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Study of the effects of docosahexaenoic acid (DHA) and a structured phospholipid containing DHA on physiological and pathological conditions of neurogenesis *in vitro*

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DOCTORAL DISSERTATION

STUDY OF THE EFFECTS OF DOCOSAHEXAENOIC ACID (DHA) AND A STRUCTURED PHOSPHOLIPID CONTAINING DHA ON PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS OF NEUROGENESIS *IN VITRO*

生理的ならびに卒中条件における神経新生に対する
ドコサヘキサエン酸（DHA）およびそのリゾリン脂質体の影響

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ABSTRACT

Docosahexaenoic acid (DHA, 22:6n-3) is an essential omega-3 polyunsaturated fatty acid (PUFA). It is specifically enriched in the brain and the retina and it is required for visual acuity, proper brain development and cerebral functions. While DHA deficiency in the brain was shown to be linked to the emergence of cerebral diseases (i.e. Alzheimer's disease or Parkinson's disease), studies showed that a dietary intake of omega-3 PUFA could prevent or attenuate neurologic disturbances linked with ageing or neurodegenerative diseases. In this context, it is primary to deliver DHA efficiently to the brain. Targeting the brain with DHA might offer great promise in developing new therapeutics for neurodegenerative diseases. The French host laboratory previously synthesized a stabilized form of lysophosphatidylcholine-DHA, which is main vector of DHA transportation to the brain, of structure 1-acetyl,2-docoshexaenoyl-glycerophosphocholine, patented and named AceDoPC®. Injection of AceDoPC or DHA after experimental ischemic stroke showed that both molecules also had neuroprotective effects. These potential neuroprotective effects are expected to be due, in part, to DHA conversion into oxygenated metabolites. This study aims to investigate the beneficial effects of DHA and its derived metabolites either unesterified or esterified within structured phospholipids on a model of neurogenesis *in vitro* under physiological or pathological conditions.

The first objective of this work was then to synthesize the DHA-containing structured phospholipid AceDoPC®, DHA oxygenated derivative protectin DX (PDX) and a novel structured phospholipid of protectin: 1-acetyl,2-protectinDX-glycerophosphocholine (AceDoxyPC). The second objective was to investigate the effects of DHA, AceDoPC and PDX on neurogenesis using an *in vitro* model of neurogenesis, namely cultures of neural stem progenitor cells (NSPCs) derived from the adult mouse brain under physiological or pathological conditions (ischemic conditions). Following this, the third objective of this work was to identify the mechanisms involved in such response to stress induced under pathological conditions.

Synthesis of the novel structured phospholipid AceDoxyPC was successfully performed by double enzymatic lipoxygenation of AceDoPC and identification of the product was possible using advanced techniques of liquid chromatography (LC)/electrospray ionization (/ESI)/mass spectrometry (/MS). Future studies on this potential neuroprotective molecule transporter are to be investigated in the near future. Neurogenesis study of cell cultures with AceDoPC showed enhanced neurogenesis compared to addition of unesterified DHA or vehicle control, especially under pathological conditions. Preliminary studies of the potential mechanisms involved in neuroprotection hinted that AceDoPC neuroprotective and regenerative effects might be due in part to its anti-oxidative effects.

Keywords: docosahexaenoic acid, 1-acetyl,2-docoshexaenoyl-glycerophosphocholine, protectin, phospholipid, neuroprotection, neurogenesis, stroke, oxidation, brain

