be possible to detect single particles assuming sufficient brightness of the fluorescent markers. In the present study, we detected the red fluorescence of TRITC, which was readily detectable throughout the tissue of the embryonic brain.

5.2.4. Cytotoxicity studies of PSi nanoparticles in embryonic brains

To investigate PSi nanoparticle interactions with cells, we performed high magnification imaging of paraffin sections of the embryonic brains injected with the nanoparticles (Figure 4a, Study II). Three days following the injection, the particles were observed inside the cell cytoplasm. Moreover, the particles could distribute inside the cell. To confirm the nanoparticles penetration to the cytoplasm and to observe the subcellular interactions of the particles, we performed electron microscopy imaging. The particle agglomerates as well as single particles were observed inside the cells near the ventricular zone (Figure 4c,d, Study II), where the injection was performed. The particles were distributed in the cytoplasm of the cell. We did not observe particles inside the nuclei. Therefore, we suggest that the particles were unable to penetrate through the nuclear pores. To confirm the identification of PSi particles by electron microscopy, we performed the electron-diffraction X-ray spectroscopy (Supplementary Figure 3, Study II). The resulting spectra contained a

readily detectable Si peak (Supplementary Figure 3b, Study II), which indicated that the observed structures were, indeed, PSi particles.

Penetration of the particles to the cell, distribution inside the cell, and accumulation in the cytoplasm or in some intracellular structures are affected by particle coating and size. For example, the PSi particles with different coating were observed in the intracellular vesicles (Santos et al., 2014) . In the present work we utilized 3-aminopropyltriethoxysilane-modified thermally carbonized porous silicon nanoparticles (APS-TCPSi NPs). Further modification of the particles could be used for targeting of the particles to the desired intra- or extracellular targets.

To estimate the possible toxic effects of the PSi particles, we stained the paraffin sections of the embryonic brains for caspase-3, which is a marker for apoptosis activation (Figure 4e,h, Study II). We did not observe a significant increase in apoptosis in the brains after the PSi particles injection (Figure 4j, Study II). Interestingly, even the strong accumulation of PSi particles inside the cell cytoplasm did not cause apoptosis activation (Figure 4i, Study II). As the relatively large particle agglomerates might affect the nearby cellular arrangements, we studied brains for gross neuronal layer aberrations by staining the slices with hematoxylin and eosin (Supplementary Figure 4, Study II). No apparent changes in layer formation were observed.

Previous *in vitro* studies have shown concentration-dependence in toxicity of PSi nanoparticles (Bimbo et al., 2011a) regarding various stress-response activation markers. In the present study, the same variety of screening tests for toxic effects as is possible *in vitro* was deemed implausible also, the toxic effects vary depending on the size and, especially, the coating of the particles. Importantly, however, the PSi particles did not produce acute apoptotic effects, thus suggesting possible biocompatibility with the embryonic brain tissues. Further behavioral tests are necessary to rule out possible adverse effects of the PSi nanoparticles. However, the relatively fast dissolution rate (in the order of several days), renders it possible that the overall toxic effects of the particles would not be significant, especially in humans,

in whom gestation lasts for 9 months, in contrast to mice, in which the embryonic development period lasts for only 19 days. Thus, in mice even a 3- to 4-day interference with the nanoparticles might produce much more significant effects.

PSi in the present study was chosen mainly as a model nanoparticle. As a low toxic nanoparticle, we assumed that PSi would not significantly interfere with the intra- and extracellular processes and would reflect well the general nanoparticle motility behavior in the growing embryonic cortex. It is possible that the nanoparticles producing acute toxic effects, such as reactive oxygen species generation, would behave differently in the cortex and perhaps propagate over different distances.

5.3. Study III

5.3.1. Spontaneous activity in embryos *in vivo*

For measurements of spontaneous calcium activity in embryos *in vivo* we slightly adapted our imaging protocol from Study I. Namely, the mice were kept under isoflurane anesthesia, and the calcium dye injection was performed manually when the embryo was already immobilized. This allowed high precision location of the imaging area, however, the variations in the amount of dye delivered inevitably increased. While the protocol in Study I was fine-tuned for high-throughput imaging of many embryos, the protocol in Study III was aimed at the continuous imaging of highly stabilized single embryos in long sessions.

