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INTRACRANIAL NIMODIPINE IMPLANT:

Feasibility and Implications for the Treatment of Subarachnoid Hemorrhage

-A Pre-Clinical Study-

Janne Koskimäki

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To my family

ABSTRACT

Janne Koskimäki

INTRACRANIAL NIMODIPINE IMPLANT: FEASIBILITY AND IMPLICATIONS FOR THE TREATMENT OF SUBARACHNOID HEMORRHAGE – A PRE-CLINICAL STUDY

From University of Turku, Faculty of Medicine, Department of Clinical Medicine, Surgery, Neurosurgical Unit, Doctoral Programme of Clinical Investigation, and University of Helsinki, Helsinki Neuroscience Center
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Intracranial aneurysmal subarachnoid hemorrhage (aSAH) is a life-threatening condition requiring immediate neurocritical care. A ruptured aneurysm must be isolated from arterial circulation to prevent rebleeding. Open surgical clipping of the neck of the aneurysm or intra-arterial filling of the aneurysm sack with platinum coils are major treatment strategies in an acute phase. About 40% of the patients suffering from aSAH die within a year of the bleeding despite the intensive treatment.

After aSAH, the patient may develop a serious complication called vasospasm. Major risk for the vasospasm takes place at days 5–14 after the primary bleeding. In vasospasm, cerebral arteries contract uncontrollably causing brain ischemia that may lead to death. Nimodipine (NDP) is used to treat of vasospasm and it is administrated intravenously or orally every four hours for 21 days. NDP treatment has been scientifically proven to improve patients' clinical outcome.

The therapeutic effect of L-type calcium channel blocker NDP is due to the ability to dilate cerebral arteries. In addition to vasodilatation, recent research has shown the pleiotropic effect of NDP such as inhibition of neuronal apoptosis and inhibition of microthrombi formation. Indeed, NDP inhibits cortical spreading ischemia. Knowledge of the pathophysiology of the vasospasm has evolved in recent years to a complex entity of early brain injury, secondary injuries and cortical spreading ischemia, instead of being pure intracranial vessel spasm.

High NDP levels are beneficial since they protect neurons and inhibit the cortical spreading ischemia. One of the drawbacks of the intravenous or oral administration of NPD is systemic hypotension, which is harmful particularly when the brain is injured. Maximizing the beneficial effects and avoiding systemic hypotension of NDP, we developed a sustained release biodegradable NDP implant that was surgically positioned in the basal cistern of animal models (dog and pig). Higher concentrations were achieved locally and lower concentrations systemically. Using this treatment approach in humans, it may be possible to reduce incidence of harmful hypotension and potentiate beneficial effects of NDP on neurons.

Intracellular calcium regulation has a pivotal role in brain plasticity. NDP blocks L-type calcium channels in neurons, substantially decreasing intracellular calcium levels. Thus, we were interested in how NDP affects brain plasticity and tested the hypothesis in a mouse model. We found that NDP activates Brain-derived neurotrophic factor (BDNF) receptor TrkB and its downstream signaling in a reminiscent of antidepressant drugs. In contrast to antidepressant drugs, NDP activates Akt, a major survival-promoting factor. Our group's previous findings demonstrate that long-term antidepressant treatment reactivates developmental-type of plasticity mechanisms in the adult brain, which allows the remodeling of neuronal networks if combined with appropriate rehabilitation. It seems that NDP has antidepressant-like properties and it is able to induce neuronal plasticity. In general, drug induced neuronal plasticity has a huge potential in neurorehabilitation and more studies are warranted.

Keywords: delayed cerebral ischemia, implant, neurosurgery, nimodipine, plasticity, rehabilitation, subarachnoid hemorrhage, vasospasm

TIIVISTELMÄ

Janne Koskimäki

KALLONSISÄINEN NIMODIPIINI-IMPLANTTI: SOVELTUVUUS JA VAIKUTUKSET SUBARAKNOIDAALIVUODON HOIDOSSA – PREKLIININEN TUTKIMUS

Turun yliopisto, Lääketieteellinen tiedekunta, Kliinen laitos, Kirurgian oppiaine, Neurokirurgian yksikkö, Turun kliininen tohtoriohjelma sekä Helsingin yliopisto, Neurotieteden tutkimuskeskus
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Kallonsisäinen aneurysmaattinen subaraknoidaalivuoto (aSAV) on henkeä uhkaava tilanne, joka vaati välitöntä neurotehohoitoa. Puhjennut aneurysma on suljettava pikaisesti verenkierrosta uusintavuodon ehkäisemiseksi, joko kraniotomian kautta kirurgisesti sulkemalla aneurysman kaula klipsillä tai verisuonen sisäisesti täyttämällä aneurysma platinakoilein. Noin 40 % aSAV potilaista kuolee vuoden kuluessa hoidoista huolimatta.

aSAV:n jälkeen potilaalle voi kehittyä henkeä uhkaava komplikaatio, vasospasmi. Suurin spasmiriski ajoittuu 5−14 vuorokauden sisään primaarivuodosta. Vasospasmissa aivovaltimot supistuvat hallitsemattomasti aiheuttaen vakavan aivokudoksen hapenpuutteen, joka voi johtaa kuolemaan. Hoitona vasospasmiin käytetään nimodipiinia (NDP), jota annostellaan laskimonsisäisesti jatkuvana infuusiona tai suun kautta neljän tunnin välein 21 vuorokauden ajan. NDP -hoidon on tieteellisesti osoitettu parantavan potilaan ennustetta.

L-tyypin kalsiumkanavan salpaajana NDP:n vaikutus perustuu aivovaltimoiden relaksoitumiseen, jolloin valtimot laajenevat. Nykytutkimukset ovat kuitenkin osoittaneet, että NDP:llä on edellä mainitun ominaisuuden lisäksi muitakin edullisia vaikutuksia, kuten hermosolujen apoptoosin esto, mikrotromboosien kehittymisen esto sekä patologisten aivosähköpurkausten vähäisempi ilmaantuminen vuodon jälkeen. Lisääntyneen tutkimuksen ansiosta vasopasmin patofysiologiaa on alettu ymmärtää monimuotoisena aivosairautena eikä vain pelkkänä verisuonten supistustilana.

Korkeiden NDP-pitoisuuksien on osoitettu olevan edullisia erityisesti hermosolujen suojaamisessa sekä patologisten aivosähköpurkausten estämisessä. Erityisenä ongelmana oraalisesti tai laskimonsisäisesti annosteltu NDP voi aiheuttaa potilaalla voimakkaan verenpaineen laskun, mikä on erityisen haitallista, kun aivot ovat vaurioituneet.

Kehitimme biohajoavan NDP-implantin, joka asennettiin kirurgisesti koe-eläinmallien (koira ja maatiaissika) basaalisiin likvortiloihin. Implanttihoidolla saavutettiin korkeampi NDP-pitoisuus likvorissa sekä matalampi pitoisuus systeemisesti kuin perinteisellä hoidolla. Tällä tavoin pystyttäisiin välttämään potilailla haitallinen verenpaineen lasku sekä tehostamaan NDP:n edullisia vaikutuksia hermosoluihin. Hoitoannoksella implanttihoidosta ei ollut osoitettavissa haittavaikutuksia koe-eläinmalleissa.

Koska hermosolun sisäisellä kalsiumin pitoisuuden säätelyllä on tärkeä rooli aivojen plastisuudessa, tutkimme NDP:n vaikutusta aivojen plastisuuteen hiirimallissa. NDP aktivoi voimakkaasti aivoperäisen hermokasvutekijän (BDNF) reseptoria TrkB:tä ja sen signalointireittejä samaan tapaan kuin masennuslääkkeet. Masennuslääkkeistä poikkeavasti neuroprotektioon liitetty Akt-signalointireitti aktivoitui voimakkaasti. Ryhmämme on aiemmin osoittanut, että TrkB:n aktivaatio liitettynä kuntoutukseen muokkaa tehokkaasti hermoverkkoja. Tällä indusoidulla plastisuudella on todennäköisesti erittäin edullisia vaikutuksia aivosairauksista kuntoutuessa.

Hakusanat: implantti, kuntoutus, lukinkalvonalainen verenvuoto, neurokirurgia, nimodipiini, plastisuus, subaraknoidaalivuoto, vasospasmi

TABLE OF CONTENTS

ABSTRACT 4

TIIVISTELMÄ 5

LIST OF ORIGINAL PUBLICATIONS 9

ABBREVIATIONS AND TERMINOLOGY 10

1. INTRODUCTION 12

2. REVIEW OF THE LITERATURE 15
 2.1. Intracranial Aneurysms 15
  2.1.1. Prevalence 15
  2.1.2. Pathobiology 15
  2.1.3. Treatment of unruptured aneurysms 16
 2.2 Subarachnoid hemorrhage 16
  2.2.1. Incidence 16
  2.2.2. Pathophysiology 17
   2.2.2.1. Early brain injury 17
   2.2.2.2. Inflammation 18
   2.2.2.3. Microthrombosis 18
   2.2.2.4. Cortical spreading ischemia and delayed cerebral ischemia 19
  2.2.3. Management and treatment of ruptured aneurysms 19
   2.2.3.1. Rebleeding 19
   2.2.3.2. Surgical clipping and endovascular coiling 20
   2.2.3.3. Delayed cerebral ischemia 20
   2.2.3.4. Other complications after aSAH 21
   2.2.3.5. Outcome 21
   2.2.3.6. Rehabilitation 22
 2.3. Intracranial treatments for vasospasm 22
  2.3.1. Concept 22
  2.3.2. Sustained release implants for intracranial treatment 23
  2.3.3. Biomaterials for intracranial treatment 23
 2.4. Neuroplasticity 24
  2.4.1. Evolution of the brain 24
  2.4.2. Neuronal plasticity 25
   2.4.2.1. Role of TrkB and BDNF in neuronal plasticity 25
   2.4.2.2. L-type calcium channels 27
   2.4.2.3. Role of calcium in neuronal plasticity 27
   2.4.2.4. Critical periods 27
  2.4.3. Induced plasticity 28
   2.4.3.1. Drug-induced plasticity 28
  2.4.4. Induced plasticity for neurorehabilitation – Current progress 28

3. AIMS OF THE STUDY 30

4. MATERIALS 31
4.1. Implants 31
4.1.1. Material 31
4.1.2. Drug concentration 31
4.2. Animal models 31
4.2.1. Dog and pig models 31
4.2.2. Mouse model 31
4.3. Drugs 31
4.3.1. Nimodipine 31

5. METHODS 32
5.1. Pharmacokinetics of nimodipine in healthy beagle dogs 32
5.1.1. Study treatment 32
5.1.2. Blood and CSF sampling 32
5.1.3. Sample handling 32
5.1.4. Nimodipine analysis 33
5.1.5. Pharmacokinetic analysis 33
5.2. Nimodipine implant study treatment and follow-up 33
5.2.1. Preparation of silica-based implants 33
5.2.2. Dissolution of implants *in vitro* 33
5.2.3. Allocation of the treatment groups and follow-up 33
5.2.4. Anesthesia and analgesia 34
5.2.5. Surgery 35
5.2.6. Sampling 35
5.2.7. Computed tomography 35
5.2.8. Histological analyses 36
5.2.9. Statistical analyses 36
5.3. Nimodipine-induced plasticity in mice 36
5.3.1. Drug treatment and tissue sampling 36
5.3.2. Western blot 36
5.3.3. BDNF ELISA 37
5.3.4. BDNF qPCR 37
5.3.5. Data processing and statistical analyses 37

6. RESULTS 38
6.1. Pharmacokinetics of nimodipine in healthy beagle dogs 38
6.1.1. Individual kinetic profiles 38
6.1.2. Pharmacokinetic parameters 38
6.1.3. CSF concentrations of nimodipine 38
6.2. Nimodipine implant treatment and follow-up 39
6.2.1. Dissolution of the implant ***in vitro* 39**
6.2.2. Surgery and recovery period 39
6.2.3. Histology of brain and meninges 40
6.2.4. Nimodipine concentrations 41
6.2.5. Computed tomography 42
6.3. Nimodipine-induced neuroplasticity 42
6.3.1. Nimodipine activates TrkB in the brain 42
6.3.2. Nimodipine induces neuroplasticity in the brain 43

6.3.3. Nimodipine induces neuroprotective signaling in the brain 44
6.3.4. Levels of BDNF protein and mRNA after acute treatment 44

7. DISCUSSION 45
7.1. Nimodipine implant treatment for vasospasm......45
7.2. Nimodipine induces neuronal plasticity 46
7.3. Limitations and strengths of the research 48
7.4. Future views 49

8. CONCLUSIONS 51

ACKNOWLEDGEMENTS 52

REFERENCES 54

ORIGINAL PUBLICATIONS (PAPERS I–III)......67

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals (I-III). The thesis contains also previously unpublished material.

I **Koskimäki J, Tarkia M, Ahtola-Sätilä T, Saloranta T, Simola O, Forsback A-P, Laakso A, Frantzén J.** Intracranial biodegradable silica-based nimodipine drug release implant for treating vasospasm in subarachnoid hemorrhage in an experimental healthy animal model. *BioMed Research International, Special Issue: Translational and Clinical Research in Surgery, 2015 Jan 22.*

II **Koskimäki J, Tarkia M, Ahtola-Sätilä T, Saloranta T, Laakso A, Frantzén J.** Absorption, elimination and cerebrospinal fluid concentrations of nimodipine in healthy beagle dogs receiving human intravenous and oral formulation. *European Journal of Drug Metabolism and Pharmacokinetics, 2015 Feb 5.*

III **Koskimäki J, Matsui N, Umemori J, Rantamäki T, Castrén E.** Nimodipine activates TrkB neurotrophin receptors and induces neuroplastic and neuroprotective signaling events in the mouse hippocampus and prefrontal cortex. *Molecular and Cellular Neurobiology, 2014 Sep 10.*

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ABBREVIATIONS AND TERMINOLOGY

ADP	Adenosine diphosphate
Akt	Protein kinase B
aSAH	Aneurysmal subarachnoid hemorrhage
BDNF	Brain-derived neurotrophic factor
CBF	Cerebral blood flow
CPP	Cerebral perfusion pressure
CREB	cAMP response element-binding protein
CRST	Clinical research services Turku
CSDz	Cortical spreading depolarization
CSDs	Cortical spreading depression
CSF	Cerebrospinal fluid
CSI	Cortical spreading ischemia
CT	Computed tomography
DCI	Delayed cerebral ischemia
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyseraldehyde 3-phosphate dehydrogenase
GDC	Guglielmi detachable coil
HC	Hippocampus
H&H	Hunt & Hess grade
IA	Intracranial aneurysm
ICP	Intracranial pressure
IP_3	Inositol trisphosphate
IVH	Intraventricular hemorrhage
LC-MS	Liquid chromatography–mass spectrometry
LTP	Long-term potentiation
LTD	Long-term depression
NDP	Nimodipine
PDGF	Platelet-derived growth factor
PFC	Prefrontal cortex
PI3K	Phosphoinositide 3-kinase
PLGA	Poly D,L-lactide coglycolide
SDDS	Sustained drug delivery system
SiO_2	Silica oxide
SSRI	Selective serotonin reuptake inhibitor
TrkB	Receptor for BDNF

Biomaterial:	Material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body
CSDz:	Cortical neuronal depolarization wave, which is associated with a total increase in cations and H_20 influx. It is seen in injured brain that cannot respond to an increased energy demand
CSDs:	Cortical spreading depression wave, as above but the brain is not injured and can respond to an increased energy demand

DCI:	Cerebral ischemia propagated by all pathophysiological cascades occurred after aSAH with peak incidence after seven days of initial bleeding
Dissolution:	Solute (in this context implant) that forms a solution by dissolving in the solvent (in this context CSF)
Foreign body reaction:	Variation in normal tissue behavior caused by the presence of foreign material
Homeostatic plasticity:	Structural or functional plastic changes that maintain the balance between excitation and inhibition
Implant:	Medical device made from one or more biomaterials that are intentionally placed within the body either totally or partially
Induced plasticity:	Form of plasticity in which any inductor (*e.g.* a drug) re-establishes the critical or sensitive periods in the brain and further enables effective remodeling of neural networks with proper input
Long-term depression:	Long-lasting decrease in synaptic strength
Long-term potentiation:	Long-lasting postsynaptic signaling enhancement after high-frequency stimulus. Increase synaptic strength
Neuroplasticity:	A strengthening in synaptic connections or alterations in the neuron soma, dendrites or axons so that neuronal synaptic or nonsynaptic behavior is altered
Synapse:	Connection between neurons that relay the communications *via* neurotransmitters or an electrical stimulus

1. INTRODUCTION

Hippocrates (c. 460 – c. 370 BC) once stated: "*When persons in good health are suddenly seized with pains in the head, and straightway are laid down speechless, and breathe with stertor, they die in seven days, unless fever comes on.*" (Hippocrates: The Genuine Works of Hippocrates. 1925 William Wood & Co New York). The aphorism is undoubtedly one of the first descriptions of aneurysmal subarachnoid hemorrhage (aSAH) and reminds us of the severity of its natural history.

aSAH occurs when an intracranial aneurysm (IA) is ruptured and the blood rapidly flows into the subarachnoid space. IAs are usually pouch-like formations at bifurcations of intracranial arteries. These kinds of aneurysms are called saccular. In rare cases the IA is a dilatation of the intracranial artery and is therefore called fusiform.

After bleeding, the mortality rate inreases up to 50% even with prompt aneurysm closure and rigorous neurocritical care (Sarti et al. 1991; Fogelholm et al. 1993). The natural history of aSAH is presented in an unselected series of 363 patients with ruptured IAs, there having been 15% mortality before admission and 60% mortality after 6 months (Pakarinen 1967). Rebleeding of the ruptured IA significantly increases mortality. The risk of rebleeding with conservative treatment is high, 4% on the first day, and the risk remains constant at a rate of 1% to 2% per day during the following two weeks (Kassell and Torner 1983). When the IAs were not eliminated from the arterial circulation, the mortality was 64% at the first recurrence and 86% at the second recurrence (Pakarinen 1967). Thus the primary aim of treating IAs is to prevent rebleeding by isolating the IAs from the arterial circulation while preserving the blood flow in the parent artery and arterial branches.

Currently, the main treatment strategies used to occlude IAs are clipping and coiling. Clipping is performed surgically through the use of a craniotomy, during which a titanium clip is applied across the neck of the aneurysm. Coiling is a less invasive method, in which a platinum coil is intra-arterially inserted into the pouch of the aneurysm, thereby occluding the aneurysm and isolating it from arterial circulation. Since aSAH is a very complex and life-threatening disease, eliminating the aneurysm from circulation is not sufficient. aSAH patients require intensive neurocritical care, which is extremely important if the best results are to be achieved for the patients.