RESUME

L'acide docosahexaénoïque (DHA, 22:6n-3) est un acide gras polyinsaturé (AGPI) oméga-3. Il est particulièrement abondant dans le cerveau et la rétine et est nécessaire pour l'acuité visuelle, le bon développement du cerveau et les fonctions cérébrales. Tandis qu'une déficience en DHA a été montrée être liée à l'émergence de maladies cérébrales (telles que la maladie d'Alzheimer ou la maladie de Parkinson), des études ont également montré qu'un apport alimentaire en AGPI oméga-3 pouvait empêcher ou atténuer les perturbations neurologiques liées au vieillissement ou aux maladies neurodégénératives. Dans ce contexte, il est primordial de transporter efficacement le DHA au cerveau. Cibler le cerveau avec du DHA pourrait offrir de nouvelles possibilités de développement de thérapies contre les maladies neurodégénératives. Le laboratoire français a synthétisé auparavant une forme stabilisée de la lysophosphatidylcholine-DHA, qui est le vecteur principal d'apport de DHA au cerveau, de structure 1-acétyl,2-docosahexaénol-glycérophosphocholine, brevetée et nommée AceDoPC®. L'injection d'AceDoPC ou de DHA après un accident vasculaire cérébral ischémique provoqué expérimentalement a montré que ces deux molécules avaient des effets neuroprotecteurs. Ces effets sont supposés être dus en partie à la conversion du DHA en métabolites oxygénés. Notre étude vise à examiner les effets du DHA et de ses métabolites dérivés sous forme non estérifiés ou estérifiés dans des phospholipides structurés sur un modèle de neurogenèse *in vitro* en conditions physiologiques ou pathologiques.

Le premier objectif de ce travail a été de synthétiser le phospholipide structuré contenant du DHA, l'AceDoPC®, le dérivé oxygéné du DHA, la protectine DX, et un nouveau phospholipide structuré contenant la protectine: 1-acétyl,2-protectine DX-glycérophosphocholine (AceDoxyPC). Le second objectif a consisté à étudier les effets du DHA, de l'AceDoPC et de la PDX sur la neurogenèse en utilisant un modèle *in vitro* de neurogenèse, constitué de cultures de cellules souches progénitrices neurales (NSPCs) dérivées de cerveaux de souris adultes, dans des conditions physiologiques ou pathologiques (conditions ischémiques). Enfin, le troisième objectif de cette thèse a été d'identifier les mécanismes impliqués dans la réponse des cellules au stress sous conditions ischémiques.

La synthèse du nouveau phospholipide structuré AceDoxyPC a été réalisée avec succès par une double lipoxygénation enzymatique de l'AceDoPC, et l'identification du produit a été possible grâce à l'utilisation de techniques avancées de chromatographie liquide couplée à la spectrométrie de masse par ionisation par électronébuliseur (LC/ESI/MS). De futures études sur ce transporteur de molécule neuroprotectrice potentielle doivent être réalisées prochainement. Les cellules incubées en présence d'AceDoPC présentent une augmentation de neurogenèse comparativement à celles cultivées avec addition de DHA non estérifié ou du véhicule contrôle, notamment sous conditions pathologiques. Les études préliminaires des mécanismes potentiellement impliqués dans la neuroprotection indiquent que les effets neuroprotecteurs et régénératifs de l'AceDoPC pourraient être en partie dus à des effets anti-oxydants.

Mots-clés: acide docosahexaénoïque, 1-acétyl,2-docosahexaénol-glycérophosphocholine, protectine, phospholipide, neuroprotection, neurogenèse, accident vasculaire cérébral, oxydation, cerveau

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FOREWORD

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A. Lo Van, N. Sakayori, N. Osumi, and N. Bernoud-Hubac. Brain targeting with phospholipids containing docosahexaenoic acid (DHA) and its derived mediators. *ELyT Workshop 2015* (07/02/2015, Sendai, JP).

INTRODUCTION

On the importance of docosahexaenoic acid for human brain

Docosahexaenoic acid (DHA, 22:6n-3) is an omega-3 (ω -3) polyunsaturated fatty acid (PUFA) essential for human brain development, function and visual acuity (Birch et al., 2000, 1992; Bourre et al., 1989; Innis and Friesen, 2008). It is specifically enriched in the brain and the retina (Bazan and Scott, 1990; Breckenridge et al., 1972). PUFA were shown to exhibit many health benefits in a great variety of diseases such as cardiovascular or neurodegenerative diseases (Chauveau et al., 2011; Colussi et al., 2014; Hashimoto et al., 2009; Thies et al., 2003). Recent studies showed that dietary intake of omega-3 PUFAs could prevent cerebral physiological disturbances due to aging or neurodegenerative diseases (Belayev et al., 2009, 2005; Gao et al., 2007; Hong et al., 2014; Peet and Stokes, 2005; Young and Conquer, 2005). Although being an essential fatty acid, DHA is only scarcely synthesized *de novo* in our metabolism, approximately 1 to 2% from alpha linolenic acid (Brenna et al., 2009). It is mainly incorporated into our metabolism through dietary intake (Sinclair, 1975).

