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Nimodipine enhances neurite outgrowth in dopaminergic brain slice co-cultures

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ABSTRACT

Calcium ions (Ca^{2+}) play important roles in neuroplasticity and the regeneration of nerves. Intracellular Ca^{2+} concentrations are regulated by Ca^{2+} channels, among them L-type voltage-gated Ca^{2+} channels, which are inhibited by dihydropyridines like nimodipine. The purpose of this study was to investigate the effect of nimodipine on neurite growth during development and regeneration. As an appropriate model to study neurite growth, we chose organotypic brain slice co-cultures of the mesocortical dopaminergic projection system, consisting of the ventral tegmental area/substantia nigra and the prefrontal cortex from neonatal rat brains. Quantification of the density of the newly built neurites in the border region (region between the two cultivated slices) of the co-cultures revealed a growth promoting effect of nimodipine at concentrations of 0.1 μM and 1 μM that was even more pronounced than the effect of the growth factor NGF.

This beneficial effect was absent when 10 μM nimodipine were applied. Toxicological tests revealed that the application of nimodipine at this higher concentration slightly induced caspase 3 activation in the cortical part of the co-cultures, but did neither affect the amount of lactate dehydrogenase release or propidium iodide uptake nor the ratio of bax/bcl-2. Furthermore, the expression levels of different genes were quantified after nimodipine treatment. The expression of Ca^{2+} binding proteins, immediate early genes, glial fibrillary acidic protein, and myelin components did not change significantly after treatment, indicating that the regulation of their expression is not primarily involved in the observed nimodipine mediated neurite growth. In summary, this study revealed for the first time a neurite growth promoting effect of nimodipine in the mesocortical dopaminergic projection system that is highly dependent on the applied concentrations.

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Abbreviations: ANOVA, analysis of variance; Arc, activity-regulated cytoskeleton-associated protein; CBPs, Ca^{2+} binding proteins; DAB, 3,3′-diaminobenzidine hydrochloride; DIV, days *in vitro*; Egr, early growth response protein; FCS, fetal calf serum; Fos, proto-oncogene c-Fos; GFAP, glial fibrillary acidic protein; HS, horse serum; IEGs, immediate early genes; Junb, transcription factor jun-B; LDH, lactate dehydrogenase; LSM, laser scanning microscope; LVCC, L-type voltage-gated Ca^{2+} channels; Mal, myelin and lymphocyte protein; MAP2, microtubule associated protein-2; Mog, myelin oligodendrocyte glycoprotein; MrpL32, mitochondrial ribosomal protein L32; NB-A, Neurobasal-A; NGF, nerve growth factor; P, postnatal day; PFC, prefrontal cortex; PI, propidium iodide; Plp1, myelin proteolipid protein 1; Pvalb, parvalbumin; qPCR, quantitative reverse transcription polymerase chain reaction; SOM, self-organizing maps; TBS, tris buffered saline; Ubc, ubiquitin; VTA/SN, ventral tegmental area/substantia nigra.

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1. Introduction

Nimodipine is a blocker of the L-type voltage-gated Ca^{2+} channels (LVCC), which regulate the intracellular Ca^{2+} concentration. Calcium ions (Ca^{2+}) play an important role in neuronal plasticity (Gispen et al., 1988). Their intracellular concentration is crucial for the regulation of axonal and dendritic growth, guidance during neuronal development and for resprouting of axons after injury (for review see Gomez and Zheng, 2006). It has been shown that proper neuronal development proceeds when the intracellular Ca^{2+} is within an optimal range (Fields et al., 1993; Kater and Mills, 1991; Kater et al., 1988). Therefore, the modulation of Ca^{2+} channels such as the LVCC is of special interest, when neurite (re)growth is desired.