Dr. Viktor Horsley (1857–1916) was the first who surgically treated an intracranial aneurysm by ligation of the carotid artery in 1885 (Kretzer et al. 2010). The method is known as Hunterian ligation, named after Dr. John Hunter who introduced proximal femoral artery ligation for popliteal aneurysms instead of an amputation (Hunter 1835). The first planned operation on an intracranial aneurysm was performed on April 22th, 1931 by Dr. Norman Dott (1897–1973) (Dott 1933). Dr. Dott used a method called wrapping, a technique which he had probably adopted from Dr. Harvey Cushing (1869–1939) during his residency training in 1923–1924 (Kretzer et al. 2010). Before the World War II on March 27th, 1937, Dr. Walter E. Dandy (1886–1946) operated ruptured right sided internal carotid artery aneurysm and introduced the method called clipping (Dandy 1938; Kretzer et al. 2010). The clipping technique is currently one of the most important treatment strategies for treating intracranial aneurysms and became safer since the 1960's when an operating microscope was introduced.

Started in 1970s, a new less invasive endovascular method for occluding IAs was developed. The revolution in neuroendovascular treatment of IAs took place after Dr. Guido Guglielmi had developed detachable platinum coils (Guglielmi detachable coil, GDC) which were approved in clinical use by Food and Drug Administration (FDA, K951256) on September 1995 (Guglielmi et al. 1991; Guglielmi et al. 1992).

Currently, surgical and endovascular aneurysm occlusion therapies can be considered equally effective and safe (Koivisto et al. 2000; Molyneux et al. 2005). Both approaches have pros and cons, thus a selection of the best treatment strategy for the patient require a highly experienced vascular neurosurgeon.

A new paradigm shift has already occurred in endovascular treatment. Flow diverters close the parent artery causing endoluminal reconstruction rather than saccular filling. This technique is effective in treating non-ruptured incurable wide neck, dissecting or giant aneurysms (Alderazi et al. 2014). Flow diverters seem to be safe, but especially the indications of flow diverters need to be confirmed in further studies to avoid complications that could be prevented with surgical clipping. (Berge et al. 2012; Cohen et al. 2014; Briganti et al. 2014; Britz).

Despite the technical development of aneurysm occlusion therapies, mortality rates are high after aSAH. A devastating complication, called vasospasm, occurs in about 30% of patients after aSAH, the peak incidence being reached seven days after the initial bleeding (Dorsch and King 1994; Macdonald 2014). Vasospasm is an intracranial arterial spasm with complicated and partly unknown pathophysiology that may lead local or even global brain ischemia. Clinically the state is observed as a delayed neurological deterioration (Macdonald 2014).

In late 1980s, L-type calcium channel blocker nimodipine (NDP) was introduced for treating vasospasm. In clinical trials, 60 mg of NDP orally every four hours improved patients' outcome (Laursen et al. 1988; Pickard et al. 1989). The mechanism of action of NDP was originally postulated to reduce angiographic vasospasm *via* dilatation intracranial blood vessels. However, angiographic vasospasm and outcome do not seem to correlate and the exact mechanism is still mainly obscure (Dorsch 2011; Woitzik et al. 2012; Etminan et al. 2013).

NDP has been shown to have favorable pleiotropic effects for neuronal survival, *e.g.* inhibition of neuronal apoptosis and excitotoxicity, inhibition of cortical spreading ischemia and reducing the number of microthombosis (Zornow and Prough 1996; Dreier et al. 2002; Vergouwen et al. 2008). On the other hand, NDP can decrease systemic blood pressure, which may restrict the NDP treatment after aSAH.

NDP binds to L-type calcium channels of neurons and alters intracellular calcium concentration. Calcium signaling has a major role in neuronal homeostasis. Activity-dependent calcium influxes *via* L-type calcium channels regulate several intracellular signaling pathways that modulate short- and long-term alterations in gene expression and synaptic plasticity in neurons (Park and Poo 2013; Bading 2013; Frank 2014). The density of L-type calcium channels are significantly higher in neurons than corresponding cerebral blood vessels (Ricci et al. 2002). However, excessive activation of L-type calcium channels leads to compromised plasticity, excitotoxicity and neurodegeneration (Choi 1994; Mattson 2007).

Drug-induced plasticity for enabling rehabilitation was introduced by Dr. Maya Vetencourt together with Prof. Castrén and Prof. Maffei in 2008 (Maya Vetencourt et al. 2008). Amblyopia was cured on an animal model using antidepressant drug fluoxetine. Fluoxetine restored so-called juvenile-like plasticity by opening the new time window for rehabilitation although the earlier developmental critical period had closed. Additionally, in clinical settings fluoxetine have shown to improve motor outcome in ischemic stroke patients (Chollet et al. 2011).

According to several studies, L-type calcium channel blockers have independent and potentiating antidepressant properties (Mogilnicka et al. 1987; Czyrak et al. 1989; Czyrak et al. 1990; Dubovsky et al. 2001). Interestingly, fluoxetine blocks also L-type calcium channels, affecting calcium signaling of the neurons (Deák et al. 2000; Kim et al. 2013).

Considering the above mentioned aspects, we developed an intracranial silica-based biodegradable NDP implant. The implant was tested in a healthy pig and dog model by surgically positioning the implant in the basal cisterns through frontotemporal craniotomy. Feasibility, tolerability, degradation, histology and pharmacokinetic properties of the implant were widely studied and evaluated. Our aim was to produce higher NDP concentration in cerebrospinal fluid (CSF) than achieved orally for maximizing the positive pleiotropic effects of NDP. Furthermore, our aim was to decrease the systemic concentration of NDP to reduce clinically relevant NDP-induced hypotension, which compromise patients' cerebral perfusion pressure (CBF).

The pivotal role of intracellular calcium signaling in the regulation of plasticity led us to investigate whether NDP induces such molecular conditions that facilitate plastic changes in neurons similarly as seen in antidepressant drugs. Lastly, we discuss the role of NDP after aSAH in a context of drug-induced plasticity and rehabilitation.

2. REVIEW OF THE LITERATURE

2.1. Intracranial Aneurysms

2.1.1. Prevalence

Intracranial aneurysm (IA) is saccular in a 98% of cases and usually forms at the branches of the intracranial blood vessels (Dashti et al. 2007). Multiple aneurysms are found in about one third of the patients with IAs (Inagawa 1990; Rinne et al. 1994; Ellamushi et al. 2001; Dashti et al. 2007). According to a recent meta-analysis, the overall prevalence of IAs reported for 83 study populations is 3.2%, and the prevalence is higher for females, their relative risk being 1.6. Smoking, hypertension and genetic factors increase the prevalence (Rinkel et al. 1998; Vlak et al. 2011).

2.1.2. Pathobiology

Histology of normal intracranial arteries differs from extracranial arteries. The innermost layer is intima, which consists of epithelial cells that surrounds the endoluminal side in the direct contact in blood. Between intima and media, a layer of elastic fibers is forming internal elastic lamina. The middle layer of the blood vessels is called media, and it consists mainly of smooth muscle cells and type III collagen (Canham et al. 1991). The outermost layer is adventitia, with the most complex structure consisting of type I collagen, nerves, fibroblasts, elastin and vasa vasorum (Smith et al. 1981; Finlay et al. 1995; Rowe et al. 2003). In extracranial arteries, media and adventitia are separated by external elastic lamina, which is not present in the structure of intracranial arteries. Another special character in the intracranial vessels are strong tendon-like formations and gaps in the media at the apexes of bifurcations (Finlay et al. 1998; Futami et al. 1998). In the 1930's, these discontinuities in the cerebral vessels were found and were considered to have pathologic nature (Forbus 1930). Further research revealed that these fibrous gaps and tendon-like formations are physiologic (Finlay et al. 1998; Rowe et al. 2003). However, the true physiological meaning of these structures, and the role in the pathogenesis of aneurysms, are still obscure.

A normal intracranial vessel has highly organized structure, which is compromised in the genesis of an aneurysm (Kim et al. 1993; Austin et al. 1993; van den Berg et al. 1997; Kondo et al. 1998). Histopathological changes seen in the wall are a loss of internal elastic lamina, myointimal hyperplasia, hypocellularity and hyalinization (Frösen et al. 2012; Chalouhi et al. 2013; Frösen 2014). The myointimal hyperplasia is a physiological response to the increased hemodynamic demand or the mechanical injury of the blood vessel (Intengan and Schiffrin 2001). However, it is not known if myointimal hyperplasia in the wall of the aneurysm is an adaptive mechanism for increased stress or if it is contributing to the degeneration and weakening of the aneurysm wall (Frösen et al. 2004; Chalouhi et al. 2013).

Organized vessel structure is resistant to the local hemodynamic stress. When the organization is lost, the critical balance between arterial hemodynamic stress and the strength of the wall is disrupted, creating favorable conditions for aneurysm formation (Stehbens 1989; Inci and Spetzler 2000). Several mechanisms that initiate and maintain the wall degeneration have been identified. The activity and expression of matrix metalloproteinases 2 and 9 are increased, causing proteolysis of elastin and collagen and leading to loss of tensile strength (Bruno et al. 1998; Caird et al. 2006). Apoptosis is markedly increased in the wall of aneurysms (Pentimalli et al. 2004). The episodes that elicit apoptosis are indistinct. However, proinflammatory cytokine tumor necrosis factor α and c-Jun amino-terminal kinase have been proposed to be the mechanisms in action (Takagi et al. 2002; Starke et al. 2014). Inflammation is extensively present in the degenerative aneurysm walls (Tulamo et al. 2010a). Every main type of human immune cells are discovered in the wall as well as antibodies (Chyatte et al. 1999). Indeed, expression of a proinflammatory chemokine called monocyte chemoattractant protein-1 is upregulated (Cao et al. 2002; Aoki et al. 2009; Wang et al. 2014).

Complement activation and inflammatory cell infiltration is higher in the ruptured than in the unruptured aneurysms (Tulamo et al. 2006; Tulamo et al. 2010b). Interestingly, high hemodynamic stress *i.e.* hypertension, causes conditions in which all above-mentioned pathological mechanisms are seen (Stehbens 1989; Inci and Spetzler 2000). Lastly, atherosclerosis can contribute to the formation of aneurysms by increasing the inflammatory response, lipid accumulation and oxidation in the wall of the aneurysms (Frösen et al. 2013). Association of the pathobiological mechanisms behind the aneurysms and the clinical risk factors for aneurysms and aSAH are mainly logical. Still, major discrepancies, *e.g.* female gender predisposition, are observed.

2.1.3. Treatment of unruptured aneurysms

Unruptured aneurysms are found either incidentally or in the screening for a familial background of aneurysms. As mentioned, the overall prevalence of IAs is 3.2% (Vlak et al. 2011). It means that in Finland alone, there are nearly 200 000 people carrying an unruptured aneurysm. If an aneurysm stays unruptured it rarely causes any medical problems.

The rupture rate is different between nations, being the highest in Finland and Japan (Linn et al. 1996; Ohkuma et al. 2002; Korja et al. 2013; Suzuki and Izumi 2014). The ability to predict the rupture of the unruptured aneurysm would be the most effective way to evaluate the need of care. Unfortunately, a lack of precise biomarkers is a current fact. However, encouraging results have been obtained for preventing the rupture of aneurysms and evaluating the risk of rupture. Vascular endothelial growth factor and transforming growth factor beta receptors have been to be associated with an increased risk of the rupturing and remodeling of IAs, making them potential targets for biological drug therapy (Frösen et al. 2006). In addition, diagnostic and prognostic biomarkers for characterizing the rupture risk of unruptrured IAs are under development (Sabatino et al. 2013).

When unruptured aneurysm is detected, a careful analysis of risk factors related to the patient and the aneurysm itself is required. Aneurysm size, location and multiplicity are risk factors for rupture as well as earlier rupture and familial background (Kissela et al. 2002; Wiebers et al. 2003; Clarke et al. 2005; UCAS Japan Investigators et al. 2012). Smoking, hypertension and alcohol abuse are treatable risk factors that increase the risk of the rupture (Knekt et al. 1991; Longstreth et al. 1992; Juvela et al. 1993).

Vast uncertainty for treating of small aneurysm is evident. It seems that aneurysms under 5 mm will rupture rarely (Brown and Broderick 2014). However, certain populations may be susceptible to developing aSAH even when the aneurysm is small (Dashti et al. 2007; Wong et al. 2013). In carefully selected cases, treatment of small aneurysms is rational.

A meticulous analysis of the afore-mentioned risk factors is critical when a decision is made concerning treatment or a follow-up strategy. If risk factors are clinically considered to be significant and the risk of aneurysm rupture is possible, an unruptured IA is eliminated from arterial circulation. The main treatment strategies are surgical clipping and endovascular coiling, as described earlier. However, in the treatment of complex unruptured IAs, new flow diverters offer a possible treatment option (see section 1).

Clinical trials directly comparing the two aneurysm occlusion modalities surgical clipping and endovascular coiling are lacking. Whether the treatment strategy is coiling or clipping, the selection is influenced by patient's age, size, morphology and location of the aneurysm, institution and neurosurgeon's experience (Brown and Broderick 2014).

2.2 Subarachnoid hemorrhage

2.2.1. Incidence

Although the prevalence of IAs is at the same level in the different nations, incidence of the aSAH is significantly higher in Finland and Japan, reaching as high as 20 cases per 100 000 (Linn et al. 1996; Ohkuma et al. 2002; Korja et al. 2013;

Suzuki and Izumi 2014). In other populations, the incidence is 6–10 cases per 100 000 (van Gijn et al. 2007). Risk factors for aSAH are smoking, hypertension, heavy alcohol consumption, age, female gender, aneurysm size and location and genetic factors (Knekt et al. 1991; 1998; Clarke et al. 2005; Feigin et al. 2005; Sandvei et al. 2009). Genetic factors have a moderate effect to the incidence of aSAH. However, in familial aSAH, the increased rupture rate is usually attributed to environmental risk factors (Korja et al. 2010).

2.2.2. Pathophysiology

Immediately after rupture of an aneurysm, several pathophysiological cascades originate in the brain. Blood flows into the subarachnoid space with high pressure. Bleeding is stopped when the ruptured vessel is constricted and the local intracranial pressure meets the systolic blood pressure (Nornes and Magnaes 1972). Bleeding itself can cause mechanical injury to the brain by flowing with high pressure in the parenchyma.

Knowledge of the pathophysiology of the aSAH has evolved tremendously in recent years. Inflammation and microthrombosis play significant role in the light of latest studies. Importantly, understanding of the delayed cerebral ischemia (DCI) and the underlying vasospasm has developed in recent years to a complex entity of early brain injury, secondary injuries and cortical spreading ischemia, instead of being pure intracranial vessel spasm (Macdonald 2014). The increased knowledge of the pathophysiology of aSAH provides the fundamental prerequisites for the development of more effective treatments.

2.2.2.1. Early brain injury

Early brain injury is a sequence of cascades that occurs during 72 hours of the initial bleeding and is initiated immediately after the rupture of an aneurysm. Mechanical injury and ionic and physiologic imbalance occur in seconds. Within an hour, ionic and physiologic imbalance proceed to cell death, inflammation, oxidative stress and vascular changes. During 72 hours, these cascades persist lead to a permanent injury of the brain. (Cahill et al. 2006; Sehba et al. 2011; Caner et al. 2012; Fujii et al. 2013).

As stated, mechanical trauma plays a pivotal role in the pathological process. Bleeding pressure may rupture the brain parenchyma causing direct damage. Directly after the rupture, intracranial pressure may exceed the pressure over 160 mmHg (Nornes and Magnaes 1972). The blood clot mechanically stretches blood vessels and promotes the vasoconstriction (Arutiunov et al. 1974).

ICP rises, compromising the cerebral perfusion pressure (CPP) and cerebral blood flow (CBF) (Brinker et al. 1992). However, decreased CPP alone does not explain the acute cerebral ischemia; direct vasoconstriction needs to be present (Bederson et al. 1995). Interestingly, two distinct patterns of ICP are distinguished and both have their unique mechanisms. The first pattern forms a rapid peak in ICP but soon returns to near basal values. The amount of bleeding is small, but edema is already present. This condition is called ischemic-edematous lesion to distinguish it from hemorrhagic-compressive lesion in which ICP remains high due to the mass of the expanding hematoma or an acute hydrocephalus (Nornes and Magnaes 1972; Nornes 1973).

Ionic imbalance of sodium, potassium, calcium and magnesium occurs and leads to vascular and electrical instability. Disruption of the ion, energy and nitric oxide (NO) homeostasis, increased oxyhemoglobin, glutamate and endothelin-1 are involved by producing measurable waves of cortical spreading depolarization (CSDz) (Dreier et al. 2002). In unfavorable conditions, such as after aSAH, CSDz progress to a cortical spreading ischemia (CSI) (Dreier et al. 2002; Dreier et al. 2009; Macdonald 2014).

Neuronal cell death *via* necrosis as well as apoptosis have been described and several cellular pathways are involved (Dreier et al. 2000; Lee et al. 2009a; Zhao et al. 2014). Cellular death is highly active during the first week and is less active on day 11 (Nau et al. 2002; Prunell et al. 2005). However, the time period of cellular death is unknown. After aSAH most patients suffer from cognitive and memory deficit. Interestingly, in the hippocampus, long-term potentiation (LTP) formation is lost after experimental aSAH independently of global ischemia and neuronal

loss, but it is associated with a decrease in the number of synapses, which compromises the plasticity of the brain (Han et al. 2014).

NO is a double-edged sword. Both increased and decreased levels of NO may be present after aSAH. NO levels decrease first, remaining low for three hours and then returning to the basal level (Sehba et al. 2000). After 24 hours, increased levels are measured (Ng et al. 2001; Yatsushige et al. 2006). Decreased levels cause vasoconstriction, platelet aggregation and decrease in CBF (Sehba et al. 2000; Sehba et al. 2005). Increased levels can cause damage to the cell membranes and mitochondria and may eventually lead to cell death (Szabó and Dawson 1998; Sehba and Bederson 2011; Zhao et al. 2014). Also, increased endothelin-1 is shown to be present when NO levels are low, generating conditions that favor vasospasm (Sehba et al. 2011).

2.2.2.2. Inflammation

Complement activation, cytokines, leukocytes, neutrophils, macrophages and adhesion molecules are behind the inflammation process after aSAH. E-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) are represented mainly on the luminal endothelial cells (Kasuya and Shimizu 1989; Handa et al. 1995; Lin et al. 2005). These molecules are upregulated after aSAH and are required for inflammatory cell migration into the central nervous system maintaining the inflammation process (Lossinsky and Shivers 2004). Interestingly, ICAM-1 is present not only on the endothelial layer but also in the medial layer (Handa et al. 1995). Inhibition of the cell adhesion molecules has been shown to improve outcome after aSAH in animal models and increased concentration is associated with poor outcome in humans after aSAH (Handa et al. 1995; Mack et al. 2002; Lin et al. 2005).