Under isoflurane anesthesia, we observed calcium oscillations in various cells, although at relatively low frequency (0.001 Hz) (Study III, Figure 1 A-D). Most of the oscillations achieved amplitudes of 10-30% of $\Delta F/F$ increase, and the most prevalent half-life of the oscillations was in the range of 10-30 s (Study III, Figure 1 E, F). The frequency of the events observed was lower than observed in slices at that age (Liu et al., 2010b), which might stem from the suppressing effect of isoflurane on the activity (Sitdikova et al., 2014) of immature neurons.

We observed two principally different modes of activity: stochastic, low correlated activity (Study III, Figure 2 F-H) and highly correlated, wave-like cluster

activity (Study III, Figure 2 A-C). The wave-like activity was observed only in half of the recordings, however, low frequency of the calcium waves has also been observed in the cortical slices *ex vivo* (Weissman et al., 2004). Interestingly, in slices the propagating calcium waves at the developmental stage E14-E15 were observed only in the radial glia (Liu et al., 2010b; Weissman et al., 2004) and were believed to appear in the differentiated neurons in slices at the later stages of E17-E18 (Crepel et al., 2007). Our results imply possible discrepancies in the coordinated calcium activity *in vivo* versus *ex vivo*. This is in line with the differing behavior of acetoxymethyl forms of calcium dyes, which are taken up only by differentiated neurons in embryos *in vivo*. The discrepancies in the calcium dye properties might also explain the differing results between *in vivo* and *ex vivo* measurements.

Spontaneous waves observed involved 10 to 14 cells observed in the imaging field, which is lower than the stimulated waves evaluated in Study I. The analysis of synchronicity implied a higher correlation of neighboring cells than distant ones in the same cluster. In contrast, there was no synchronicity observed in the stochastic events (Study III, Supplementary Figure 1).

The possibility of measurements and analysis of spontaneous activity of cellular populations in embryos *in vivo* presents a powerful tool for investigating the drugs and agents affecting intercellular connectivity. This may be further combined with measurements of stimulated activity (Study I), thus facilitating the elucidation of molecular mechanisms involved by gaining higher statistical power.

5.3.2. NMDA receptor involvement in spontaneous calcium activity

The *ex vivo* studies of developing brains have shown the presence of active NMDA receptors already at the early stage of development (Flint et al., 1997; Komuro and Rakic, 1993; LoTurco et al., 1991). However, there has been no confirmation in embryos *in vivo* of the functionality of NMDA receptors. In Study I, we observed almost no spontaneous activity under ketamine/xylazine anesthesia. Since the

calcium recordings were performed in differentiated cells, and ketamine is known to block NMDA receptors, we hypothesized that the spontaneous activity might depend on the activation of NMDA receptors. To test this, we injected ketamine intraventricularly, which led to an almost complete shut-down of spontaneous calcium activity (Study III, Figure 3). To confirm that ketamine can penetrate the placental barrier, we measured calcium in the embryos, which were connected to mothers anesthetized by the ketamine/xylazine mixture. We did not observe spontaneous calcium activity in this case (Study III, Figure 4), thus confirming the penetration of placental barrier by the anesthetics.

The involvement of NMDA receptors in spontaneous activity in the developing cortex has long been studied in the *ex vivo* and *in vitro* conditions (LoTurco et al., 1991; Luhmann et al., 2015). However, the direct *in vivo* confirmation had been lacking thus far. Although the action of glutamate on activity in the developing cortex is commonly accepted, there are many ongoing debates regarding the action of another crucially important neurotransmitter GABA *in vivo* (Ben-Ari et al., 2012). Various disagreements have stemmed from differences in results obtained in *ex vivo*, *in vitro* and *in vivo* experiments (Kirmse et al., 2015; Valeeva et al., 2016; Zilberter, 2016). Thus, the direct confirmation of the action of NMDA receptors *in vivo* is an important step towards understanding the real picture of the developing brain activity. Moreover, since we were able to test the effects of the different agents on the activity in embryos *in vivo* (ATP and caffeine in Study I, ketamine in Study III), the developed *in vivo* imaging method might help to elucidate the real *in vivo* action of neurotransmitters and other basic regulating molecules.