Deficiency in DHA level in the brain was shown to be linked with the emergence of neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease (Calon and Cole, 2007). Efficient delivery of DHA into the brain is therefore necessary to prevent brain dysfunction. This is a particularly important topic for Western countries where the dietary intake of omega-3 is greatly overshadowed by intake of omega-6 PUFAs (Simopoulos, 2002). One should note that omega-6 PUFAs are strong competitors against omega-3 PUFAs for the conversion of DHA from precursors (Smink et al., 2012) and to derived active mediators.

Introduction of stabilized brain targeting vector containing DHA

Several groups evidenced the preferential form of physiological transport of DHA through the blood-brain-barrier (BBB) to be lysophosphatidylcholine-DHA (lysoPC-DHA) compared with its non-esterified form (Lagarde et al., 2001; Nguyen et al., 2014). The French host laboratory has previously shown in rat and in an *in vitro* model of the BBB that lysoPC with DHA esterified at the *sn*-2 position is a privileged form of transport of PUFAs, especially DHA, to the brain (Thies et al., 1994). This preferential uptake of lysoPC-DHA was specific to the brain as it was not observed for other organs. DHA migrates easily from the *sn*-2 position of lysoPC, which could be considered as the physiological form of polyunsaturated lysoPC since DHA is stored in tissues at the *sn*-2 position, to the *sn*-1 position, which is more stable (Croset et al., 2000). Therefore to prevent the migration of DHA, the French host laboratory synthesized a molecule called 1-acetyl,2-docosahexaenoyl-glycerophosphocholine (AceDoPC; patented; Hachem et al., 2016; Lagarde et al., 2015), which retains the main physico-chemical properties of the physiological carrier. Thus, AceDoPC is a stabilized form of lysoPC-DHA.

DHA effects on brain functions and brain neuroprotection were covered by numerous studies. Notably, one collaboration between the French host laboratory and another group investigated the effects of AceDoPC and unesterified DHA injection after the induction of focal cerebral ischemia *in vivo* in rats (Chauveau et al., 2011). We showed that DHA and AceDoPC could lessen the lesion size due to the ischemia, 24 hours after its induction, compared to control condition. Moreover, the decrease in lesion size was more important when DHA was esterified within AceDoPC rather than unesterified. Lipid peroxidation was also decreased in the treated group. An *in vitro* model of cerebral ischemia named oxygen/glucose deprivation (OGD) was first established by Goldberg and Choi in 1993. The authors observed that murine neocortical cells cultured in hypoxic conditions and in a medium deprived of glucose triggered rapid neuronal death through mechanisms similar to ischemia-induced pathogenic mechanisms (i.e. extracellular calcium and glutamate increases). Comparable neuroprotection of DHA was shown in neuronal cell cultures subjected to OGD (Zhang et al., 2014). One mechanism involved is the activation of nuclear factor E2-related factor 2 (Nrf2) by DHA, allowing for the upregulation of hemeoxygenase-1 (HO-1), active anti-oxidant enzyme (Panahian et al., 1999). Other studies also confirmed this finding (Chang et al., 2013; Ishikado et al., 2013; Q. Liu et al., 2014). Other potential neuroprotective mechanisms include anti-inflammatory and anti-apoptotic properties of DHA (Lo Van et al., 2016).