Inflammatory cytokines interleukine-6, tumor necrosis factor α and interleukine-1 antagonist are increased and have been shown to elicit ischemic deficits, and are associated with poor outcome (Mathiesen et al. 1997; Gruber et al. 2000). Activated complement proteins C3a and C5a are also upregulated rapidly after aSAH, mainly due to activated coagulation and thrombin formation (Kasuya and Shimizu 1989; Rittirsch et al. 2008). The inhibition of the complement system reduces the delayed vasospasm (German et al. 1996).

Inflammation processes after aSAH are extremely complex. Current scientific evidence refers to the substantial role of the inflammation in the pathophysiology of aSAH. These cascades may provide important drug targets against aSAH.

2.2.2.3. Microthrombosis

Pathological cascades that occur during early brain injury promote the development of microthrombosis in the blood vessels of the brain. After initial bleeding, platelet activation occurs in seconds and platelet aggregates are found in the arterioles after minutes in aSAH models and in humans after days in autopsy studies (Sehba et al. 2005; Stein et al. 2006; Ishikawa et al. 2009). Platelet aggregates may produce the state called “no-reflow phenomenon” in which blood flow in arterioles is permanently compromised (Abumiya et al. 2000; Sehba et al. 2011). Activated platelets release serotonin, adenosine diphosphate (ADP) and platelet-derived growth factor (PDGF), and subsequent decrease in NO levels causes further constriction of the cerebral vessels (Friedrich et al. 2010b; Sabri et al. 2012; Shiba et al. 2013). Activated complement protein C5a activates tissue factor and decreases plasmin levels *via* activating plasminogen inhibitors -1 and -2, thus contributing to the clot formation (Rittirsch et al. 2008). In addition, ischemia causes a decrease in pH that further aggravates coagulation. Platelets have also a pathological role in causing inflammation response and increasing collagenase activity disrupting the matrix, especially collagen IV (Sehba et al. 2004; Friedrich et al. 2010a). Lastly, the number of microthrombosis has been shown to correlate in neuronal cell death and severe DCI (Suzuki et al. 1990; Sabri et al. 2012; Andereggen et al. 2014).

In general, microthrombosis seems to be an inevitable consequence of aSAH. Complex pathophysiological cascades that start from initial bleeding generate the conditions in which clot formation occurs. Complexity of the pathophysiology of microthrombosis favors multimod-

al drug targeting, in which the clotting itself, but especially inflammation, may serve as a target.

2.2.2.4. Cortical spreading ischemia and delayed cerebral ischemia

Cortical spreading ischemia (CSI) initiates from cortical spreading depolarization (CSDz) when brain homeostasis is severely disturbed by an injury. CSDz wave arises from a population of cortical neurons that depolarize together at the near-complete level (Dreier 2011; Pietrobon and Moskowitz 2014). The wave spreads across the cortex with velocity of 2–5 mm/min (Ochs and Hunt 1960). CSDz induce reactive hyperemia in conditions where ionic and energy balance is sufficient (Lauritzen 1994). The increase in the extracellular potassium levels above critical value seems to act as a key factor for the initiation of the CSDz wave (Reid et al. 1988; Pietrobon and Moskowitz 2014).

When brain is progressively damaged, CSDz leads to an inverse hemodynamic response *via* metabolic and vascular reactivity impairment that leads to neurovascular uncoupling (Wahl et al. 1987; Seitz et al. 2004; Piilgaard and Lauritzen 2009; Lauritzen et al. 2011). Consequently, vasoconstriction and hypoperfusion are measured, although neurometabolic coupling can remain unaffected (Piilgaard and Lauritzen 2009). The worst endpoint is anoxic depolarization (also known as terminal spreading depression) when metabolic disturbance is severe (Dreier 2011). In this condition, neurons do not repolarize and neuronal death is an inevitable consequence (Saito et al. 1997; Farkas et al. 2010; Dreier 2011).

Cortical spreading depolarization is not measured only after aSAH but also seen in traumatic brain injury and ischemic stroke (Nakamura et al. 2010; Lauritzen et al. 2011; Hinzman et al. 2014). In migraine, a cortical spreading depression (CSDs) wave is similarly propagated on the cortex at 2–5 mm/min in the difference that metabolic disturbance is minimal if present at all (Pietrobon and Moskowitz 2014; Cui et al. 2014). CSDs leads to the suppression of the spontaneous brain activity that leads shortly to reactive hyperemia (Leao 1947; Cui et al. 2003).

In conclusion, early brain injury, secondary injuries, vasospasm and microthombosis together induce the environment in which the cortical spreading depolarization waves are formed. These waves together with previously discussed pathophysiological cascades generate a vicious cycle of cortical spreading ischemia, and that forms the delayed cerebral ischemia (DCI). Clinically, DCI is observed as a delayed neurological deterioration (see section 2.2.3.4).

2.2.3. Management and treatment of ruptured aneurysms

Sudden onset of explosive headache is a major indicator of aSAH, which is in the most of the cases followed by altered consciousness and focal neurological deficit. Typical patient is female over 50 years old with the medical history of hypertension and smoking. The natural history of ruptured IAs makes an early treatment of these lesions mandatory. For a historical unselected series of 363 patients with ruptured IAs, Pakarinen reported 15% mortality before to hospital admission. Mortality was 32% at day one, 46% at week one, 56% at month one and 60% at month six (Pakarinen 1967). Rebleeding causes a significant increase in mortality, as described in the next section. Thus, the treatment goal is to prevent rebleeding by occluding the ruptured aneurysm. In general, the treatment must focus on the following four major aspects: rebleeding, perfect aneurysm closure, delayed cerebral ischemia, and other complications.

2.2.3.1. Rebleeding

After a sudden explosive headache and neurological deterioration, the patient is transported to a hospital for immediate computed tomography (CT) or CT-angiography (CTA). If aSAH is detected, tranexamic acid is immediately infused to prevent rebleeding, and the process is repeated every six hours until the aneurysm is closed or three days have elapsed since the primary bleeding (Nibbelink et al. 1975; Hillman et al. 2002; Larsen and Astrup 2013). Prospective, randomized and open-label study of ultra-early tranexamic acid after subarachnoid hemorrhage (ULTRA) is currently ongoing (Germans et al. 2013). The study is of importance, since 50%–90% of rebleeds occur in the first six hours (Larsen and Astrup 2013).

Susceptibility for rebleeding is highest during the first 24 hours, being from three to seven percent (Inagawa et al. 1987; Starke et al. 2011; Larsen and Astrup 2013). The cumulative frequency of rebleeding is as follows: 7%, 16%, 23%, and 33% at week one, week two, week three and week four, respectively (Pakarinen 1967). Recently, cumulative risk of rebleeding is reported to be as high as 23% (8–23%) (Larsen and Astrup 2013). Rebleeding is a severe complication; up to 60% of the patients who have suffered from rebleeding will die (Juvela 1989; Larsen and Astrup 2013). In Pakarinen's series, the mortality at first recurrence was 64%, and it is 86% after a second recurrence (Pakarinen 1967). Lastly, hypertension is the only another manageable risk factor for rebleeding, thus it is of great interest to control blood pressure (Tang et al. 2014).

2.2.3.2. Surgical clipping and endovascular coiling

After the patient's ruptured aneurysm is confirmed with either CTA or cerebral angiography, rapid treatment by occluding the ruptured IA is necessary to prevent rebleeding. The two occlusion therapies currently available are surgical clipping and endovascular coiling (see section 1). Early intervention by either means is effective in preventing rebleeding, thus decreasing the mortality rate and also improving the patient's quality of life and outcome (Fogelholm et al. 1993; Starke et al. 2011; Larsen and Astrup 2013; Zhou and Song 2014).

In the light of current research data, neither treatment strategy is superior to another. The choice depends on the location, size and morphology of the aneurysm as well as the institute, patient's age and the experience of the neurosurgeon (Brown and Broderick 2014; Santiago-Dieppa et al. 2014). Currently, these two techniques supplement each other, and the vascular neurosurgeon is transforming into a hybrid mastering the both techniques (Peschillo and Delfini 2012; Sorkin et al. 2014). According to a study conducted before the endovascular treatment modality was available, early surgery with NDP reduces both the rebleeding rate and the emergence of DCI (Ohman and Heiskanen 1989).

Further, the patient is transferred to an operating room for microsurgical clipping or to an angiography room for coiling. The result of coiling and clipping is confirmed by CTA or cerebral angiography. Imperfection in ligation or coiling may lead in severe complications (Brisman et al. 2005; Bulters et al. 2011; Starke et al. 2013; Cho et al. 2014). After the closing of the aneurysm, the patient is relocated to a neurocritical care unit for monitoring.

2.2.3.3. Delayed cerebral ischemia

Although the rebleeding rate decreases after a surgical or endovascular procedure, there is another severe complication threatening the patient's life. Angiographic vasospasm is monitored for 50% to 90% of patients after aSAH, and it is defined as arterial narrowing, which may lead to local or even global ischemia (Kassell et al. 1985; Dorsch and King 1994). A total of 35% of the patients develop symptoms such as fluctuation in body temperature, hypertensive periods and neurological deterioration (Dorsch and King 1994; Dorsch 2011). These symptoms vary from mild to severe but 15% lead to death (Haley et al. 1992; Dorsch 2011). Susceptibility to developing a symptomatic vasospasm is highest after one week from the initial bleeding (Heros et al. 1983). Clinical biomarkers for predicting the incidence of vasospasm are presented in the table 1 (Conway and Tamargo 2001; Frontera et al. 2006; Yin et al. 2011).

Table 1. *Risk factors for vasospasm after aSAH. Modified Fisher grades are described as follows: 0 = no aSAH, no IVH, 1 = thin aSAH, no intraventricular hemorrhage (IVH), 2 = thin aSAH, IVH, 3 = thick aSAH, no IVH, 4 = Thick aSAH, IVH (Conway and Tamargo 2001; Frontera et al. 2006; Yin et al. 2011).*

modified Fisher grade 0	No risk
modified Fisher grade 1	Low risk (24%)
modified Fisher grade 2	Moderate risk (33%)
modified Fisher grade 3	Moderate risk (33%)
modified Fisher grade 4	High risk (40%)
History of hypertension	
Hunt-Hess grade on admission	
Fever	
Aneurysm location	
Intraventricular blood	
Smoking	
Cocaine use	

For the prevention of DCI, the patient is usually treated with NDP. NDP is an L-type calcium channel blocker, which has been shown to improve outcome after aSAH. (Laursen et al. 1988; Pickard et al. 1989). Mechanisms of its actions are mainly obscure, but pleiotropic effects such as inhibition of neuronal apoptosis, inhibition of microthrombi formation and inhibition of CSI are involved (Lazarewicz et al. 1990; Dreier et al. 2002; Vergouwen et al. 2008; Pluta et al. 2009). Pure vasodilatation theory is mainly abandoned since the correlation between the angiographic vasospasm and outcome is in conflict (Woitzik et al. 2012; Etminan et al. 2013; Shen et al. 2013).

In severe symptomatic cases, triple-H (hypertension, hemodilution, hypervolemia) therapy is used but the evidence of its benefits is controversial (Lee et al. 2006; Muench et al. 2007; Treggiari and Deem 2009; Dabus and Nogueira 2013). The last option is a mechanical or pharmacological angioplasty that in some cases saves the patient's life (Sayama et al. 2006; Dabus and Nogueira 2013).

2.2.3.4. Other complications after aSAH

Several additional complications can occur after aSAH. Markedly, 25% of patients develop acute hydrocephalus (Milhorat 1987; Mehta et al. 1996). In addition, expanding intracerebral hematomas and infarcts are seen in patients after aSAH. Other common complications are hyponatremia and seizures (Hart et al. 1981; O'Connor et al. 2014). Rare complications are cardiac arrhythmias and dysfunction, neurogenic pulmonary edema, hepatic dysfunction, and renal dysfunction (Chen et al. 2014; Hannon et al. 2014; Kumar et al. 2014; Hubner et al. 2014).

2.2.3.5. Outcome

Subarachnoid hemorrhage is a fatal disease with about 45% mortality rate despite the decrease in the fatality rates in the last decades (Fogelholm et al. 1993; Hop et al. 1997; Pobereskin 2001; Stegmayr et al. 2004; Koffijberg et al. 2008). Interestingly, the one explanation of observed decline in the mortality of aSAH was dependent on gender with the respect of decreased incidence for men and decreased case-fatality for women (Stegmayr et al. 2004).

Factors that predict poor outcome are age, poor neurological condition on admission (Hunt & Hess), amount of blood on admission included intracerebral or intraventricular hemorrhage on CT (Fisher grade), and aneurysm location (Rosen and Macdonald 2005; Lindvall et al. 2009). Hunt & Hess grading has the greatest impact on outcome (table 2). Grades 1 and 2 favor good outcome (Kassell et al. 1990; Osawa et al. 2001). The turning point occurs when even a mild focal neurological deficit is present on admission that dramatically increases the risk of poor outcome (Hunt and Hess 1968; Säveland et al. 1992). Second important factors are age and the rupture of posterior circulation aneurysm that are related to unfavorable outcome (Rosengart et al. 2007).

Table 2. *Hunt & Hess grading (I–V) and mortality after aneurysmal subarachnoid hemorrhage (Hunt and Hess 1968).*

Category	Criteria	Deaths %
Grade I	Asymptomatic, or minimal headache and slight nuchal rigidity.	11
Grade II	Moderate to severe headache, nuchal rigidity, no neurological deficit other than cranial nerve palsy.	26
Grade III	Drowsiness, confusion, or mild focal deficit.	37
Grade IV	Stupor, moderate to severe hemiparesis, possibly early decerebrate rigidity and vegetative disturbances.	71
Grade V	Deep coma, decerebrate rigidity, moribund appearance.	100

Of those who survive from aSAH, only 25% have completely favorable outcome, but still they carry the increased risk to die earlier than their healthy compeers (Olafsson et al. 1997; Ronkainen et al. 2001; Wermer et al. 2007; Wermer et al. 2009; Huttunen et al. 2011; Nieuwkamp et al. 2014).

Outcome is measured usually using the modified Rankin scale (mRS) or Glasgow outcome scale (GOS) (Rankin 1957; Jennett and Bond 1975). Currently in most of the studies seen in the literature, outcome is recorded using three or six months GOS or mRS (Lees et al. 2012). These categorized, relatively insensitive scales combined with selection bias make comparison of the different patient series difficult. In addition, variation in inter-rater reliability is a significant factor (Wilson et al. 2005; Quinn et al. 2009). However, the scales are simple, universal, easy and cost-effective to use (Banks and Marotta 2007). It is evident, seen in both literature and clinical work, that recovery occurs far beyond six

month follow-up (Samra et al. 2007; Macdonald 2013). One of the most important questions in any stroke study, but especially in neurorestorative treatment studies, is how long the follow-up period should be (Macdonald 2013).

2.2.3.6. Rehabilitation

Neuropsychological disorders are common after aSAH (Haug et al. 2007). They significantly cause morbidity for the patients that is seen *e.g.* as increased number of divorces and unemployment (Wermer et al. 2007). Motor deficits tend to recover in the first six months, but verbal memory takes a longer time (Haug et al. 2007). Especially, anterior circulation aSAH may cause a decrease in executive functions (Fontanella et al. 2003; Sheldon et al. 2012). Over 90% of patients with Hunt & Hess grade I and II on admission show a favorable (4 and 5) outcome classified on Glasgow Outcome Scale. However, 70% of patients show neuropsychological deficits (Bjeljac et al. 2002). Thus, it is of great importance to recognize these patients and provide the proper treatment.

Unfortunately, the pathophysiologies of neuropsychological disorders after aSAH are poorly understood. It is known that DCI increases the risk of neurological deficits over 6-folds (Stienen et al. 2014). Molecular alterations and compromised neuronal plasticity in the hippocampus are occurring after aSAH and may lead to the memory deficit and impaired executive functions (Han et al. 2014). However, the long-term consequences of aSAH to the brain plasticity remain to be studied (Han et al. 2014).

There are only few studies for drug treatments. However, rivastigmine has shown promising preliminary results (Wong et al. 2009). For now, neuropsychologists provide rehabilitation using different training batteries depending on which brain function is impaired, and drugs for symptoms can be prescribed by a rehabilitation neurologist or psychiatrist.

A substantial number of patients with aSAH are suffering from a motor deficit. Physiotherapy is standard of care but must be intensive and repetitive in all phases of poststroke rehabilitation (Veerbeek et al. 2014). However, new rehabilitation modalities are emerging. Robotics, stem cell therapies and brain-computer interfaces are future options for stroke patients (Chang and Kim 2013; Boninger et al. 2014).

Drug treatments for improving the rehabilitation results have also been tested and the results have been encouraging (Chollet et al. 2011). Some of these new treatment modalities are currently available (Mirbagheri et al. 2011; Sale et al. 2014). In clinical use, exercise robots are available for the patients. For example, with a walking robot, a paralyzed patient is able to take one-hour walking trips in the virtual park or train sensory-motor skills of the paralyzed hand with an assisting robot. This kind of training not only induces plastic changes in the brain but also increases self-esteem and relieves spasticity, limb edema and depression (Gómez-Pinilla et al. 2002; Kim et al. 2005; Griesbach et al. 2009; Mirbagheri et al. 2011; Zabukovec et al. 2013; Trompetto et al. 2013; Bechara et al. 2013; Yang et al. 2014; Calabrò et al. 2014; Chisholm et al. 2014). Additionally, transcranial magnetic stimulation is currently under active research and has been shown to induce positive neuroplastic changes among stroke patients (Shah et al. 2013).

In conclusion, a substantial portion of aSAH patients has neuropsychological, psychiatric and motor disorders. Active recognition, treatment and rehabilitation are required for the best results. Treatment is multi-professional, where doctors, psychologists, occupational and physiotherapists and family are intensively present. In the future, neurorestorative therapies as well as robotics play a significant role for improving patients' outcome measures and quality of life.

2.3. Intracranial treatments for vasospasm

2.3.1. Concept

The merciless nature of the complications caused by DCI has resulted in the search of new routes to administrate NDP for aSAH patients. Intra-arterial, intrathecal, intraventricular and intracranial routes have been described (Kawashima et al.

2000; Hänggi et al. 2008b; Hänggi et al. 2008a; Barth et al. 2011; Zhang et al. 2013). Dr. Kawashima and colleagues, using a dog model, first described intracranial administration of nicardipine in 1998 and the first patient received intracranial nicardipine implant therapy in October 1999 (Kawashima et al. 1998; Kasuya et al. 2005). The idea of this concept is to obtain higher concentrations of NDP in the CSF and lower concentrations in the peripheral blood (Macdonald et al. 2012), thus reducing the risk of DCI and the incidence of NDP-induced hypotension effectively. About 15% of aSAH patients suffer from NDP-induced hypotension (Dorhout Mees et al. 2007). By achieving higher concentration of NDP in the CSF it may be possible to increase the pleiotropic effects of NDP in central nervous system.