Being one of the examined LVCC blockers, the dihydropyridine derivate nimodipine has been intensively studied. Its beneficial effects have been confirmed in clinical studies (Liu et al., 2011, Scheller and Scheller, 2012) and in diverse experimental studies, where it has been applied to models of various disorders. These experimental studies, analyzing the effect of nimodipine (and other dihydropyridine LVCC blockers), focused on (i) the neuroprotective properties after different kinds of neuronal injury (Harkany et al., 2000; Krieglstein et al., 1996; Lecht et al., 2012; Li et al., 2009; Rami and Krieglstein, 1994; Weiss et al., 1994), for example by reducing the consequent intracellular free Ca^{2+} overload after *e.g.* ischemic injury and excitotoxic lesion (Kobayashi and Mori, 1998) and (ii) the neurite growth promoting characteristics, mainly investigated in the peripheral nervous system (Angelov et al., 1996; Lindsay et al., 2010; Mattsson et al., 2001).

LVCC are expressed in a couple of brain regions of the central nervous system, among them the mesencephalon (Mercuri et al., 1994; Nedergaard et al., 1993; Takada et al., 2001), the cortex, and the hippocampus (Dolmetsch et al., 2001; Quirion et al., 1985; Tang et al., 2003). In the cortex, LVCC are located on the entire postnatal neuron including dendrites and axonal processes (Tang et al., 2003).

Although the beneficial outcomes of LVCC modulation by nimodipine are well established, the exact cellular and molecular mechanisms by which this modulation occurs are still unclear. Nevertheless, results of some previously published studies may in part explain how neurite growth after nimodipine treatment is accomplished. An upregulation of Ca^{2+} binding proteins (CBPs, parvalbumin (Pvalb), S100b, calbindin) has been observed in cortical neurons after nimodipine administration (Buwalda et al., 1994; Luiten et al., 1994). Furthermore, the expression of the glial fibrillary acidic protein (GFAP) was enhanced following long-term nimodipine treatment (Guntinas-Lichius et al., 1997). An increase in myelination was found surrounding the recovering neurons after unilateral facial crush injury and interestingly also around neurons located in the contralateral nonlesioned site in nimodipine treated animals (Mattsson et al., 2001). Moreover, microglial-mediated oxidative stress and inflammatory response was attenuated by nimodipine (but not by other LVCC blockers) in dopaminergic neurons/microglial co-cultures (Li et al., 2009).

In this study, we wanted to investigate whether nimodipine directly influences neurite growth. We further aimed to elucidate which genes are potentially involved in the nimodipine mediated growth effects. To address these questions, we determined the neurite density in the border region of organotypic co-cultures of the mesocortical dopaminergic system, which culture together brain slices of the ventral tegmental area/substantia nigra (VTA/SN) and prefrontal cortex (PFC) (Heine et al., 2007; Heine and Franke, 2014), and analyzed gene expression patterns of nimodipine treated samples and control samples.

Our findings indicate that nimodipine, at appropriate dosages, is a promising substance to enhance neurite outgrowth as shown in the applied co-culture model of the dopaminergic (CNS)-projection system.

2. Experimental procedures

2.1. Materials

The solvent ethanol was purchased from AppliChem GmbH (Darmstadt, Germany) and VWR International (Dresden, Germany), respectively. Glutamate, nerve growth factor (NGF), and staurosporine were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Nimodipine (pure substance) was a gift from Bayer Vital GmbH (Leverkusen, Germany). 1000-fold stock solutions of nimodipine were prepared in absolute ethanol and finally added to 1 ml of incubation medium (resulting ethanol concentration 0.1%, details see Section 2.3). Untreated cultures ("untreated") or cultures treated with the vehicle ethanol ("ethanol", resulting end concentration 0.1%) were used as control groups.

2.2. Animals

Neonatal rat pups (WISTAR RjHan, own breed; animal house of the Rudolf Boehm Institute of Pharmacology and Toxicology, University of Leipzig) of postnatal day 1–4 (P1-4) were used for the preparation of the organotypic slice co-cultures. The animals were housed under standard laboratory conditions of 12 h light–12 h dark cycle and allowed free access to lab food and water *ad libitum*.