On the potential of protectins, DHA derivatives

DHA neuroprotective effect is expected to be due in part to its conversion into active oxygenated derived mediators. Being highly unsaturated, DHA has the potential to be oxygenated by various lipoxygenases to produce diverse oxylipins that regulate several biological processes within the brain. Although being scarcely identified *in vivo* in the brain, these mediators are mainly produced by lipoxygenase action and include hydroxylated DHA (HDHA), and some of them can be metabolized into potent mediators, such as protectin D1 (PD1), resolvins and maresins (Bannenberg and Serhan, 2010; Spite et al., 2014). One isomer of PD1 was identified by our group and named protectin DX (PDX, Chen et al., 2009).

PD1 is also named neuroprotectinD1 (NPD1) when it is found in neural tissue. The name protectin comes from the potent neuroprotective properties of such molecules. It was found to be present in human blood, mouse brain and glial cells (Hong et al., 2003). PD1 exhibited anti-inflammatory, anti-apoptotic and anti-oxidative effects in pathological model such as Alzheimer's disease model (Lukiw et al., 2005), ischemic renal injury (Hassan and Gronert, 2009), oxidative stress induced in human retinal pigment epithelial cells (Mukherjee et al., 2004), and brain ischemia-reperfusion (Marcheselli et al., 2003). Diverse neuroprotective effects of PD1 were reported in numerous reviews (Bazan et al., 2005; Levy, 2010; Serhan et al., 2006).

Stereoisomer PDX also exhibited anti-inflammatory and anti-aggregatory properties, notably by inhibiting cyclooxygenase, which are responsible for the formation of pro-

inflammatory derivatives from arachidonic acid (Chen et al., 2011; M. Liu et al., 2014). PDX also showed beneficial effects in the regulation of adipogenesis (White et al., 2015) and in glycoregulation in a model of obese diabetic mice (White et al., 2014). PDX reduced endoplasmic reticulum stress in astrocytes following *in vitro* ischemia (Begum et al., 2012). PDX also exhibited effects on virus such as reduction of Influenza virus replication in mice (Morita et al., 2013).

DHA influence on neurogenesis

The Japanese host laboratory highlighted the beneficial effect of DHA on neurogenesis of embryonic neural stem/progenitor cells (NSPCs) (Sakayori et al., 2011) and another group also observed similar results (Katakura et al., 2009). Such neurogenesis-promoting effects were also shown in both *in vitro* and *in vivo* models, i.e., in cultures of NSPCs from embryonic rats and in the hippocampus of eighteen-month-old rats, respectively (Kawakita et al., 2006). In adult mammalian brains, neurogenesis mainly occurs in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus (Ming and Song, 2011). Adult neurogenesis may play a role in regeneration of diseased brain as well as in learning and memory (Shivraj Sohur et al., 2006). Experiments on transgenic fat-1 mice, which are able to convert omega-3 fatty acids from omega-6 fatty acids and thus having elevated levels of omega-3 fatty acids in their tissues, showed that high brain DHA levels increase neurogenesis and improve spatial learning (He et al., 2009).

The proposed pathway for action of DHA on neurogenesis includes decreased expression of Hes1 (hairy and enhancer of split 1, a basic helix-loop-helix transcription factor), inducing an elevation of p27kip1, a cyclin-dependent kinase inhibitor (Katakura et al., 2009). p27kip1 was shown to provoke cell cycle arrest, shifting the cellular states from proliferation to differentiation (Dyer and Cepko, 2001; Kawauchi et al., 2013; Legrier et al., 2001). NeuroD (helix-loop-helix transcription factor) and MAP2 (microtubule-associated protein 2) expression levels were also increased with the addition of DHA, hinting at the involvement of protein kinase C-dependent mechanisms in neurogenesis induction with DHA. Another candidate pathway is the activation of G-protein coupled receptor 40 by DHA (Ma et al., 2010).

These results are of particular interest since one therapeutic approach to cerebral diseases is to promote neurogenesis to counteract neuronal loss (i.e. in stroke or Alzheimer's disease among others). These results could hint at a double effect of DHA on neurodegenerative diseases: (1) DHA exhibits neuroprotective effects against damage induced during cerebral ischemia for instance; and (2) DHA improves recovery of cognitive functions through an increase of neurogenesis to repair damaged region of the brain. Such effects are expected to be due in part to DHA lipid mediators (Bazan et al., 2012; Begum et al., 2012; Marcheselli et al., 2003).