2.3.2. Sustained release implants for intracranial treatment

For the intracranial drug treatment of aSAH, only poly-D,L-lactide coglycolide (PLGA) microparticles and ethylene-vinyl acetate copolymers (EVAc) were described in the literature when our research with silica-based implants was published (table 3). Nicardipine and PLGA microparticles are the most studied combination. In one experimental study on rats, PLGA and NDP were mixed *in situ* with Tisseel® -fibrin sealant, which is often used in neurosurgery as a hemostat (Hänggi et al. 2012). EVAc formulation was used in only one study as well, in which the approach to the inhibition of the vasospasm was completely different compared to the traditional calcium channel blockers (Pradilla et al. 2005). Since ibuprofen has been shown to reduce inflammation response after aSAH by inhibiting ICAM-1, the local intracranial treatment was tested (Kapiotis et al. 1996). According to the study results, ibuprofen significantly reduced vasospasm (Pradilla et al. 2005).

In conclusion of the data shown in the table 3, the intracranial treatment of vasospasm and delayed neurological deficits seem to significantly reduce vasospasm and even improve outcome without adverse events or histological toxicity (Kawashima et al. 1998; Shiokawa et al. 1998; Kawashima et al. 2000; Sasahara et al. 2000; Kasuya et al. 2002; Kasuya et al. 2005; Pradilla et al. 2005; Barth et al. 2007; Krischek et al. 2007; Barth et al. 2009; Schneider et al. 2011; Barth et al. 2011; Hänggi et al. 2012; Cook et al. 2012). However, larger trials are needed to verify these results and compare them with the conventional treatment. Lastly, novel tentative therapies are currently being actively researched for the treatment of aSAH in addition to intracranial therapies. For example, small interfering RNA molecules have successfully been used to inhibit early brain injury after aSAH in rats by silencing the CHOP gene that regulates importantly endoplasmic reticulum stress pathways (He et al. 2012).

2.3.3. Biomaterials for intracranial treatment

PLGA microparticles for intracranial therapy have been widely studied and safety has been validated in human use (Krischek et al. 2007). PLGA forms microspheres in which active drug is bound (Shive and Anderson 1997). Size of the particles is a few micrometers (Bege et al. 2013). In addition, nanoscale particles are developed (Mehta et al. 2007). Degradation of PLGA occurs through hydrolysis. Since PLGA is constituted of monomers of lactide and glycolide, only modest foreign body reactions are observed (Menei et al. 1993). In formulation, lactide proportion related to glycolide determines the properties of the PLGA matrix such as degradation time and viscosity (Paakinaho et al. 2011).

Silica-based biodegradable implant or gel can be formed using the sol-gel method. In this method, alkoxysilanes such as tetraethoxsysilane (TEOS) are hydrolyzed using water and thereby forming a polymer in which the active compound is bound (Kortesuo et al. 2001; Quintanar-Guerrero et al. 2009). Silica-based sustained drug delivery system (SDDS) dissolves in the human body in the presence of water.

Calcium channel blockers formulated in the silicate matrix for testing intracranial dissolving properties have not been described in the literature. Currently silica is under active research due to its extensive scale of applications for drug delivery. Silica-based SDDS are competent for pure sustained release systems but can be functional-

Table 3. *Published intracranial implant or gel treatment studies for DCI in the literature.*

Study	Model (no.)	Implant	Follow-up	Results
Kawashima et al 1998	Dog (12), direct clot placement *via* craniotomy	PLGA + nicardipine	7d	Reduction in vasospasm, no adverse effects, no signs of meningoencephalitis
Shiokawa et al 1998	Dog (32), direct clot placement *via* craniotomy	PLGA + papaverine	7d	Reduction in vasospasm, only high dose implants
Kawashima et al 2000	Dog (18), direct clot placement *via* craniotomy	PLGA + nicardipine	14d	Reduction in vasospasm even at a low dose
Sasahara et al 2000	Dog (10), double hemorrhage model	PLGA + nicardipine	7d	Reduction in vasospasm, no histological toxicity
Kasuya et al 2002	Human (20)	PLGA + nicardipine	7-12d	Reduction in vasospasm, no short term adverse effects
Kasuya et al 2005	Human (97), mainly Fisher 3	PLGA + nicardipine	7 and 12d	Reduction in delayed neurological deficits, no adverse effects
Pradilla et al 2005	Monkey (5+14)	EVAc + ibuprofen	3m + 7d	Reduction in vasospasm, no histological toxicity
Krischek et al 2007	Human (100), mainly Fisher 3 (same patient cohort as Kasuya et al 2005)	PLGA + nicardipine	3m GOS	Outcome improvement?
Barth et al 2007	Human (32), mainly Fisher 3, prospective, randomized, double-blind	PLGA + nicardipine	15m mRS	Outcome improvement (control group did not receive any calcium antagonist treatment)
Barth et al 2009	Human (18), the same cohort as Barth et al 2007	PLGA + nicardipine	10-20m	No improvement in quality of life
Schneider et al 2011	Human (81), case-control study	PLGA + nicardipine	1y mRS	Superior to endovascular coiling
Barth et al 2011	Human (17 clipped + 14 coiled, intraventricular administration)	PLGA + nicardipine	1w	Reduction in vasospasm only in clipped patients, no increase in shunting
Cook et al 2012	Dog & Monkey (6+13), double hemorrhage in dogs and direct clot placement in monkeys	PLGA + NDP (dog) or nicardipine (monkey)	7 and 14d	Reduced angiographic vasospasm, no reduction in microthrombi, sustained release profile
Hänggi et al 2012	Rat, double hemorrhage model	PLGA + NDP + Tisseel® (gel)	5d	Reduction in vasospasm, no adverse effects or toxicity

mRs = modified Rankin Scale, GOS = Glasgow Outcome Scale, y = year, m = months, w = week, d = day, no. = number of subjects

ized to respond to changes in pH, redox potential, temperature, biomolecules, or even magnetism and luminescence (Simovic et al. 2011; Yang et al. 2012). Silica-based SDDS can be formulated into a solid implant as well as gel with different viscosity (Quintanar-Guerrero et al. 2009).

2.4. Neuroplasticity

2.4.1. Evolution of the brain

Evolution depends on alteration in gene structures, mutations, that lead to a new phenotype (Gilbert et al. 2005; Olson-Manning et al. 2012; Paaby and Rockman 2014). Evolution of the human brain can be tracked as far as 400 million years past (Kaas 2005). A significant epoch occurred about 200 million years ago when early mammals evolved neocortex (Northcutt and Kaas 1995; Kaas 2005; Kaas 2013). Current evidence suggests that the neocortex of mammals has developed from the primitive thin dorsal cortex of reptiles (Aboitiz et al. 2003; Kaas 2005; Kaas 2013). The main theories for neocortical development are the protomap and protocortex theories (Aboitiz et al. 2003; Puelles 2011). The protomap theory is composed of the radial and concentration-dependent component, whereas the proto-

cortex theory is uprising from the importance of the arriving thalamocortical ascending fibers during the development (Aboitiz et al. 2003; Mallamaci and Stoykova 2006; Puelles 2011).

The neocortex has six distinct layers and covers 80–90% of the brain surface area (Fatterpekar et al. 2002; Alfano and Studer 2013; Arai and Pierani 2014). During the development of the neocortex, different areas are formed with different functions and cellular architecture. The process is called arealization and, areas are usually referred to as the Brodmann areas according to the cytoarchitecture (Zilles and Amunts 2010). Arealization is regulated by genes but also by environment (Arai and Pierani 2014). Most interestingly, in the perspective of modulating neuronal plasticity in adults, the relative size of cortical areas can vary during an individual's lifetime (Larsen and Krubitzer 2008).

Comparing the neocortical areas between different mammals refers to highly conserve genetic mechanisms underlying the neocortical arealization (Bayatti et al. 2008; Alfano and Studer 2013; Arai and Pierani 2014). Interestingly, the impressive ability of the neocortex to rewire and reorganize the cortical areas and structures infers to more complex mechanisms (Alfano and Studer 2013).

The other cortical areas are the allocortex and mesocortex (Reep 1984). The allocortex is divided into three subgroups, called the periallocortex, the paleocortex, and the archicortex (Reep 1984; Smart 1984; Braak and Braak 1985; Granger et al. 1995). These cortexes have three to five distinct neuronal layers and are represented in the regions such as entorhinal cortex, hippocampus, parahippocampus, olfactory system and parts of the insula (Cordero et al. 1982; Granger et al. 1995; Olafsson et al. 1997; Vyas et al. 2003; Nieuwenhuys 2012).

2.4.2. Neuronal plasticity

Hebbian plasticity is the most fundamental theory of brain plasticity. The theory is reduced to the widely known phrase *"neurons that fire together, wire together"* and *vice versa "neurons that fire apart, wire apart"* (Hebb DO 1949). Of course, this phrase should not be interpreted literally.

Neuronal plasticity is traditionally divided in synaptic and nonsynaptic plasticity (Cotman and Nieto-Sampedro 1984; Bliss and Collingridge 1993; Kato et al. 2009). Synaptic plasticity refers directly to a synaptic connection and firing rate, whereas nonsynaptic plasticity refers to modifications in the perikaryon and dendrites as well as axons that alter neuronal synaptic behavior (Abraham and Williams 2003; Kato et al. 2009; Mozzachiodi and Byrne 2010). Interestingly, and often depreciated, glial cells are important in regulating neuronal functions but also in rewiring and synaptic plasticity (Vernadakis 1996; Perea and Araque 2010; Ota et al. 2013; Benarroch 2013; Morris et al. 2013; Pirttimaki and Parri 2013; Bernardinelli et al. 2014).

Overall, neuronal networks are constantly under active remodeling (Katz and Shatz 1996). During development, immature neurons are overproducing neuronal structures that increase adaptation even if the total number of structures is not increased (Castrén and Hen 2013). This is due to a simultaneous structural elimination (Changeux and Danchin 1976). In adult neurons, the optimized function of neural network is strengthened by learning, and can be further remodeled by plasticity (Katz and Shatz 1996; Castrén and Hen 2013). Plastic changes of neurons occur by several different mechanisms and at different levels. Neurogenesis, axonal and dendritic sprouting, synaptic strengthening and genomic plasticity are the main mechanisms that promote plastic changes in the neuronal networks (Alsina et al. 2001; Lisman 2003; Hua and Smith 2004; Borrelli et al. 2008; Ming and Song 2011; West and Greenberg 2011). Notably, functional changes in the neuronal networks always require structural changes at some level. Consequently, allocation structural and functional plasticity in different categories is factitious (Castrén and Hen 2013).

2.4.2.1. Role of TrkB and BDNF in neuronal plasticity

Neurotrophin receptors consist of four different receptors; TrkA, TrkB, TrkC and p75NTR (Lewin and Barde 1996). Trk receptors (Tropomyosin related kinase) belong to a tyrosine kinase receptor family whereas p75NTR is interestingly a

member of tumor necrosis factor (TNF) receptor super family (Lewin and Barde 1996; Park and Poo 2013). Ligands of the receptors, the neurotrophins, are brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). BDNF and also NT-4/5 are the main ligands for TrkB (Barbacid 1994; Lewin and Barde 1996).

Activation of TrkB by its primary ligand BDNF regulates multiple forms of neuronal plasticity. Main signaling cascades and neuronal functions after TrkB activation is presented in the figure 1. After ligand binding, specific intracellular tyrosine residues within TrkB are phosphorylated, which leads to the activation of intracellular signaling pathways implicated in neuronal survival (e.g. Akt, protein kinase B), neuronal differentiation (e.g. ERK, extracellular regulated kinase), synaptic plasticity (e.g. CREB, cAMP related element binding protein) and syn-

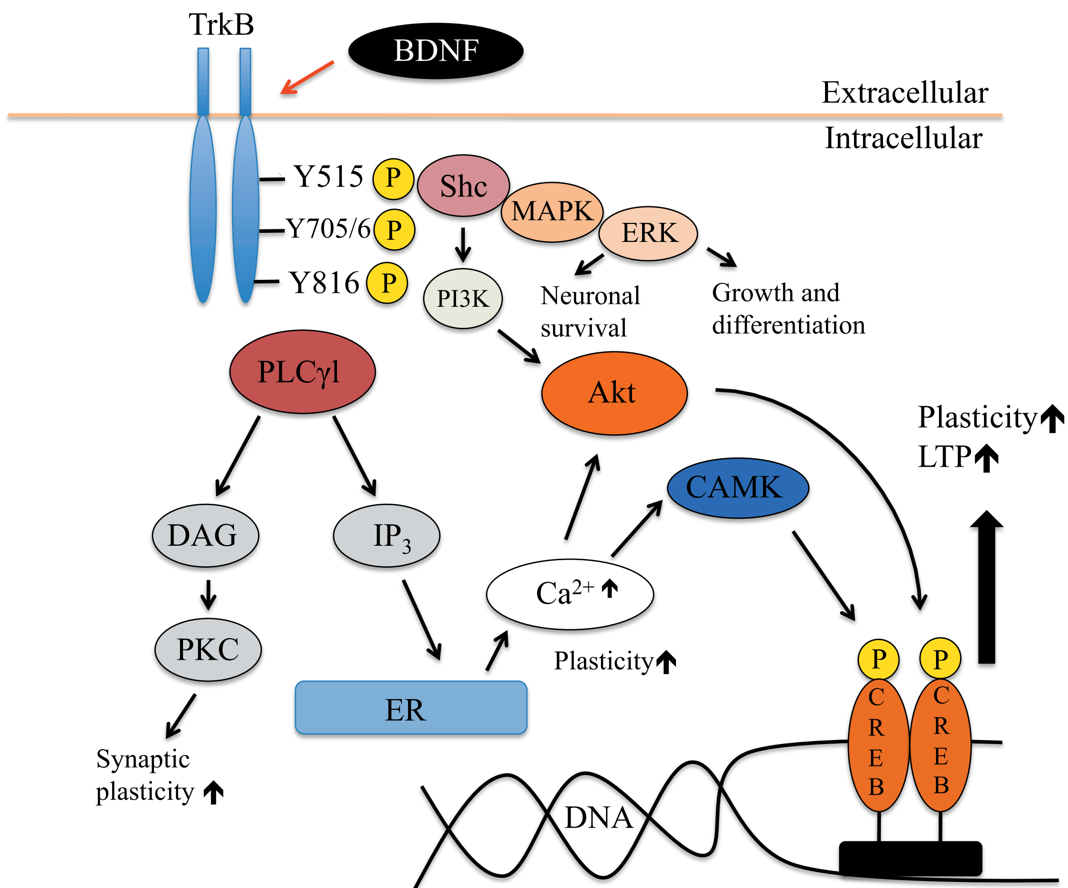

FIGURE 1. The signaling cascades in the neurons after TrkB activation by its primary ligand BDNF. *TrkB$^{Y705/6}$, TrkB^{Y816} and Shc binding domain TrkB^{Y515} sites are phosphorylated after BDNF binding further activating phospholipase Cγ1 (PLCγ1) pathway, mitogen-activated protein kinase (MAPK) pathway and phosphoinositide 3-kinase (PI3K) pathway. PLCγ1 induces degradation of phospholipid phosphaditylinositol 4,5 bisphosphate (PIP_2, not shown) to diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) pathway that is further capable of increasing synaptic plasticity. IP_3 binds to receptors on the surface of endoplasmic reticulum (ER) releasing calcium (Ca^{2+}) to the intracellular space. Increased Ca^{2+} concentration activates different forms of CAMKs and Akt. MAPK further activates extracellular regulated kinase (ERK), consequently leading to the signaling of neuronal survival, differentiation and growth. In addition, phosphorylation of the Shc binding domain TrkB^{Y515} enables the activation of PI3K that is capable of activating Akt signaling. These changes further lead to the phosphorylation of cAMP response element-binding protein (CREB), which critically regulates transcription of the genes that are implicated in plasticity, LTP formation and neuroprotection. BDNF = Brain derived neurotrophic factor, Bcl-2 = B-cell CLL/lymphoma 2. (Modified from Minichiello 2009)*

apse formation (e.g. mTor, mammalian target of rapamycin; p70S6k) (Huang and Reichardt 2001; Minichiello 2009; Gómez-Palacio-Schjetnan and Escobar 2013; Park and Poo 2013).

The actions of neurotrophins are not always trophic. Pro-isoforms of neurotrophins, pro-NTs, have an important role for activating Trk or p75NTR receptors. One of the most intriguing aspects is that pro-NTs can activate p75NTR receptor that may lead to apoptosis of neurons (Lee et al. 2001; Teng et al. 2005; Dicou 2007). Moreover, pro-NTs have higher biochemical affinity to bind p75NTR receptors than mature NTs (Lee et al. 2001). The cleavage of the pro-NTs to NTs is regulated most importantly by tissue plasminogen activator and matrix metalloproteinases (Lee et al. 2001). Thus, intracellular as well as extracellular proteinase activity regulates the function of NTs, neuronal spine formation and sprouting, and eventually can determine the fate of neurons. Notably, when these structural brakes of matrix are off, brain plasticity is enhanced in adults (Bavelier et al. 2010).

Protease activity regulates also functional outcome of the neurons. Cleavage of pro-BDNF to mature BDNF is required for long-term potentiation (LTP) whereas pro-BDNF induces long-term depression (LTD) in the synapse (Pang et al. 2004; Woo et al. 2005). However, the eventual biological and pathological consequences of LTP and LTD on plasticity are extremely complex (Castrén and Hen 2013; Hulme et al. 2013; Creed and Lüscher 2013; Toyoizumi et al. 2013; Hensch 2014).

2.4.2.2. L-type calcium channels

L-type calcium channels are allocated in four subgroups (CaV1.1-CaV1.4.) (Calin-Jageman and Lee 2008). The major isoforms CaV1.2 and CaV1.3 are expressed throughout the brain both presynaptically and postsynaptically (Hell et al. 1993; Lipscombe et al. 2004). Furthermore, widely expressed in neuronal tissue CaV1.2 constitutes of four different subgroups (Hofmann et al. 2014). Activity-dependent calcium entry to the intracellular space through these complex channels regulates numerous important intracellular signaling pathways that modulate short- and long-term alterations in gene expression, synaptic plasticity and homeostasis in neurons (Bhat et al. 2012; Bading 2013; Park and Poo 2013; Frank 2014). Interestingly, calcium channels can regulate their own function *via* calcium by facilitating calcium dependent inactivation or activation (Hofmann et al. 2014). However, excess activation of L-type calcium channels may lead to compromised plasticity, excitotoxicity or even neurodegeneration (Choi 1994; Mattson 2007).

2.4.2.3. Role of calcium in neuronal plasticity

Intracellular calcium signaling in the neurons has important effects in neuronal functions. Nonetheless, excess amount of intracellular calcium is toxic and may facilitate neuronal death (Choi 1994; Mattson 2007). Thus, intracellular calcium concentration is strictly controlled.