All of the animal use procedures were approved by the Committee of Animal Care and Use of the relevant local governmental body in accordance with the law of experimental animal protection. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.3. Preparation, culture and treatment of slice co-cultures

Dopaminergic brain slice co-cultures were prepared from P1-4 rats and cultured according to the "static" culture protocol described previously (Heine et al., 2007; Heine and Franke, 2014). In brief, coronal sections (300 μm) were cut at mesencephalic and forebrain levels using a vibratome (Leica, Typ VT 1200S, Nussloch, Germany). For details see also supplementary Fig. S1. After separation of VTA/SN and PFC, respectively, the slices were transferred into petri dishes, filled with cold (4 °C) preparation solution (Minimum Essential Medium (MEM) supplemented with glutamine (final concentration 2 mM) and the antibiotic gentamicin (50 μg/ml); all from Invitrogen GmbH, Darmstadt, Germany). Thereafter, the selected sections were placed as co-cultures (VTA/SN + PFC, 4 co-cultures per well) on moistened membrane inserts (0.4 μm, Millicell-CM, Millipore, Bedford, MA, USA) in 6-well plates (Fig. S1.A3), each filled with 1 ml incubation medium (50% Minimal Essential Medium, 25% Hank's Balanced Salt Solution, 25% heat inactivated horse serum (HS), 2 mM glutamine and 50 μg/ml gentamicin; all from Invitrogen GmbH supplemented with 0.044% sodium bicarbonate, Sigma-Aldrich; pH was adjusted to 7.2), referred to as "25% HS incubation medium".

Supplementary Fig. S1 can be found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2014.10.005.

The preliminary gene expression studies (microarray analysis, quantitative reverse transcription polymerase chain reaction, qPCR) have been conducted after culture under serum-free culture conditions. Therefore, after 4 days *in vitro* (DIV) the 25% HS incubation medium was replaced by serum-free medium (Neurobasal-A (NB-A) medium, 1 mM glutamine supplemented with 2% B27 and 50 μg/ml gentamicin; all from Invitrogen GmbH), referred to as "NB-A incubation medium". Nimodipine (final concentration 10 μM) or the vehicle ethanol (0.1%), respectively were added to the incubation medium at DIV4, 6, 8 and 11.

To determine the concentration of nimodipine that would be appropriate, we first analyzed previous published studies and the therein used concentrations (10 μM: Blanc et al., 1998; Krieglstein et al., 1996; Lecht et al., 2012; Tang et al., 2003; 20 μM: Martínez-Sánchez et al., 2004; Pisani et al., 1998; Pozzo-Miller et al., 1999; 50 μM: Bartrup and Stone, 1990, toxic at higher concentrations: Turner et al., 2007). Second, we quantified nimodipine uptake into the co-cultures with applied nimodipine concentrations ranging from 10 μM to 40 μM in a preliminary experiment. We found the lowest dose applied (10 μM nimodipine in the culture medium) yielded a sufficiently high concentration in the tissue (unpublished results). Therefore, the following studies were at first conducted with 10 μM nimodipine.

The analysis of neurite growth in the organotypic brain slice co-cultures is a well-established technique in our lab (Heine et al., 2013; Heine et al., 2007). Therefore, the neurite growth analysis has been performed after culture in 25% HS incubation medium. For the neurite growth quantification, slice co-cultures were divided into different experimental groups and were treated with nimodipine at different concentrations (first 10 μM, later 0.1 μM and 1 μM) or the vehicle control ethanol. A second control group was left untreated. Nimodipine and ethanol were added directly after the preparation and at each medium change at DIV1,4,6 and 8 (Fig. 1). In a second study the effect of 1 μM nimodipine alone or in combination with 50 ng/ml NGF (final concentration) was compared to 50 ng/ml NGF alone. These culture conditions (25% HS incubation medium, substance application five times as indicated in Fig. 1) were also applied for toxicity tests and later gene expression studies. In addition, tissue slices of the nimodipine treated groups were dissected

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