Objectives of thesis and result overview

Considering above-mentioned findings, it is thus relevant to evaluate the potential of DHA and derived mediator PDX, either unesterified or esterified within structured phospholipids (respectively AceDoPC and AceDoxyPC), as a prevention (i.e.: neuroprotective agent) and/or treatment (i.e.: activation of neuronal production) to neurodegenerative diseases such as stroke. These molecules are potentially efficient for therapeutics against such cerebral pathologic conditions, as they were shown to elicit interesting properties such as anti-inflammatory, anti-oxidative and anti-apoptotic properties. Investigating their effects on *in vitro* models would allow us to evaluate said potentiality and to identify mechanisms involved in the neuroprotective response of DHA, PDX and structured phospholipids AceDoPC and AceDoxyPC.

Therefore, our first objective was to synthesize brain-targeting vectors of DHA or active oxygenated mediator PDX, namely structured phospholipid AceDoPC and another phospholipid named AceDoxyPC for 1-acetyl,2-PDX-glycerophosphocholine (Lo Van et al., submitted). To this intent, we used synthesis techniques from the French host laboratory to produce AceDoPC. AceDoxyPC was then synthesized with a protocol similar to PDX synthesis, using a double enzymatic lipoxygenation on AceDoPC. Reaction products were analyzed and identified using liquid chromatography electrospray ionization coupled with mass spectrometry (LC/ESI/MS).

The second objective was to study effects of DHA, AceDoPC and PDX on NSPCs derived from the adult mouse brain according to the protocol used in the Japanese host laboratory as a model of adult neurogenesis *in vitro* (Lo Van et al., to be submitted). To relate this model to conditions of stroke *in vitro*, we cultured adult NSPCs under both physiological and pathological conditions, using OGD as a model of ischemia *in vitro*. We evaluated proliferation, neurogenesis and astrogenesis rates of the cell cultures through immunocytochemistry and fluorescence microscopy.

Finally, we further studied mechanisms involved in neuroprotection by focusing on markers of oxidative stress and inflammation.

In this study, we successfully synthesized and identified the protectin-containing structured phospholipid AceDoxyPC from AceDoPC. This novel molecule contains the potent neuroprotective PDX and also combines the structure of the stabilized brain targeting vector AceDoPC. Therefore, we believe that further experiments on the potential use of AceDoxyPC against neurodegenerative diseases might be of utmost interest. Moreover, we showed that AceDoPC enhanced neurogenesis of adult NSPCs, especially when the cells were subjected to pathological conditions of culture. The effect of AceDoPC on neurogenesis was even higher than the effect of unesterified DHA. These results provide us further reasons to consider the use of DHA derived compounds or containing phospholipids as neuroprotective agents or treatments to neurodegenerative diseases.

This work was conducted as a double-thesis between INSA Lyon and Tohoku University Graduate School of Medicine, within the context of the international associated laboratory ELyT Lab (CNRS). Experiments were done in CarMeN Laboratory (Lyon, France) and the Department of Developmental Neuroscience, Center for Neuroscience and Translational Research (Sendai, Japan).

This report is committed to personal work and is composed of three distinctive parts. First part is the Materials and methods. The second one is the Results and discussions, divided into two chapters: **1) Synthesis of AceDoxyPC from AceDoPC and identification using LC/ESI/MS;** and **2) Study of the neuroprotective effects of DHA, AceDoPC and PDX and adult neurogenesis.** The final part is the Conclusion and perspectives.