Calcium influx through NMDA and AMPA receptors with consequent phosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII) provides the basic mechanism for LTP formation (Bloodgood and Sabatini 2007; Asrar et al. 2009; Lisman et al. 2012; Nicoll and Roche 2013). CaMKII further activates Rho GTPases which have an abundant role in plasticity (Murakoshi et al. 2011). Interestingly, CaMKII interacts also with L-type calcium channels and activation of the channels can phosphorylate CaMKII specifically in dendritic spines (Hudmon et al. 2005; Lee et al. 2009b). Indeed, activation of L-type calcium channels is an important modulator in the formation of LTP (Magee and Johnston 1997; Remy and Spruston 2007; Lee et al. 2009b).

2.4.2.4. Critical periods

Critical periods are time windows during neuronal development that require different environmental and intrinsic inputs for proper maturation of a particular neuronal network, or otherwise the function is thought to be permanently compromised (Hensch and Bilimoria 2012). Typical critical periods are seen in the development of vision, hearing, language and also higher cognitive functions (Hensch and Bilimoria 2012). For example, the critical period for developing normal sense of hearing closes during the third year of life (Cardon et al. 2012). Thus, for children suffering from congenital deafness, cochlear

implantation is performed before the time period of maximal plasticity is lost (Cardon et al. 2012). However, since the critical period is not always "critical" and further neuronal network remodeling can occur after the critical period is closed, it is sometimes more appropriate to speak of sensitive periods (Maya Vetencourt et al. 2008). This is undoubtedly true especially when the human brain is discussed, due to the neuronal network complexity and higher cognitive functions.

In early development, neuronal connections are mainly excitatory. During maturation of the brain, an increase in the inhibitory transmission occurs, activating the critical period plasticity (Hensch and Bilimoria 2012). Much of the exact mechanisms for opening critical periods are not known, but inhibitory interneurons called parvalbumin-positive basket cells (PV cells) and methyl-CpG-binding protein 2 (MeCP2) expression in the PV cells seem to have important role (Hensch 2005; He et al. 2014; Hensch 2014). Interestingly, the GABAergic diazepam infusion can restore the critical period plasticity if the maturation of inhibitory circuits is genetically prevented (Fagiolini and Hensch 2000; He et al. 2014).

2.4.3. Induced plasticity

Induced plasticity may refer to any kind of increase in neuronal plasticity or learning ability, but usually it is referred to as reopening the critical or sensitive periods in mature brain induced by different stimuli. The term "iPlasticity" is launched referring to the latter (Castrén 2013).

Opening of the critical periods can be induced in animal models by exercise, environmental enrichment and nutritional deprivation as well as different drug treatments (Sale et al. 2007; Blumenthal et al. 2007; Maya Vetencourt et al. 2008; Morishita et al. 2010; Spolidoro et al. 2011). The connection, or rather communication, between plastic processes of LTP formation in learning and reopening the critical windows is absolutely intriguing. Underlying control mechanisms are referring to a PV cell controlled adjustment of excitation/inhibition ratio (Tao et al. 2014). However, open questions remain. Interestingly, nutritional deprivation reopens the critical period plasticity, probably due to the increase in cortisone level. However, cortisone inhibits LTP formation in the hippocampus (Pavlides et al. 1993; Spolidoro et al. 2011; Hensch and Bilimoria 2012; Fa et al. 2014).

2.4.3.1. Drug-induced plasticity

Fluoxetine, a traditional selective serotonin reuptake inhibitor (SSRI), was the first drug demonstrated to open a critical period. Fluoxetine restored the critical period of primary visual cortex by opening ocular dominance plasticity that had previously been thought to be impossible to reopen (Maya Vetencourt et al. 2008), leading to recovery of visual acuity of an amblyopic eye. The mechanism of fluoxetine-induced plasticity is not known, but alteration of balance between inhibition and excitation in favor of inhibition is suggested as well as increase in BDNF synthesis and spine density of interneurons (Huang et al. 1999; Maya Vetencourt et al. 2008; Castrén and Rantamäki 2010; Hensch and Bilimoria 2012; Guirado et al. 2014). In addition, fluoxetine has been shown to open the critical period in amygdala during fear extinction training (Karpova et al. 2011).

The acetylcholinesterase inhibitor physostigmine has also been studied in the context of amblyopia (Morishita et al. 2010). Physostigmine treatment in wild-type mice reopened the visual cortex plasticity and normal function of the amblyopic eye was recovered. Lynx1, endogenous inhibitor of nicotinic acetylcholine receptor, is a molecular cholinergic inhibitor that restricts plasticity in the mature visual cortex (Morishita et al. 2010), whereas physostigmine is able to override the inhibition. Interestingly, Lynx1 deficient mice spontaneously recover from amblyopia merely by reopening the closed eye (Morishita et al. 2010). In addition, clinically used acetylcholinesterase inhibitors galantamine and donepezil activate TrkB receptor and downstream signaling that may partially lie behind the opening of the critical period (Autio et al. 2011).

2.4.4. Induced plasticity for neurorehabilitation – Current progress

The most recent hard evidence of the benefits of drug-induced plasticity was published in 2011.

In a randomized placebo-controlled setting, fluoxetine was demonstrated to improve motor outcome after ischemic stroke in humans (Chollet et al. 2011). Furthermore, fluoxetine is under research for rehabilitation after intracerebral hematoma (Marquez-Romero et al. 2013). For curing residual amblyopia with donepezil, a clinical drug trial is ongoing in Massachusetts Children's Hospital (Trial number: NCT01584076, http://clinicaltrials.gov/ct2/show/NCT01584076).

It is shown that an increase in BDNF-TrkB signaling improves the clinical outcome in different brain pathologies (Nagahara and Tuszynski 2011). Pharmacologically diverse antidepressant drugs such as fluoxetine induce TrkB signaling and further increase in BDNF levels in the brain (Saarelainen et al., 2003; Rantamäki et al., 2007). These effects may underlie antidepressant-induced neuroplastic effects that are beneficial against numerous nervous system conditions such as neuropsychiatric and neurodegenerative disorders, *e. g.* Alzheimer, Parkinson, ALS and depression as well as stroke and spinal cord injury (Castrén and Rantamäki 2010; Chollet et al. 2011; Castrén et al. 2012; Castrén 2013; Castrén and Hen 2013; Nagahara and Tuszynski 2011).

Importantly, active rehabilitation should be part of the treatment strategy (Maya Vetencourt et al. 2008; Castrén and Hen 2013; Castrén 2013). Certain drugs may reopen the critical or sensitive periods but external, or internal, stimulus is required for the active neuronal network remodeling and rewiring. Shortly, the drug enables the neurorehabilitation, but does not perform it. However, additional studies are required for understanding the mechanisms and consequences of the induced plasticity thus securing the current progress in the field of neurorehabilitation.

3. AIMS OF THE STUDY

Intracranial implant study started as a clinician-initiated project for the fact that current treatment strategies against DCI are relatively ineffective. The implant study was performed in a successful collaboration with Orion Corporation and Delsitech Ltd. Discrepancy between angiographic vasospasm and outcome included with evidence of pleiotropic actions of NDP led to the intriguing idea that the calcium channel blocker NDP may induce plastic changes in the brain. Current evidence has shown that antidepressants induce juvenile-like plasticity by activating BDNF receptor TrkB. In addition, antidepressants such as fluoxetine inhibit L-type calcium channels in neurons. Not forgetting the fact that intracellular calcium signaling plays an important role in mediating neuronal plasticity, it was evident that the effects of NDP on TrkB signaling needed to be studied.

Consequently, the aims of the presented work were set as follows:

1. To develop a new sustained release biodegradable silica-based NDP implant.
2. To test the surgical feasibility of the NDP implants through pterional craniotomy in pig and dog models.
3. To evaluate histological effects of the NDP implants on brain parenchyma and meninges.
4. To follow and measure the degradation of the NDP implants into the subarachnoid space in dogs and pigs using computed tomography.
5. To measure pharmacokinetics of oral and intravenous NDP in healthy beagle dogs as comparative data for implant-treated dogs.
6. To measure pharmacokinetics in the plasma and CSF of the surgically placed NDP implants in the dog and pig models.
7. To evaluate effects of NDP treatment on neuroplastic and neuroprotective signaling in a healthy mouse model.

4. MATERIALS

4.1. Implants

4.1.1. Material

Implants were prepared from silica oxide (SiO_2). SiO_2 has the ability to form micropores where the active drug NDP was embedded to form the active implants. Inactive ingredient glucose was similarly embedded in the SiO_2 matrix to form the placebo implants. Image of the silica-based biodegradable NDP implant is presented in the publication I.

4.1.2. Drug concentration

The concentrations of NDP in the implants were 10 weight (wt) % (Implant A, implanted to pigs) and 15 wt % (Implant B, implanted to dogs) corresponding to the amount of SiO_2. Total absolute quantity of NDP in implants was 5 mg in implant A and 8.5 mg in implant B. Each placebo implant contained 25 mg of glucose.

4.2. Animal models

4.2.1. Dog and pig models

Implant studies were performed using a domestic landrace pig and a Beagle dog model. In total, nine animals were included in both groups. The Beagle dogs were bred by Harlan-Winkelmann GmbH Hundezucht, Germany. The pigs were purchased from a local farm following a strict health monitoring program.

The dog was selected because of the possibility of being the species for safety evaluation studies. In the field of aSAH and DCI research, pig models are rarely established. New landrace pig model was tested considering the ethical and economical aspects. All procedures were made according to European Community Guidelines for the use of experimental animals and approved by Finnish National Animal Experiment Board (licenses ESAVI/2246/2011 and ESAVI/2643/2012).

Pharmacokinetic study was performed using six beagle dogs (age four years, weight 12 ± 1.0 kg). The dogs were bred by Harlan-Winkelmann GmbH Hundezucht, Germany as well. All procedures were made according to European Community Guidelines for the use of experimental animals and approved by Finnish National Animal Experiment Board (license ESLH-2009-08809/Ym-23).

4.2.2. Mouse model

Neuroplastic effects of NDP were studied using totally 18 healthy male adult C57BL/6J mice (8 weeks of age, Harlan, The Netherlands). A mouse model was selected because our laboratory had extensive experience for research plasticity in a mouse model. Mouse is also relatively inexpensive and ethical issues are widely approved. All experiments were conducted according to the guidelines of the European Communities Council Directive (86/609/EEC) and were approved by the County Administrative board of Southern Finland. These guidelines comply with the guidelines established in the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, national Research council. Washington, DC: National Academy Press, 1996).

4.3. Drugs

4.3.1. Nimodipine

The formulations used in the pharmacokinetic study of dogs were Nimotop® (Bayer Schering Pharma) 30 mg tablets and Nimotop® (Bayer Schering Pharma) infusion 0.2 mg/ml solution. In the formulation of implants, NDP from Shandong Xinhua Pharmaceutical Co., Ltd., Batch: 1109161 was used. Lastly, for the mice we used NDP from Santa Cruz Biotechnology (SCB), CA, USA.

5. METHODS

5.1. Pharmacokinetics of nimodipine in healthy beagle dogs

5.1.1. Study treatment

NDP doses were determined as described in publication II. The selected doses were not expected to cause any toxic or overt cardiovascular effects. The treatment groups and sampling are presented in a table 4. Sampling intervals after the dosing were determined to be very frequent due to the rapid kinetic of NDP. The concentration of NDP in the CSF was measured after one hour of administration of two 30 mg NDP tablets, exactly the same single dose as used in humans.

5.1.2. Blood and CSF sampling

Blood and cerebrospinal fluid samples were collected for determining NDP concentrations. Blood samples were collected from the jugular vein and CSF samples were taken from cisterna magna through atlanto-occipital space. The exact sampling time points were recorded and are presented in table 4. Anesthesia for CSF sampling was inducted using dexmedetomidine 20 µg/kg *i.v.* and a slow propofol bolus 3 mg/kg *i.v.*

Dexmedetomidine is an alpha-2 agonist with sedative and possible neuroprotective properties, developed by Orion Pharma. Dexmedetomidine has been used in dogs since the 1970's and the drug received a centralized marketing authorization from European Commission in September 2011 for human use. In human patients, dexmedetomidine is used for mild to moderate sedation after different types of brain injury. Propofol is the most used sedative after different types of brain injuries. It is cost-effective and all side effects are known. Propofol does not increase intracranial pressure. After *i.v* bolus, the clinical effect is short because of the rapid distribution to peripheral tissues.

5.1.3. Sample handling

Blood samples of 3 ml were taken into pre-cooled K2-EDTA tubes and kept on ice. Samples were protected from light until centrifugation (1500 g, 10 min, at +4°C). 700 µl of the separated plasma was used as primary sample stored in an amber-colored polypropylene tube (Eppendorf® Safe-Lock® microcentrifuge tubes volume 1.5 ml amber) and the rest was stored as a spare sample.

Cerebrospinal fluid samples (~1500 µl) were let to drop freely from a sterile cerebrospinal fluid needle into a sterile amber-colored polypropylene collection tube (Eppendorf® Safe-Lock® tubes volume 2.0 ml). Cerebrospinal fluid samples were kept protected from light, until the separation of supernatant by centrifugation (1500 g, 10 min, at +4°C). The separated CSF was divided into two amber-colored polypropylene tubes. 700 µl was taken to NDP analysis and any excess CSF was stored as a spare sample.

Since NDP is very photosensitive, all samples were handled so that exposure to light was minimized. The samples were stored frozen at -80°C until shipping to the laboratory

Table 4. *Treatment and sampling for the kinetic study (Koskimäki et al II).*

	Treatment	Animal no	Dosing route	Dose mg/dog	Formulation	Blood sampling time post dose	Cerebrospinal fluid sampling time
1	Nimotop® infusion 0.2mg/ml	1–3	*i.v.*	0.72 (3.6ml)	Slow *i.v.* bolus (in 30 seconds, with covered syringes due to light sensitivity)	predose, 10, 20, 30, 45, 60 & 90 min, 2h, 3h, 5h, 7h & 24h	-
2	Nimotop® 30mg tablet	1–3	*p.o.*	30	1x 30 mg tablet	predose, 20, 40, 60 & 90 min, 2h, 3h, 5h, 7h & 24h	-
3	Nimotop® 30mg tablet	4–6	*p.o.*	60	2x 30 mg tablet	1h	1h
4	Nimotop® 30mg tablet	4–6	*p.o.*	60	2x 30 mg tablet	predose, 20, 40, 60 & 90 min, 2h, 3h, 5h, 7h & 24h	-

5.1.4. Nimodipine analysis

Liquid chromatography mass spectrometric (LC-MS) method was used for determination of NDP concentration in dog plasma and CSF samples (II). Sample preparation was performed by liquid-liquid extraction. The extracts were analyzed using reverse-phase chromatography followed by mass spectrometry. The lower limit of quantification (LLOQ) of the method was 0.01 ng/ml for both sample matrices.

5.1.5. Pharmacokinetic analysis

The pharmacokinetics of NDP was characterized by using non-compartmental methods for oral administration and two-compartmental model for *i.v.* administration. Pharmacokinetic analysis was performed using the WinNonlin 5.0.1 program (Pharsight Corporation, Mountain View, CA, USA). The apparent maximum concentration of NDP in plasma (C_{max}) and time to apparent maximum plasma concentration of NDP in plasma (T_{max}) were obtained directly from the observed data. The areas under the NDP plasma concentration-time curves (AUC) were estimated by using the trapezoidal method. The parameters estimated in the two-compartmental analysis included systemic clearance (Cl), steady state volume of distribution (Vss), distributional half-life ($t_{1/2\alpha}$) and elimination half-life ($t_{1/2\beta}$).

5.2. Nimodipine implant study treatment and follow-up

5.2.1. Preparation of silica-based implants

The silica implants were prepared from tetraethoxy silane (TEOS 98%, Sigma-Aldrich, USA), deionized water (Millipore, Milli-Q® >17.5 MΩ cm), ethanol (EtOH, Etax AA 99.5%, Altia, Finland) and hydrochloric acid (HCl, Merck, USA) as catalyst using the sol-gel method (Ciriminna et al. 2013). The molar H_2O/TEOS ratio was 2 and the molar EtOH/TEOS ratio was 1. After hydrolysis, the sols were aged at 60˚C temperature. After ageing of the sols, the NDP (or glucose) was dissolved into the sols. The pH of the sols was adjusted by sodium hydroxide solution (NaOH, Merck, USA). The final pH of the sols was 5.5. Sols were sterile filtered (Whatman™, 0.22μm) and cast into polytetrafluoroethylene molds. The sols were gelled for 6 days at room temperature (RT). Formed implants were dried in a desiccator at RT.

5.2.2. Dissolution of implants *in vitro*

Degradation of silica matrices was measured in 50 mM TRIS (Trisma® -pre-set crystals, Sigma-Aldrich) + 0.1% (wt/wt) sodium dodecyl sulfate (SDS, Merck) buffered at pH 7.4 (37 ˚C). SiO_2 concentrations in the dissolution medium were kept below 30 ppm to ensure *in sink* condition (free dissolution of the SiO2 matrix). If needed, the whole dissolution medium was changed to fresh medium in order to keep SiO_2 concentrations < 30 ppm. Degradation of silica matrix was also measured with flow-through dissolution method. In the flow-through dissolution method, one implant was moved into a sample container with 150 ml dissolution medium and the dissolution medium was changed continuously by pumping dissolution medium through sample container at 347 μl/min (c.a. 500 ml/day). Purpose of the flow-through method was to mimic CSF dynamics in the human brain for estimating the dissolution rate of the implant *in vivo*.

The silica concentrations were measured with UV/VIS spectrophotometer (V-560, Jasco) analyzing the molybdenum blue complex absorbance at λ = 820 nm (Koch and Koch-Dedic 1974). Release of NDP from the SiO_2 matrices was analyzed with high-pressure liquid chromatography (HPLC-UV) by CRST in Turku. The chromatographic separation was obtained on a Gemini 5μ C18 110A, 150 x 2.0 mm (Phenomenex) analytical HPLC column. The mobile phase consisted of a mixture of acetonitrile and 15 mM hydrogen phosphate buffer (60:40 volume/volume).

5.2.3. Allocation of the treatment groups and follow-up

Nine eight-weeks-old male pigs, weighing 18.6–26.1 kg, and nine four-year-old male dogs,

weighing 10.0–14.0 kg, were randomly selected for the operation. Board-certified neurosurgeons were blinded for the content of the implants in all surgeries. Five implants were determined to be the number of implants for testing toxicity of the NDP silica implant (Active 1). The group receiving one implant was considered as a regular treatment group at expected pharmacological dose and any adverse effects were not expected (Active 2). Schedule of the study and allocation to treatment groups are presented in table 5 for the pigs and table 6 for the dogs. Day 1 indicates the operation day.