5.3.3. Measurements of cellular motility *in vivo*

Using *in vivo* imaging, it was possible to track the same cells over a time period long enough to detect cell displacement. Furthermore, it was possible to detect the same cells after the injection of substances to the embryo during imaging, however, only for the initially visible image. Whole field of view tracking before and after the injection was challenging because of the tissue directionality change due to the

change in the volume of liquid inside the ventricle. We measured the response of the same cells in the same embryo to the injected NMDA-blocking drug ketamine. As described in the literature review, the NMDA receptors are involved in the neuronal migration process and blocking NMDA receptors causes a reduction in migration speed (Komuro and Rakic, 1993). This was confirmed in our experiments, migration speed being decreased in moving cells following ketamine injection. The motility reduction in the Fluo4-AM loaded cells corresponded to an almost complete block of the calcium activity in the cells, as well as in the surrounding area. Interestingly, not only somatic translocation of the cells was affected, but also fine protrusion motility on the tip of the cells (Figure 6). The resolution of the method did not allow us to study the effects on actin machinery in detail, however, the bulk effect on motility could be readily assessed by measurement of pixel displacement over the motion period (Figure 5 D) (Bertling et al., 2012). While the effects of calcium activity on the intercellular processes, involving actin, are well studied *in vitro*, the *in vivo* mechanisms await elucidation.

In the future, the *in vivo* imaging techniques developed should enable study of the cell-to-cell interaction in the context of surrounding calcium activity. Also, it is still unclear how the activity of neighboring cells affects the migration of the cells, e.g. how activity in pyramidal neurons affects the positioning of interneurons (Hoerder-Suabedissen and Molnar, 2015).

The *in vivo* imaging of neuronal migration is slowly becoming a routine method in the field of brain development, as an alternative to the common *ex vivo* brain slice technique. Imaging of migration of GABAergic neurons was performed *in vivo* in embryos as early as in 2003 by the Pasko Rakic group (Ang et al., 2003) in the marginal zone, and later by (Yokota et al., 2007). These experiments were focused on the random-walk behavior of migrating cells. The *in vivo* imaging of the Golgi complex in the migrating neurons was demonstrated by (Yanagida et al., 2012). The imaging of early postnatal mice (Inada et al., 2011) revealed differences in the migration behavior in GAD67-GFP and VGAT-GFP mice since the GAD67-GFP model

lacks one allele, thus having lower levels of intrinsic GABA. However, deep imaging of unidirectional migration of GABAergic neurons has been achieved only recently (Higuchi et al., 2016). This might reflect, on the one hand, the optical limitations for deep tissue imaging in E16 and later stages of mouse development, and, on the other hand, the difficulties in harmless live embryo immobilization at the earlier stages.

Conclusions

The main conclusions of this thesis are as follows:

1. System for *in vivo* two-photon imaging in brains of mouse embryos connected to the mother was developed. The system allows measurements of intracellular calcium oscillations, cellular motility, and motion of the externally introduced nanoparticles.
2. Calcium waves could be evoked with high laser irradiation in the embryonic cortex *in vivo*. The evoked waves are ATP-dependent and could be enhanced by facilitation of intracellular calcium release by caffeine.
3. Spontaneous calcium activity observed in mouse embryos at the E14 developmental stage could be divided into two distinct types: sporadic activity in single cells and propagating calcium waves.

   Spontaneous activity and cellular motility are suppressed by blocking NMDA receptors with ketamine.
4. Porous silicon nanoparticles can reach the embryonic cortex of mice upon injection of the mother. In embryonic brains, the nanoparticles are highly motile and can propagate deep into the cortex, without significant acute toxic effects.

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