MATERIALS AND METHODS

I. MATERIALS

Biochemical synthesis and analysis

DHA was purchased from Sigma Chemical (St.Louis, MO, U.S.A.). Sn-glycerophosphocholine (GPC) was from Bachem (Budendorf, Switzerland). Analytical grade chloroform, methanol, dimethylformamide (DMF) as well as 4-(dimethylamino)pyridine (DMAP), 1,3-dicyclohexylcarbodiimide (DCC), immobilized enzyme *Candida antarctica* (Novozym 435), soybean lipoxygenase (sLOX, EC 1.13.11.12, Type 1-B, 205,716 units/mg solid) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All chemicals used were reagent grade or with the highest quality available and purchased from CARLO ERBA Reagents (Val-de-Reuil, FR). Analysis was carried out using high performance liquid chromatography (HPLC) system coupled to a triple quadrupole mass spectrometer (AB SCIEX QTRAP® 4500 system).

Cell culture

Animal experiments were performed in both France and Japan. All mice were either maintained at Tohoku University School of Medicine (Japan) or at INSA Lyon (France) in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and with the European Communities Council Guidelines for the Care and Use of Laboratory Animals (22 September 2010, 2010/63/EU) in conformity with the Public Health Service (PHS) Policy on Human Care. All animal experiments were approved by the Tohoku University's committee for animal experiments (MED#2013-114) and carried out in compliance with French Ministry of Agriculture guidelines (n° 87–848).

NSPCs were obtained from brains of C57BL/6 wild-type (WT) mice (Clea Japan, Tokyo, Japan; Envigo, Gannat, France).

Materials used for cell culture are listed in Table 1.

Incubators used were from Heraeus (Hanau, Germany), Thermo Fisher Scientific and Astec (Fukuoka, Japan). LC/ESI/MS was performed on AB SCIEX QTRAP® 4500 system. Spectrometer used was from Shimadzu.

II. BIOCHEMICALS SYNTHESIS

Preparation of AceDoPC

AceDoPC was synthesized as previously described (Hachem et al., 2016). Briefly, 1-acetyl-GPC was formed by acetylation of *sn*-glycerophosphocholine. GPC (1 eq) dissolved in water (20 eq) was added to immobilized enzyme *Candida antarctica* (5 eq), vinyl acetate (40 eq), and 50 % (v/v) *t*-butanol/substrate solution. After stirring the mixture for 52 h, the lipase was filtered off and the filtrate was evaporated. An uncolored oil was obtained by addition of diethyl ether. After freeze-drying overnight, 1-acetyl-GPC (1 eq) was solubilized with anhydrous DMF and incubated with DCC (2 eq), DMAP (1 eq) and DHA (2 eq) and the mixture was incubated for 52 h at room temperature in anhydrous chloroform under N₂. AceDoPC was extracted using a C18 solid-phase cartridge. Purity was checked by thin-layer chromatography (TLC) on silica gel G (Merck, Darmstadt) using chloroform/methanol/water (65:25:4 vol/vol/vol). AceDoPC synthesis protocol is illustrated on Figure 1.

Preparation of PDX

PDX synthesis was done as previously described (Chen et al., 2009). Briefly, DHA (100 µM) was incubated with sLOX type 1B (40,000 U/mL) in 0.02 M sodium borate, pH 9.0, for 1 h on ice. Hydroperoxides were reduced by NaBH₄ for 15 min. pH was adjusted to 3 with glacial acetic acid. Hydroxylated fatty acids were extracted on a C18 solid-phase and dried under nitrogen. Hydroxylated products were analyzed by reverse phase high performance liquid chromatography (RP-HPLC) on a Waters XBridge C18 column (4.6 x 250 mm, 3.5 µm). Solvents used were acetonitrile/water (10:90, v/v, pH3) and 100% acetonitrile. PDX was detected using a diode array detector set at 270 nm and collected to be stored at -20°C until use.

Preparation of AceDoxyPC

AceDoxyPC synthesis (Figure 2) was inspired from PDX synthesis. AceDoxyPC was synthesized from AceDoPC by a sLOX type 1B treatment. AceDoPC (100 µM) was incubated with sLOX (5,000 U/ml) in 0.05 M sodium borate, pH 9.0, for 1 h on ice. The reaction products were subjected to sodium borohydride reduction for 15 min. Lipids were extracted on a C18 solid-phase cartridge and stored at -20°C until analysis.