5.2.4. Anesthesia and analgesia

At day 1, pigs and dogs were sedated, anesthetized, intubated and connected to a ventilator for surgery. Pigs were sedated with midazolam 1 mg/kg *i.m.* and xylazine 3 mg/kg *i.m.* and anesthesia was maintained with propofol infusion 10 mg/kg/h *i.v.* Dogs were sedated using dexmedetomidine 20 µg/kg *i.v.*, anesthesia was induced with slow propofol bolus 3 mg/kg *i.v.* and maintained with a propofol infusion 9 mg/kg/h *i.v.* Heart rate, oxygen saturation and rectal temperature were observed and recorded throughout the procedure. As a prophylactic antibiotic, cefuroxime was administered before operation 250 mg *i.v.* for dogs and 750 mg *i.v.* for pigs. For analgesia, fentanyl was administered to pigs intraoperatively 8–13 µg/kg *i.v.* and 3–7 days postoperatively using a 50 µg/h transdermal patch. For the dogs, fentanyl was administered intraoperatively 3 µg/kg *i.v.* and 3 days postoperatively using a 50 µg/h transdermal patch.

Pigs were sedated for blood and CSF sampling with midazolam 1 mg/kg *i.m.* and xylazine 3 mg/kg *i.m.* For CSF sampling, anesthesia was induced with propofol bolus 3 mg/kg *i.v.* for pigs. Sedation of dogs for CSF sampling was done using dexmedetomidine 20 µg/kg *i.v.* and a slow propofol bolus 3 mg/kg *i.v.* Sedation or anesthesia was not required for dogs when only venous blood samples were collected.

Table 5. *Allocation of treatment groups and schedule of the pig study (Koskimäki et al I).*

Group	Procedure	Parenchyma implant	Animals per group	Animal IDs
Sham	Sham operation	No implant	3	22A, 22B, 23C
Placebo	Placebo implant 25mg glucose x1	Placebo implant	3	22C, 23A, 23F
Active	Nimodipine implant 5mg x1	Placebo implant	3	23B, 23D, 23E
Samples	**Day 1**	**Day 4**	**Day 5[1]**	**Day7**
Plasma (Ear vein)			x	x
Plasma (Vena jugularis)	x	x	x	x
Cerebrospinal fluid			x	x
CT	x			x[2]

[1]Day 6 on animals 22A, 22B and 22C.
[2]Only animal 23A and 23D underwent CT-imaging.

Table 6. *Allocation of treatment groups and schedule of the dog study (Koskimäki et al I).*

Group	Procedure	Parenchyma implant	Animals per group	Animal IDs	
Active 1	Nimodipine implant 8.5mg x 5 (total 42.5 mg)	NA	3	1, 2, 3	
Active 2	Nimodipine implant 8.5mg x 1	NA	3	4, 5, 6	
Sham	Sham operation	NA	3	7, 8, 9	
Samples	**Day 1**	**Day 5**	**Day 7**	**Day 14**	**Day 21**
Plasma (Vena jugularis)	1h, 1.5h, 2.5h, 4h, 6h and 22h (on Day 2)	x	x	x	x
Cerebrospinal fluid	1h		x	x	x
CT			x	x	x

*NA = Not applicable

Pigs were sedated for CT imaging procedures with midazolam 1–2 mg/kg *i.m.*, xylazine 3–6 mg/kg *i.m.* and anesthesia was maintained with propofol 8–15 mg/kg/h *i.v.* infusion. For the dogs, dexmedetomidine 20 µg/kg *i.v.* was used for the sedation and anesthesia induced with slow propofol bolus 3–5 mg/kg *i.v.* and maintained as needed with propofol boluses during CT imaging.

5.2.5. Surgery

During the surgical procedures, the heart rate, the rectal temperature and the oxygen saturation were recorded every fifteen minutes. Similar pterional surgical approach was used for both species. The animals were positioned in a lateral-prone gesture, and a longitudinal incision was made between the left eye and ipsilateral ear. The scalp was retracted away from the zygomatic arch. The temporal muscle was cut vertically and retracted to expose the lateral frontotemporal bone at the level of the anteromedial skull base. A lateral frontotemporal craniotomy, approximately 3 cm in diameter, was made using a trephine and a craniotome in order to reach the anterior and middle skull base. The dura was opened and basal cisterns were exposed under the operating microscope. In the pigs, one placebo implant, which contained 25 mg glucose, or one NDP implant, which contained 5 mg NDP was placed towards the scull base in the basal cisterns. One placebo implant with glucose was placed into the brain parenchyma in the temporal lobe in the Placebo and Active groups. Among the dogs, group Active 1 was treated with five implants containing 8.5 mg of NDP and group Active 2 with one implant containing 8.5 mg of NDP. For both pig and dog study, a sham group was included, where exactly the same procedure was performed including opening the arachnoid membrane and visualization of the optic chiasm but no implant was placed. In all groups, dura was closed using artificial graft (TachoSil®, Baxter, USA, Deerfield, IL 60015-4625) and fibrin tissue glue (TISSEEL, Baxter, USA, Deerfield, IL 60015-4625). The soft tissues and wound were closed in layers. After the surgery, viability of the animals was recorded at least twice a day. During the recovery period of 7 days for pigs and 21 days for dogs, the animals were observed daily. Body weight, food and water consumption were recorded once a day. Clinical signs and rectal temperature were recorded at least twice a day.

5.2.6. Sampling

Plasma and CSF samples were collected for determining NDP concentration. Schedules for the sampling are presented for the pigs in table 5, and for the dogs in table 6. Blood samples were collected from the external jugular vein into K2-EDTA tubes. In pigs, the samples were collected also from ear vein. CSF samples were collected from cisterna magna through the atlanto-occipital space. The samples were centrifuged (1200 G, 10 min, RT) and plasma was separated. CSF samples were centrifuged as well and supernatant was collected. Since NDP is very photosensitive, all samples were handled with minimal light exposure. The samples were stored frozen at -80°C nominal until transferred for analysis.

Liquid chromatography mass spectrometric (LC-MS) method was used for determination of NDP in dog and pig plasma and CSF samples. Sample preparation was performed by liquid-liquid extraction. The extracts were analyzed using reverse-phase chromatography followed by mass spectrometry. Lower limit of quantification (LLOQ) of the method was 0.01 ng/ml for both sample matrices.

5.2.7. Computed tomography

The schedule for CT imaging is shown in table 5 for pigs and table 6 for dogs. CT studies were performed with Discovery STE PET/CT scanner (General Electric Medical Systems, Milwaukee, WI, USA) operated in a helical mode.

Cerebral CT imaging was performed using an ultrafast CT protocol (335 mA, 100 kVp). Voxel size was 0.625 mm × 0.625 mm × 0.625 mm. The volume of interest (VOI) was drawn for the implant(s). A calibration phantom (13002 Model 3 CT Calibration Phantom, Mindways Software, Inc, San Francisco, CA, United States) was used prior to CT imaging and densities of the implants were calculated and corrected using the phantom data. CT image analysis was done with Carimas

2.5 software (Turku PET Centre, Turku, Finland; http://www.turkupetcentre.fi/carimas). Density and volume of the implant were defined using cerebral CT imaging and implant degradation was evaluated. Implant degradation was calculated as the change in density and volume of the implant at different time points (for pigs days 1 and 7, for dogs days 7, 14 and 21).

5.2.8. Histological analyses

At the end of the study period, the pigs were sedated with midazolam 1 mg/kg *i.m.*, xylazine 3 mg/kg *i.m.* and euthanized with propofol *i.v.* overdose and exsanguinated. Dogs were sedated with dexmedetomidine 20 μg/kg *i.v.* and euthanized with pentobarbital *i.v.* overdose and exsanguinated.

The cranium of the animals was opened to expose the operation site. Meninges and brain tissue were observed for any macroscopic changes and the presence of implants was evaluated. For the histopathological examination, samples from the meninges and brain tissue were taken from the vicinity of the implant site and from corresponding area on the opposite hemisphere. All samples were fixed in a buffered 4% formaldehyde solution. The tissue samples were embedded in paraffin, cut to a thickness of approximately 4 μm and stained with hematoxylin & eosin (HE) for histopathology. All slides were examined by a pathologist and histopathological changes were recorded and scored with a 5-step scale from minimal to severe.

5.2.9. Statistical analyses

All the results are presented as means±SD. Mixed model for repeated measurements was applied to the implant volume and density data (SAS 9.2 SAS Institute Inc., Cary, NC, USA). P-values <0.05 were considered statistically significant.

5.3. Nimodipine-induced plasticity in mice

5.3.1. Drug treatment and tissue sampling

For the drug treatments, animals were gently immobilized and injected subcutaneously (*s.c.*) with either NDP (10 mg/kg, Santa Cruz Biotechnology (SCB), CA, USA) or vehicle (0.5% Tween-40 in saline) (N=6 per group) into the neck pouch. All the animals were injected on the same day by the same researcher using a 5-minute interval between each animal. All the animals were sacrificed after 90 minutes of injection. This is shown to be a lag period that corresponds to TrkB phosphorylation induced by pharmacologically diverse antidepressant drugs (Rantamäki et al. 2007). Mice were stunned with carbon dioxide and sacrificed with rapid cervical dislocation. Next, the hippocampus and medial prefrontal cortex were isolated for extraction of protein and total RNA. Samples for total RNA were frozen immediately with dry ice and stored at -80˚C. For protein extractions, the hippocampus and medial prefrontal cortex were collected on a cooled dish and homogenized in lysis buffer (137 mM NaCl, 20 mM Tris, 1% NP-40, 10% glycerol, 48 mM NaF, 2X Complete inhibitor mix (Roche Diagnostics, Hertforshire, UK) and 2 mM Na_3VO_4). After incubation on ice for 15 min, samples were centrifuged (16100 *g*, 15 min, +4˚C) and the supernatant collected for further analysis.

5.3.2. Western blot

The exact description is presented in the publication III. Shortly, sample protein concentrations were measured using a commercial kit (Bio-Rad DC protein assay) and next, the total protein was separated in a SDS-PAGE under reducing conditions and transferred onto a PDVF membrane (300 mA for 1 h at 4˚C). The membranes were blocked with 3% bovine serum albumin (1 h, room temperature) and incubated with the selected primary antibodies that were anti-p-TrkB^{Y816}, anti-p-TrkA^{Y490}/TrkB^{Y515}, anti-Trk, anti-p-CREB^{S133}, anti-CREB, anti-p-Akt^{T308}, anti-AKT, anti-p-44/42 MAPK$^{T202/Y204}$, anti-p44/42 MAPK, anti-p-mTOR^{S2481}, anti-mTOR, anti-p-p70S6 kinase$^{T421/S424}$, anti-p70S6 kinase and anti-GAPDH. After washing, the membranes were incubated in

HRP-conjugated secondary antibody (1:10000, BIO-RAD; 1 h, room temperature) followed by visualization with an enhanced chemiluminescence kit (ECL+, Amersham Biosciences) and the detection of luminescence with Fuji LAS-3000 camera. Phosphoprotein detection was always conducted first after which the filter was stripped and probed with the corresponding total protein antibody. GAPDH immunoblotting was used to control equal loading and for the quantification of TrkB protein expression.

5.3.3. BDNF ELISA

Mature BDNF protein levels were assessed using ELISA method as previously described (Karpova et al. 2010). The assay shows no cross-reactivity with other neurotrophins (Karpova et al. 2010) and has been further validated using tissues obtained from $BDNF^{+/-}$ mice (~50% expression) and conditional $BDNF^{-/-}$ mice (undetected). That was the reason why we selected ELISA method for mature BDNF detection. Shortly, transient acidified brain lysates, BDNF standards (7.8–1000 pg/ml in Hanks; Promega), and POD-conjugated secondary BDNF antibody were transferred to pre-blocked (300 µl; Hanks buffer, 2% BSA, 0.1% Triton X-100, 2 h, RT) Maxisorb® ELISA plates that were previously coated with the primary BDNF antibody. The following day the plates were washed with PBS-T and the POD substrate was added to the wells according to manufacturer's instructions (BM Blue; Roche). The colorimetric reaction was stopped within 20 min with 1 N H_2SO_4 (50 µl), and absorbance was immediately measured at 490 nm. BDNF protein was normalized to total protein levels. The r^2 for the standard curve was ≥0.99 in all experiments.

5.3.4. BDNF qPCR

We conducted RNA extraction followed by real-time quantitative PCR as previously described (Uutela et al. 2014). Total RNA was extracted from frozen tissues by using QIAzol (Qiagen, Valencia, CA) and treated with DNaseI (Thermo Fisher Scientific Inc, Rockford, IL) according to the manufacturer's instruction. cDNA was synthesized with 1 µg of total RNA by using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, Rockford, IL). Real-time quantitative PCR was performed using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc, Rockford, IL) and the CFX96 Touch™ detection system (Bio-Rad, Hercules, CA). The primers described previously (Karpova et al. 2011) were used to amplify specific cDNA regions of transcripts. The coding region in the exon IX of the *Bdnf* gene for the total *Bdnf* mRNA and a housekeeping control gene are precisely described in the publication III. Ct and quantitative values were calculated from each sample using CFX Manager™ software (Bio-Rad, Hercules, CA) and the quantitative values were normalized to the control *Gapdh* levels.

5.3.5. Data processing and statistical analyses

Protein bands were quantified using ImageJ program (NIH, National Institutes of Health). Phospho-protein band intensities were divided by corresponding total protein band intensities. Final values were divided by the control group average and multiplied by 100. All the data are expressed as mean ± SEM (Standard Error of Mean; standard deviation divided by the square root of sample size) and as percentage of control. Statistical tests were performed using the two-tailed Student t-test. The criterion for significance was set to $p < 0.05$.

6. RESULTS

6.1. Pharmacokinetics of nimodipine in healthy beagle dogs

6.1.1. Individual kinetic profiles

Individual kinetic profiles of NDP in plasma after *i.v.* and oral administration are presented in the publication II. Kinetic profile of NDP in beagle dogs follows the kinetics seen in humans as expected. A typical rapid increase in the NDP concentration is seen that is followed by a steep decrease in few hours. Thus, oral administration of NDP requires several dosing times per day for achieving sufficient concentration.

6.1.2. Pharmacokinetic parameters

The individual and mean ±SD pharmacokinetic parameters for NDP in dog plasma after a single intravenous dose are presented in table 7 and after oral administration in table 8. During NDP treatment, no adverse effects were observed in dogs.

The mean terminal elimination half-life of NDP after i.v. bolus dose 0.72 mg was 1.8 h and mean plasma clearance was 40.3 l/h and 3.4 l/h/kg. After oral administration of 60 mg NDP, the mean terminal elimination half-life was 0.89 h. The absolute bioavailability (calculated for the animals dosed intravenously and orally) was 22%. Dose proportionality was evaluated by comparing the exposure parameters C_{max} and AUC_{0-24} at oral doses of 30 mg and 60 mg. C_{max} and AUC_{0-24} increased in a dose-proportional manner. The same individuals were not used in the groups tested at oral doses of 30 mg and 60 mg. Individual variation in the kinetic profile of NDP was measured.

6.1.3. CSF concentrations of nimodipine

At one hour after oral administration, NDP can be measured in the CSF in concentrations of about 1-2% of the measured plasma concentrations. Plasma and CSF concentrations of NDP after oral dosing of 60 mg are presented in table 9.

Table 7. *Mean pharmacokinetic parameters of NDP after i.v. bolus dosing of 0.72 mg (Koskimäki et al II).*

Animal no	AUC (h*ng/ml)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (h)	Cl (l/h)	Vss (l)
1	22.7	11	1.4	31.7	37.6
2	13.3	9	1.6	54.0	98.8
3	20.4	10	2.5	35.3	58.9
Mean	18.8	10	1.8	40.3	65.1
SD	4.9	0.02	0.6	12.0	31.1

Table 8. *Mean pharmacokinetic parameters of NDP after oral dosing of 30 mg or 60 mg (Koskimäki et al II).*

	Dose (mg)	Animal no	C_{max} (ng/ml)	t_{max} (h)	AUC24h (h*ng/ml)
	30	1	46.8	0.67	104.9
	30	2	212.4	0.67	342.0
	30	3	50.7	1.00	123.3
Mean			103.3	0.78	190.0
SD			94.5	0.19	131.9
	60	4	238.8	1.00	486.8
	60	5	81.2	1.00	273.2
	60	6	345.0	0.67	569.9
Mean			221.7	0.89	443.3
SD			132.8	0.19	153.1

Table 9. *Plasma and CSF concentrations of NDP after oral dosing of 60 mg (Koskimäki et al II).*

Animal no	Time (h)	Matrix	Conc. (ng/ml)	CSF/plasma -ratio
4	1	CSF	1.37	0.0134
	1	Plasma	102	
5	1	CSF	0.68	0.0125
	1	Plasma	54.6	
6	1	CSF	0.97	0.0132
	1	Plasma	73.4	

6.2. Nimodipine implant treatment and follow-up

6.2.1. Dissolution of the implant *in vitro*

Degradation rates of the silica implant formulation were measured from three parallel samples. The implant formulations were totally degraded after 3.5–4 days *in sink* dissolution and 24 days in flow-through dissolution. The cumulative release profiles of NDP were measured from the same sample solutions (pH = 7.4 at 37˚C) as used for the SiO_2 degradations measurements. NDP was mainly released from the implant formulations by degradation of silica matrices. According to dissolution results there were 5.0 mg and 8.5 mg of NDP in implant A and implant B, respectively. Degradation of the implant in 24 days in flow-through dissolution was in line with the clinical treatment period of NDP.

6.2.2. Surgery and recovery period

During the surgery, heart rate, oxygen saturation and rectal temperature were within the normal range, except for one dog (No. 3), which suffered from a decrease in oxygen saturation (down to 70%). This persisted for three minutes at the end of the operation due to a malfunction of the ventilator. Postoperatively, signs of systemic infection were not manifested and rectal temperatures were within a normal range throughout the recovery period. All clinical findings of the dogs during the 21-day recovery period are presented in table 10. During the 7-day recovery period of the pigs, no clinical signs of morbidity or changed behavior were noted. Body weights of the dogs were slightly decreased in each group: on average, in group Active 1 a decrease of 7% (n=2) was seen, in Active 2, a decrease of 4% and, in the sham group a decrease of 2% was noted. Food consumption in all dog groups remained normal during the 21-day recovery period. In pigs, body weight gain was 7%–18% and can be considered normal growth rate in each group.

Table 10. *Summary of the clinical signs of the dogs during the 21-day recovery period (Koskimäki et al I).*

Group	Dog number	Epileptic seizure	Other/notice:
Active 1	1	No	-
	2	Yes	Epileptiformic seizure on day 4. Treated with phenobarbital medication 2.5mg/kg twice a day on days 4-12. The medication was reduced to 2.5mg/kg once a day on day 13 and ended on day 20.
	3	Yes	Epileptiformic seizure 6 hours post-operatively. Epileptiformic seizures were refractory to phenobarbital medication. The animal had to be euthanized on day 3 according to the protocol. It was not replaced.
Active 2	4	No	24 h post-operatively conjunctivitis.
	5	No	24 h post-operatively vomiting x 1.
	6	No	24 h post-operatively conjunctivitis.
Sham	7	No	-
	8	No	24 h post-operatively conjunctivitis.
	9	No	-

One animal deceased during the non-clinical experiment. Animal number 3 (dog) from group Active 1 developed refractory complex partial epilepsy and had to be euthanized on day 3. Another dog from the same group (No. 2) had epileptic seizures on Day 4 and was treated with phenobarbital medication. The dog responded well to the treatment. Post-operative eye infections were treated with local ophthalmic ointment containing fusidic acid and the infections responded well to the treatment.

6.2.3. Histology of brain and meninges

In the macroscopic examination of the pigs, no abnormalities were detected at the implantation area. In dogs, local attachment of meninges to the implant site was seen, which prevented the evaluation of actual implants. This lesion was not seen in the Sham group. In the figure 2, microscopic histology of the sham group and the implant group is presented (Koskimäki et al, unpublished).

Summary of the histopathological examination is shown in table 11. Dog number 3 (Active 1) was sacrificed on day 3 and therefore excluded from these results. In histopathological examination of the operation site, local inflammatory reaction characterized by infiltration of mononuclear and granulocytic cell was seen in the meninges of both species. In pigs, there was an eosinophilic component whereas in dogs the inflammation was more neutrophilic. Multinucleated giant cells indicating foreign body reaction and foreign implant material were seen

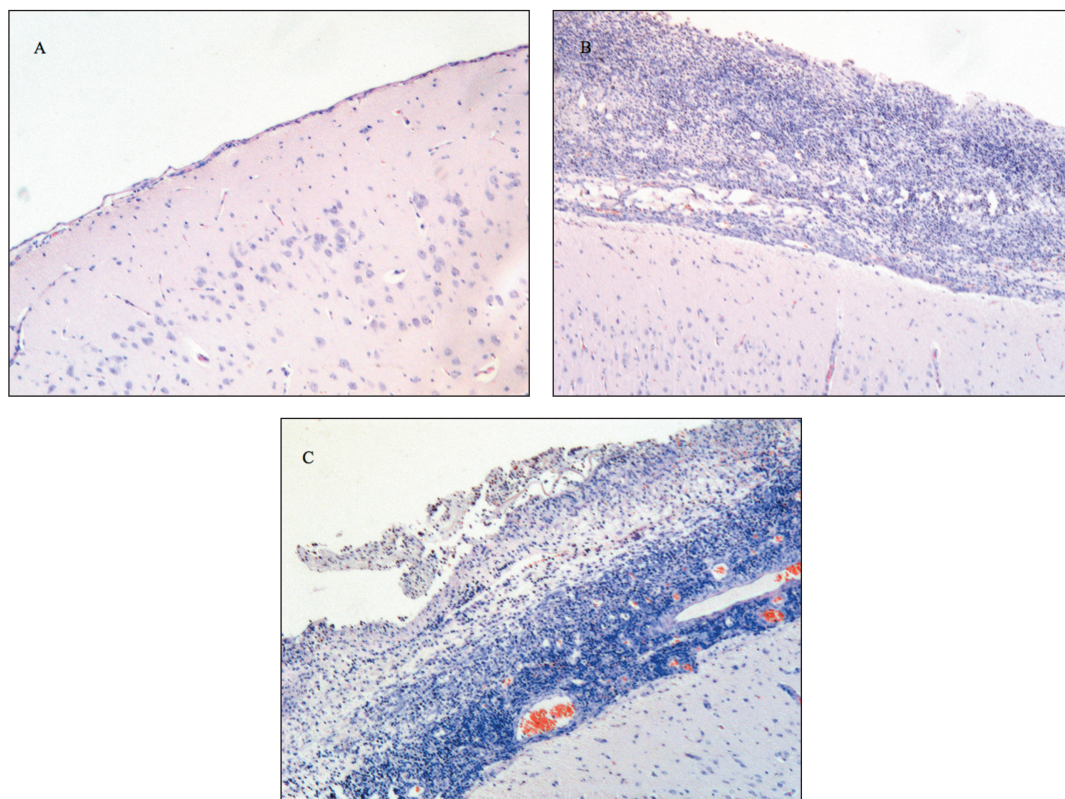

FIGURE 2. Histological findings of operated dogs (Koskimäki et al, unpublished). A. *The leptomeninges from non-operated area. The meninges are thin and few small intracranial blood vessels are detected with sparse inflammatory cells. Sham operated (Dog 7), magnification x50, HE stain.* **B and C.** *On the operation site the leptomeninges are heavily infiltrated by mononuclear inflammatory cells and a few polymorphonuclear cells. Importantly, the inflammation is not extending to the brain parenchyma. No clear difference can be detected between implanted (***B***; Dog 6) and sham operated (***C***; Dog 8) dogs. Magnification x50, HE stain.*

mainly in dogs receiving five implants. Additional findings were mild to moderate fibrosis, vascular regeneration and mild hemorrhage on the meninges in all groups. In general, the changes were more frequent and severe in the dogs, but no clear differences in the severity of changes could be seen between sham groups and implanted groups. Furthermore, there was no difference in changes between the two implant types (glucose versus NDP as active ingredient). In all groups, the underlying brain parenchyma was only mildly affected by perivascular mononuclear cuffing or spreading of inflammatory infiltrate from the meninges. In dogs, a single dog in the Sham group and in the Active 1 group showed focal degeneration and gliosis in the deeper parenchyma with no direct connection to implant site. No significant abnormalities were observed in the samples from opposite hemisphere.

The implant site located in the brain parenchyma was present in the samples from two pigs (Nos. 23D and 23F). The local changes around the parenchymal implant site did not show major signs of foreign body reactions. There was local inflammatory reaction with foamy macrophages and minimal to marked degeneration in the surrounding parenchyma, but not more that could be expected from the procedure itself and intraparenchymal implantation.

6.2.4. Nimodipine concentrations

In pigs, calculated CSF/peripheral venous plasma ratio was at day 5 1.31 ± 1.34 and at day 7 0.886 ± 0.255. Correspondingly in dogs receiving active implant, after one hour in 5x 8.5 mg NDP group the CSF/jugular plasma ratio was 0.002 ± 0.0005 and in 8.5 mg NDP group 0.149 ± 0.174. The sustained release profile of NDP in CSF was achieved for 21 days in dogs and 7 days in pigs (table 12).

The best achieved CSF/plasma ratios and the highest CSF concentrations after the dosing with the 60 mg tablet and the 8.5 mg implant after one hour of administration in our study are presented in table 13. The CSF/plasma ratio was 25 times higher after the implant treatment. In addition, the systemic concentration after the implant treatment was 12 times lower and achieved CSF concentration was over two times higher compared to the traditional treatment.

Table 11. *Summary of histopathological findings and severity in pigs and dogs in each group (Koskimäki et al I). Number of affected animals in each group is shown. Severity score indicates the recorded minimum and maximum severity in each group. Only one score is shown if all animals had same severity or only one animal was affected. N=3 in each group.*

Lesion	Pigs			Dogs		
	Sham	Placebo	Active	Sham	Active 1*	Active 2
Infiltration of mononuclear cells (meninges)	1	3	3	3	2	3
*Severity score, min/max^*	++++	++/+++	+/++++	++++	++++	+++/++++
Infiltration of granulocytes (meninges)	1	1	1	3	2	3
Severity score, min/max	++++	+	++	+/+++	+++	+/++
Multinucleated giant cells and foreign material (meninges)	0	0	0	0	2	1
Severity score, min/max	-	-	-	-	++	++
Fibrosis (meninges)	1	3	1	3	2	3
Severity score, min/max	++	+	++	++/+++	+++/++++	++/++++
Vascular regeneration (meninges)	0	2	0	3	1	3
Severity score, min/max	-	+++	-	++	++	++
Hemorrhage +/- hemosiderophages (meninges)	1	2	1	2	1	3
Severity score, min/max	+	+/+++	++++	++	++	++
Perivascular cuffing (parenchyma)	0	0	1	3	2	3
Severity score, min/max	-	-	+	+/+++	++	+/++

*In this group, *n* = 2. Animal number 3 was excluded due to preterminal sacrifice.
^ + minimal, ++ slight, +++ moderate, ++++ marked, - not present

Table 12. *Concentrations of NDP at different time points after surgery in dogs and pigs (ng/ml) (Koskimäki et al I).*

	1h		Day 7		Day 14		Day 21	
	Jugularis	CSF	Jugularis	CSF	Jugularis	CSF	Jugularis	CSF
Nimodipine 8.5 mg implant in dogs	17.79 ± 15.17	2.19 ± 1.90	6.13 ± 4.66	0.025 ± 0.009	11.51 ± 3.77	0.030 ± 0.008	3.05 ± 1.53	0.013 ± 0.004
Nimodipine 5x 8.5 mg implant in dogs	63.9 ± 18.7	0.110 ± 0.005	37.8 ± 15.8	0.095 ± 0.008	36.5 ± 9.97	0.083 ± 0.009	32.9 ± 8.77	0.034 ± 0.004

	Day 5			Day 7		
	Jugularis	Ear	CSF	Jugularis	Ear	CSF
Nimodipine 5 mg implant in pigs	0.045 ± 0.008	0.027 ± 0.005	0.027 ± 0.022	0.054 ± 0.006	0.045 ± 0.020	0.038 ± 0.017

Table 13. *The best achieved CSF/plasma ratios and the highest CSF concentrations after the dosing with the 60 mg tablet and the 8.5 mg implant after one hour of administration. (Modified from I and II)*

Route	Matrix	Conc. (ng/ml)	CSF/plasma -ratio
Oral	CSF	1.37	0.0134
	Plasma	102	
Implanted	CSF	2.94	0.341
	Plasma	8.62	

6.2.5. Computed tomography

Clinical analyses of the CT data showed the location and degradation of the implants in the subarachnoid space in pigs and dogs. The implants were correctly placed at the base of the cranium in the Sylvian fissure in the subarachnoid space in all pigs. This was noted in the dogs as well except for dogs number 2 and 3 in group Active 1. Dog number 3 had to be euthanized at day 3 and CT imaging was therefore not performed. CT imaging of dog number 2 showed a too rostral positioning of the implants, and five implants had formed a pile-like formation protruding 6.5 mm into the parenchyma (I). Further evaluation of radiographic data indicated the importance of the location of the craniotomy in order that the required space and location was reached without increasing the risk of complication.

In dogs, implant size and density were decreased between weeks 1 and 3. Degradation was very linear as presented in the publication I. Based on slopes of the degradation curves, density was decreased faster than volume.

6.3. Nimodipine-induced neuroplasticity

6.3.1. Nimodipine activates TrkB in the brain

Signaling cascades after NDP treatment are concluded in the figure 3. The first and essential post-translational modification is autophosphorylation of the TrkB autocatalytic domain ($TrkB^{Y705/6}$) that activates the kinase activity of TrkB (Segal et al. 1996). Indeed, we saw autophosphorylation of the $TrkB^{Y705/6}$ after a single NDP treatment. Phospho-$TrkB^{Y705/6}$ levels were significantly increased in the prefrontal cortex (PFC) and hippocampus (HC) 90 minutes af-

ter injection. Nonetheless, the phosphorylation state of the Shc binding domain ($TrkB^{Y515}$) remained unaltered after the treatment. The Shc binding domain is not phosphorylated after traditional antidepressant treatment. However, Shc site of TrkB is phosphorylated by its endogenous ligand BDNF. Lastly and the most importantly, the phosphorylation status of the PLCγl-binding tyrosine within TrkB ($TrkB^{Y816}$) was significantly increased after NDP treatment. Interestingly, these NDP-induced changes on TrkB receptor activation resemble those produced by antidepressant drugs (Saarelainen et al. 2003; Rantamäki et al. 2007).

6.3.2. Nimodipine induces neuroplasticity in the brain

The phosphorylation of $TrkB^{Y515}$ serves as a docking site for Shc adaptor proteins that further regulate the phosphorylation and activity of downstream signaling molecules implicated in neuronal apoptosis and survival (Akt) and neuronal differentiation (ERK1/2) (Huang and Reichardt 2001; Ahn 2014). In line with the

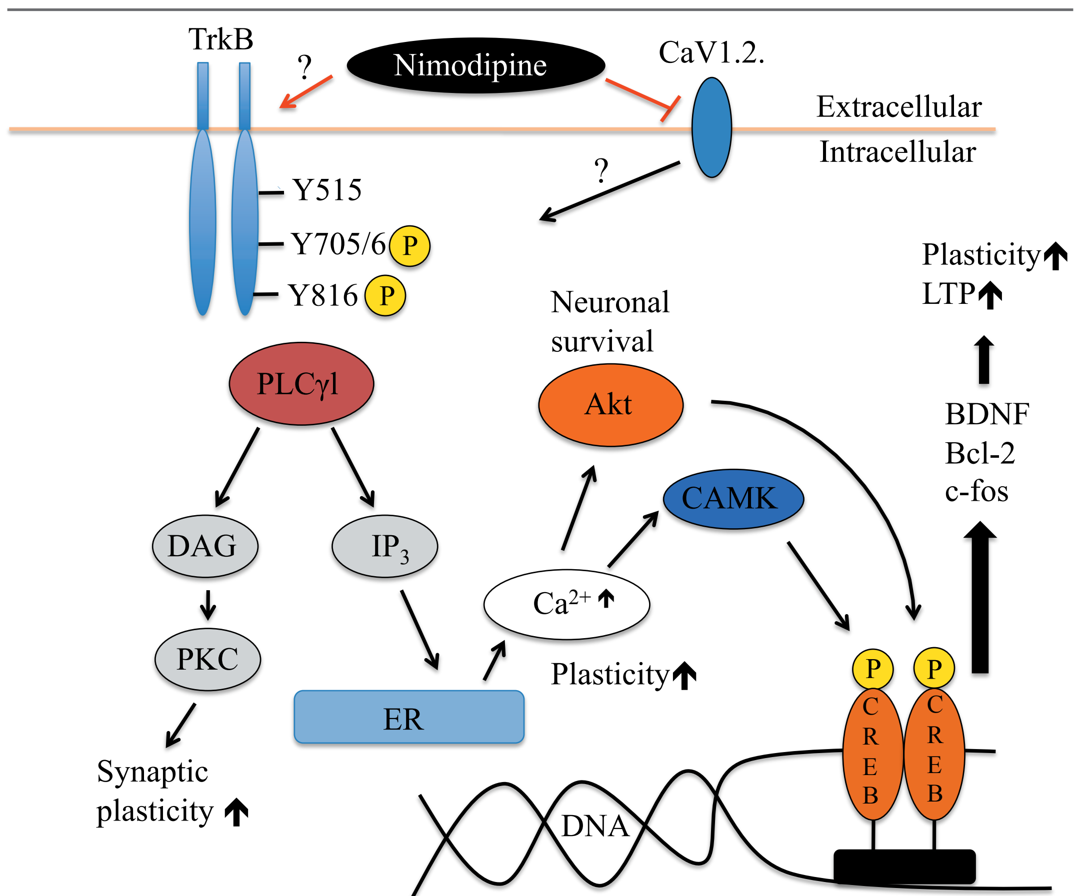

FIGURE 3. The signaling cascades in hippocampus and prefrontal cortex after NDP treatment. *$TrkB^{Y705/6}$ and $TrkB^{Y816}$ sites are phosphorylated further activating PLCγ1. PLCγ1 induces degradation of phospholipid phosphatidylinositol 4,5 bisphosphate (PIP_2, not shown) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) pathway that is further capable of increase synaptic plasticity. IP_3 binds to receptors on the surface of endoplasmic reticulum (ER) releasing calcium (Ca^{2+}) to the intracellular space. Increased Ca^{2+} concentration activates different forms of CAMKs and Akt signaling. These changes further lead to the phosphorylation of cAMP response element-binding protein (CREB), which critically regulates transcription of the genes that are implicated in plasticity and LTP formation. BDNF = Brain derived neurotrophic factor, Bcl-2 = B-cell CLL/lymphoma 2.*

unaltered levels of phospho-$TrkB^{Y515}$, phosphorylation levels of ERK1/2 in the HC and the PFC were indistinguishable in the control and NDP-treated animals. However, even though the phosphorylation of $TrkB^{Y515}$ is important for activating neuroprotective Akt signaling, there is evidence that IP_3 pathway and further increase in the intracellular calcium concentration, particularly released from intracellular stores, regulates Akt signaling possibly through phosphoinositide 3-kinase (PI3K) (Zheng et al. 2008).

Phosphorylation of $TrkB^{Y816}$ leads to the activation PLCγ1 that consequently increases intracellular calcium mobilization from intracellular calcium stores *via* IP_3 signaling (Huang and Reichardt 2001). This signaling event has been tightly associated with the phosphorylation and activation of CREB, a transcription factor that critically regulates the transcription of genes implicated in synaptic plasticity (e.g. long-term potentiation) (Minichiello 2009). In line with observed changes in phospho-$TrkB^{Y816}$ levels, the phosphorylation levels of CREB were significantly increased after NDP in both brain areas investigated.

6.3.3. Nimodipine induces neuroprotective signaling in the brain

NDP treatment increased the phosphorylation levels of Akt in the PFC and a trend was also seen in the HC (p=0.055). Apart from regulating neuronal apoptosis and survival, Akt has also been linked with the activation of mTor-p70S6k pathway that is implicated in dendritic spine formation and morphology (Kumar et al. 2005). However, the phosphorylation levels of mTor and its downstream target kinase remained unaltered after acute NDP administration. The activation of Akt signaling by NDP treatment is most probably conveyed *via* IP_3 signaling and increased mobilization of calcium from intracellular calcium stores (Zheng et al. 2008).

6.3.4. Levels of BDNF protein and mRNA after acute treatment

The levels of the BDNF protein and total mRNA remained at the normal level in the medial PFC and HC after a single dose of NDP. This finding is in line with results seen after acute antidepressant drug treatment (Rantamäki et al. 2011). However, it is not known whether chronic NDP administration induces translation of BDNF mRNA and further expression of BDNF protein.

7. DISCUSSION

7.1. Nimodipine implant treatment for vasospasm

We formulated a new silica-based intracranial NDP implant. The implant is feasible for surgical application in the subarachnoid space, and no major histological foreign body reactions were observed. Most importantly, with the use of the new implant treatment, it was possible to achieve significantly lower systemic concentrations, higher CSF concentrations, and higher CSF/plasma ratios of NDP than when the conventional oral NDP treatment is administrated. A sustained realease profile of the NDP implants was measured, *in vivo* as well as *in vitro*. In addition, CT imaging was feasible for monitoring the degradation of the implants *in vivo*.

Intracranial treatment for DCI is an emerging treatment modality (Kasuya et al. 2002; Kasuya et al. 2005; Krischek et al. 2007). PLGA implants have been studied the most, and the results have thus far been encouraging (Kasuya et al. 2005; Schneider et al. 2011; Cook et al. 2012). Calcium channel blockers are interesting candidates for intracranial treatment because of proven pleiotropic effects (Nuglisch et al. 1990; Zornow and Prough 1996; Kobayashi and Mori 1998; Dreier et al. 2000; Liu et al. 2004; Vergouwen et al. 2008; Cook et al. 2012; Scheller and Scheller 2012; Macdonald 2014). Higher concentrations of NDP in the CSF can be achieved with intracranial therapy, and these higher concentrations can be more beneficial for inhibiting the pathological cascades occurring after aSAH (Dreier 2011; Cook et al. 2012; Macdonald 2014). With intracranial therapy, lower systemic drug concentration is achieved, as our study also proved (Macdonald et al. 2012; Cook et al. 2012). This is of interest in the case of inhibiting NDP-induced hypotension but can be extremely beneficial for other drugs as well.

The sol-gel method was used in the formulation of the new implant, and NDP was the drug of choice as the most convincing evidence for treating DCI (Laursen et al. 1988; Pickard et al. 1989; Kortesuo et al. 2001; Dorhout Mees et al. 2007; Ciriminna et al. 2013). As stated, NDP is a calcium channel blocker with high specificity to intracranial blood vessels, but interestingly, neurons possess L-type calcium channels with higher density (Langley and Sorkin 1989; Ricci et al. 2002). Undisputedly, dilatation of cerebral vessels is not the only method by which NDP improves patients' outcome. Indeed, we demonstrated the new potential neuroplastic mechanism behind the therapeutic effects of nimodipine (see the next section).

We met our aim to deliver the NDP biodegradable silica-based implants into the subarachnoid space through pterional craniotomy in pigs and dogs. The recovery periods went without any complications in pigs. In the dogs, epileptic seizures were present in the group of five implants. It was evident already by the time of the surgery that five implants may cause morbidity since the space of basal cistern was too limited for numerous implants, even though an operative microscope was used. One of the dogs had to be euthanized due to refractory epileptic seizures, whereas the other responded well to the phenobarbital treatment and the medication could be terminated. However, one dog that received five implants did not show any distress. In the CT imaging, the dog that received phenobarbital medication with good response showed improper placement of implants and protrusion of the implants into parenchyma, which most probably caused the seizures. In histological examination of this dog focal parenchymal degeneration was seen as well, caused probably by the protrusion of implants in the parenchyma.

In histopathological examination of dogs and pigs, no major differences were distinguished between groups in spite of the presence of implants. Most of the findings were present also in the sham-operated animals, demonstrating that these findings were related to the procedure itself. Foreign body type reaction was mainly seen in dogs receiving five implants, which can be directly related to the larger amount of implant material or compression of the brain parenchyma. However, the implantation directly into the brain parenchyma of pigs did not produce wide-

spread tissue damage around the implant. Therefore silica-based biodegradable materials can be considered for use in the release of the therapeutic agents for an intracranial treatment of DCI. Compared to histopathological studies for PLGA implants, similar minimal foreign body type reaction profile is observed as well (Menei et al. 1993; Shive and Anderson 1997).

According to the NDP concentrations in the CSF, a sustained release profile of NDP was achieved for 21 days in dogs and for 7 days in pigs. After the dosing with the 60 mg tablet and the 8.5 mg implant, the CSF/plasma ratio was 25 times higher after the implant treatment, and the systemic concentration was 12 times lower. In addition the achieved CSF concentration was over two times higher than with the traditional treatment (table 13). Thus, significantly higher concentrations can be achieved in the CSF with the use of the intracranial delivery system. A significant reduction in the systemic concentration of NDP was also achieved in our study, and may inhibit NDP-induced hypotension after aSAH (table 13).

Interestingly, despite the intracisternal location of the implants, the increase of NDP concentration in CSF was modest. The samples were taken through atlanto-occipital space from cisterna magna and therefore the low concentrations could be due to a net flow of CSF towards arachnoid villi and sinuses, resulting in a lower concentration in CSF located extracranially or cisterna magna. In addition, in our dog study, the plasma samples were taken from the external jugular vein, not from a peripheral vein, which may result in a higher measured NDP concentration since the NDP is not totally distributed. Our data in pigs suggests that after five and seven days, the external jugular plasma and peripheral plasma ratio is about two. However, the early data from the CSF kinetics is lacking and cannot be directly extrapolated to the different species.

Specific features in the implant or formulated NDP amount can cause a decrease in dissolution rates. For example, Cook et al. reported significantly lower NDP concentration in CSF when used 30 mg loaded NDP PLGA microparticles compared to 10 mg loaded PLGA microparticles (Cook et al. 2012). However, according to the NDP implant *in vitro* dissolution curves presented in our study, it seems that the implant itself is not culpable. The reason of relatively low CSF concentration and high plasma concentration is unclear.

The physical degradation of the silica implant and prediction of the implant behavior *in vivo* was feasible to follow using CT imaging. High dissolution rates in the basal cisterns can be induced by increased circulation of spinal fluid. Of course, after aSAH, early hydrocephalus is a considerable problem for patients, but also considering the dissolution of the implant (Milhorat 1987; Germanwala et al. 2010). Thus, the dissolution profile needs to be studied also when CSF kinetics is pathologically altered. A quick decrease of the density is in line with the physical properties of the implant matrix. The degradation of silica matrix occurs constantly throughout the implant and therefore the implant size decreases slower than its density.

7.2. Nimodipine induces neuronal plasticity

For the first time, our study showed that acute nimodipine treatment activates BDNF receptor TrkB sites $TrkB^{Y705/6}$ and $TrkB^{Y816}$, leaving the Shc site unaltered (figure 3). Phosphorylation of $TrkB^{Y816}$ leads to the activation of PLCγ1, which was activated in the HC and PFC. In addition, the phosphororylation status of Akt and CREB was significantly increased without any alteration in the BDNF mRNA and protein levels.

The ability of NDP to improve the clinical outcome after aSAH was established already in 1988 (Laursen et al. 1988; Pickard et al. 1989; Dorhout Mees et al. 2007). However, the exact mechanisms of actions of calcium channel blocker NDP remain obscure.

Pathophysiology of aSAH is a complex combination of early brain injury, vasospasm, secondary injuries, inflammation and cortical spreading ischemia (Macdonald 2014). Nonetheless, the therapeutic actions of NDP have been considered to be principally arbitrated by its vasoactive properties, while clinical data, as well as pre-clinical data, indi-

cate that also additional mechanisms are involved (Etminan et al. 2011; Macdonald 2014).

NDP holds extensive neuroprotective properties by reducing neuronal and glial apoptosis, increasing fibrinolysis of microthrombosis and inhibiting cortical spreading ischemia (Lazarewicz et al. 1990; Dreier et al. 2002; Dreier et al. 2009; Pluta et al. 2009; Etminan et al. 2011; Vergouwen et al. 2011; Hashioka et al. 2012; Sehba et al. 2012; Woitzik et al. 2012). Chronic administration of NDP has also been reported to induce synaptogenesis (de Jong et al. 1992).

As an L-type calcium channel blocker, NDP effectively affects intracellular calcium signaling. NDP binds to CaV1.2 and CaV1.3 that are the major isoforms expressed in the nervous system both presynaptically and postsynaptically (Langley and Sorkin 1989; Hell et al. 1993; Lipscombe et al. 2004; Frank 2014). Thus, NDP partially inhibits activity-dependent calcium influx that is mainly conveyed thorough activation of NMDA and AMPA receptors (Abraham and Williams 2003; Asrar et al. 2009). In physiological conditions, calcium influx seems to have a role of strong modulator that has local (neurotransmission, LTP) but also broad-ranged (gene transcription, synaptic plasticity, homeostatic plasticity) effects (Magee and Johnston 1997; Hudmon et al. 2005; Remy and Spruston 2007; Lee et al. 2009b; Frank 2014).

Under pathophysiological conditions, neuronal membrane and energy homeostasis are disrupted, leading to broad-range alterations in synaptic and neuronal functions (Choi 1994). Due to the membrane disruption and energy failure, excess calcium enters to the cells causing excitotoxicity and eventually leading to neuronal death *via* a variety of mechanisms (Choi 1994; Gepdiremen et al. 1997; Hashioka et al. 2012).

NDP inhibits L-type calcium channels by reducing the intracellular calcium concentration (Choi 1994; Gepdiremen et al. 1997). Furthermore, NDP also increases the expression of an antiapoptotic factor Bcl-2, and as our study showed, increased activation of neuroprotective Akt signaling (Liu et al. 2004). Due to the lipophilic nature of NDP it easily passes through cell membrane (Langley and Sorkin 1989). However, its direct actions in the intracellular space are not known. Indeed, on the surface of reticular formation in neurons, different types of calcium channels are expressed (Hasan and Venkiteswaran 2010).

How do calcium channels and signaling modulate synaptic plasticity, spine formation, or how can they open plastic periods in adult brain? Understanding these mechanisms requires detailed information of plastic processes and broad-ranged integration of available data, thus the main aspects are discussed.

Homeostatic plasticity is partly regulated through calcium channels. Chronic inhibition of CaV.1 channels in the neurons increases the profusion of NMDA receptor, and intriguingly, decreases the abundance of inhibitory receptors in gabaergic synapses (Kilman et al. 2002; Swanwick et al. 2006; Saliba et al. 2007; Frank 2014). This is intuitive due to the fact that activity-dependent excitation and inhibition are essentially in balance for preventing immoderate silencing or generating epileptiformic activity. In this context, it is necessary to understand that TrkB activation modulates independently gabaergic transmission *via* IP_3 pathway by releasing calcium for intracellular stores (Tanaka et al. 1997). Furthermore, BDNF blocks the activity of deprivation-induced decrease in the gabaergic neurotransmission in interneurons, thus shifting the balance of the particular neural network to more inhibitory (Rutherford et al. 1997). Intriguingly, the maturation of inhibitory interneurons reactivates the juvenile-like plasticity by opening critical periods (Hensch 2005; Bavelier et al. 2010; Hensch and Bilimoria 2012). Acute fluoxetine and NDP treatment rapidly activates TrkB receptor and its downstream signaling leading to the increased release of intracellular calcium.

Intracellular calcium release from endoplasmic reticulum is an interesting step in the pathway. Pathway is suggested to control metaplasticity, which is a form of plasticity controlling the direction of plasticity in an integrative and temporal manner (Abraham and Bear 1996). Importantly, it seems that in controlling of the plastic changes, intracellular release of calcium is the critical step, not the total increase of intracellular calcium (Zheng et al. 2008; Maggio and Vlachos 2014). However, albeit NDP treatment decreases the extracellular calcium influx, NDP as well as

fluoxetine increases the release of intracellular calcium that is clearly dependent of phosphorylation of PLCγ1 and further activation IP_3-pathway. Additionally, PV neurons and VIP (vasoactive intestinal peptide) neurons importantly regulate the experience-dependent plasticity, however, the relations in extracellular and intracellular calcium signaling are not well understood (Donato et al. 2013).

Considering the fact that BDNF and its receptor TrkB regulate neuroprotection and neuroplastic changes in the healthy brain and under pathological conditions, it was an extremely interesting question whether NDP might also regulate TrkB receptor signaling. Intriguingly as stated, systemic administration of NDP induces the autophosphorylation of TrkB and the activation of downstream signaling that is seen in neuronal survival (Akt) and plasticity (CREB). Furthermore, the NDP-induced changes on TrkB phosphorylation closely resemble those previously seen after antidepressant drug treatment (Rantamäki et al. 2007; Di Lieto et al. 2012). Both NDP and antidepressant drugs specifically induce the phosphorylation of TrkB autocatalytic domain and the PLCγ1 phosphorylation status of the Shc site unaltered. Additionally, the levels of total BDNF mRNA and mature BDNF protein remained unaltered after acute NDP treatment. This observation is suggesting that NDP does not induce rapid changes in BDNF synthesis. Similarly, although a single antidepressant drug treatment is sufficient to activate TrkB (Rantamäki et al. 2007; Rantamäki et al. 2011), the levels of BDNF mRNA and BDNF protein are augmented only after weeks of continuous treatment (Nibuya et al. 1995).

Our findings indicate that common molecular mechanisms mediate TrkB activation after acute NDP and antidepressant drug treatments. Interestingly, conventional antidepressant drugs, such as fluoxetine, block L-type calcium channels and suppress intracellular calcium spikes (Deák et al. 2000; Kim et al. 2013). Moreover, L-type calcium channel antagonists facilitate antidepressant effects of conventional medication, and even show independent antidepressant effects in rodents that appear to be dependent on the CaV1.2 channel (Mogilnicka et al. 1987; Czyrak et al. 1989; Czyrak et al. 1990; Cohen et al. 1997; Dubovsky et al. 2001; Taragano et al. 2001; Sinnegger-Brauns et al. 2004; Taragano et al. 2005). Furthermore, although L-type calcium channel blockers have been shown to block activity-dependent BDNF synthesis, our recent data support a BDNF-independent mechanism underlying rapid antidepressant-induced TrkB activation. (Zafra et al. 1990; Poulsen et al. 2004; Rantamäki et al. 2011). In contrast to antidepressant drugs, however, NDP activates Akt, a major survival-promoting factor (Ahn 2014). The mechanisms behind the differential effects of NDP and antidepressants on Akt remain unknown, but these findings may suggest stronger neuroprotective properties for NDP than for antidepressant drugs (Zhao et al. 2006; Ahn 2014).

This molecular study provides good basis to investigate the role of TrkB behind the neuroprotective effects of NDP in translationally relevant animal models of brain trauma and compromised plasticity. Current findings also suggest that NDP may activate synaptic plasticity in a manner reminiscent to that induced by antidepressant drugs. Our previous findings demonstrate that long-term antidepressant treatment reactivates developmental-type of plasticity mechanisms in the adult brain, which allows the remodeling of synaptic connectivity if combined with appropriate rehabilitation (Maya Vetencourt et al. 2008; Karpova et al. 2011). In addition, sustained antidepressant treatment after ischemic stroke improves motor function when combined with physiotherapy (Chollet et al. 2011).

7.3. Limitations and strengths of the research

Significant limitation in the implant study was lack of aSAH modeling (Study I). However, studies with healthy animals provided important data of the new silica-based biodegradable implant. Especially the histological data indicated the feasibility of the implant material

that would be rather hard to interpret by using an aSAH model. The mortality after aSAH induction would have required significantly larger animal cohort and would have aroused ethical issues. Furthermore, dissolution of the silica-based implant in the CSF has never been described before. Consequently, dissolution of the implant during normal CSF dynamics was important to evaluate. Dissolution of the implant requires at least partial flow of the CSF. It is known that hydrocephalus after aSAH is compromising about 25% of patients (Milhorat 1987; Germanwala et al. 2010). Acute hydrocephalus is treated with temporal drainage or shunting recovering the CSF flow. Thus, dissolution of the implant is not inhibited. However, dissolution of the implant may be disturbed and this issue remains to be studied by using an aSAH model. The total number of treated animals was 18. Further evaluation of the safety of the implants requires a larger sample size. However, the study provided important experience for intracranial surgery of dog and pig models.

Pharmacokinetics of NDP was evaluated also with traditional oral administration in dogs (Study II). Thus, conclusions between implant and oral treatment modalities were able to be made accurately.

Effects of NDP on TrkB signaling were studied by using a healthy mouse model (Study III). We clearly demonstrate the antidepressant-like activation of TrkB and downstream signaling and additional activation of neuroprotective signaling after acute NDP treatment. BDNF protein and mRNA levels were quantitatively analyzed to confirm rapid TrkB activation independently of BDNF. We did not study effects of chronic NDP administration. As discussed above, the evidence of independent antidepressant action of NDP treatment is already described in the literature. However, effects of chronic NDP treatment on BDNF levels need to be studied. In a pathological point of view, the major restriction in our study was a lack of aSAH model. Consequently, direct conclusions about the effects of NDP on TrkB signaling or induced plasticity after aSAH cannot be made.

7.4. Future views

Silica-based formulation is a feasible candidate for an intracranial drug delivery system. However, further formulation of the implant to a gel is of interest since a rigid implant material is potentially hazardous and could cause compression of the brain parenchyma or even intracranial vessels. The treatment study needs to be performed with an aSAH model for further analysis of safety and efficacy. PLGA implants are already proven to be safe in human trials. However, the clinical use of PLGA nicardipine implants is extremely limited. It is interesting to see if implant or gel treatments will take its place in the treatment of DCI. In addition, since pathological mechanisms are extremely complex after aSAH, multitarget therapy with different drug combinations can be beneficial. Further studies are needed to determine how different combinations of drugs act in intracranial therapy. In addition, more studies are needed to understand the pharmacokinetics of intracranial NDP delivery systems especially after pathologically altered CSF kinetics, *e.g.* aSAH model.

Drug-induced plasticity is of great interest among neurorehabilitation researchers. Inducing plasticity by reopening sensitive periods and enhancing neuronal network remodeling including appropriate rehabilitation can open new doors for neurorehabilitation. Enabling the development of new effective treatment strategies requires diligent basic research and active translation in the clinical settings. Furthermore, as showed in our studies, rapid TrkB activation and downstream signaling after NDP treatment is reminiscent to the conventional antidepressant treatment whereby it may have beneficial effect in remodeling injured neural network. However, the effects of chronic NDP administration need to be studied. The questions by which mechanisms NDP or fluoxetine induce phosphorylation of TrkB are interesting ones and remain to be further

studied as well. Whether the therapeutic effects of NDP, in aSAH and other nervous system conditions that benefit from induced plasticity and neuroprotection, can be facilitated by more prolonged administration and active rehabilitation remains to be studied in different relevant animal models, e.g. aSAH model and compromised plasticity.

Lastly, our results arise one hypothetical question. May aSAH patients benefit from more prolonged administration of NDP than the traditional 21 days?

8. CONCLUSIONS

On the basis of presented pre-clinical experiments, the following conclusions can be made:

1. The new silica-based biodegradable implant was developed and surgical feasibility was successfully confirmed in pig and dog model (I).
2. *In vitro* dissolution modeling of the implants was a feasible method for predicting the dissolution properties *in vivo* (I). *In vivo* dissolution of the implants can be followed by using CT (I).
3. Silica-based nimodipine implant treatment does not induce any major histological foreign body reaction in the brain or meninges (I).
4. Higher nimodipine concentration in the CSF and lower systemic nimodipine concentration is achieved by using the implant treatment compared to the traditional oral nimodipine treatment (I and II).
5. Nimodipine rapidly activates BDNF receptor TrkB and downstream signaling reminiscent to antidepressants independently without the increase in BDNF protein and mRNA levels (III).
6. Nimodipine activates neuroprotective Akt signaling that differs from the actions of antidepressants (III)

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