Analysis of AceDoPC and AceDoxyPC by LC/ESI/MS/MS

AceDoPC and AceDoxyPC were analyzed by LC/ESI/MS/MS in the negative ion mode using a Waters 2.1 × 150 mm C18 column and a water/methanol gradient containing 0.1% acetic acid at 0.5 ml/min. The HPLC system was coupled to a triple quadrupole mass

spectrometer (AB SCIEX QTRAP® 4500 system). The auxiliary gas pressure was 20 p.s.i., and the sheath gas pressure was 40 p.s.i. The capillary voltage was set to -4500 V. The capillary temperature was 250 °C.

III. CELL CULTURE

NSPC extraction and maintenance

NSPCs were obtained by primary culture with a protocol described previously with some modifications (Reynolds et al., 1992). Wild-type mice (aged of 8 to 10 weeks old) were sacrificed by cervical dislocation and were decapitated. The brain was removed, and the subventricular zone of the wild-type mouse brain were dissected and collected into an artificial cerebral spinal fluid (aCSF) containing: pure water, NaCl (0.12 M), KCl (5 mM), MgCl₂ (3 mM), CaCl₂ (1 mM), NaHCO₃ (2.19 mg/mL), D-glucose (1.79 mg/mL), penicillin/streptomycin (50 U/mL), trypsin (1.33 mg/mL), hyaluronidase (0.67 mg/mL) and kynurenic acid (0.1 mg/mL). Tissues were incubated at 37°C for 30 minutes under shaking motion. They were collected and dissociated mechanically into media hormone mixture (MHM) composed as follows: DMEM-F12 (1:1), 0.6% D-glucose, L-glutamine (2 mM), HEPES buffer (5 mM), insulin (25 µg/mL), apo-transferrin (100 µg/mL), progesterone (20 nM), putrescine (60 µM), and selenite (30 nM). Additional bFGF (10 ng/mL), EGF (20 ng/mL), and heparin (2 µg/mL) were added to MHM resulting in MHM containing growth factors (MHM_{w/GF}). Dissociated cells were filtrated with 40 µm nylon mesh. Filtrated cells were seeded in uncoated petri dishes filled with MHM_{w/GF} (2.6×10^3 cells/cm²) and maintained in a humidified incubator at 37°C with 95% atmospheric air and 5% CO₂.

Cells proliferated for 7 days *in vitro* (DIV) and formed neurospheres (clusters of NSPCs). The neurospheres were collected and dissociated with a mixture of 0.25% trypsin-EDTA/HBSS (1:1) containing recombinant DNase 1 (4.17 U/mL) activated by two successive incubations at 37°C and mechanical dissociation. Dissociated NSPCs were plated in monolayers onto poly-L-ornithine- and laminin-coated wells at density 3.5×10^4 cells/cm² with MHM_{w/GF} and incubated in a humidified incubator at 37°C with 95% atmospheric air and 5% CO₂ for further proliferation or differentiation assays.

Proliferation assays

DHA, AceDoPC or PDX were dissolved in a vehicle containing fatty acid-free BSA (10 g/L), 0.1% ethanol and α-tocopherol (70 µM). DHA, AceDoPC or PDX solutions were incubated at 37°C for 30 min. 2 hours after monolayer plating, DHA, AceDoPC or PDX were added to the culture medium. The cells proliferated for 1 DIV after plating before fixing. 4 hours before fixing the cells, BrdU was added to the medium (10 µM).

Differentiation assays

DHA, AceDoPC or PDX solutions were prepared as described in the proliferation assays. After monolayer plating, the cells were cultured for 1 DIV. After 1 DIV, the culture medium was changed to MHM to induce differentiation of the cells. DHA, AceDoPC or PDX

were added during medium change. The cells differentiated for 4 DIV after medium change before fixing.

***In vitro* hypoxia/ischemia**

To mimic pathological conditions *in vitro*, we chose to apply OGD to induce hypoxia/ischemia to the cells. Upon addition of DHA, AceDoPC or PDX in either the proliferation or differentiation assays, the medium was changed to PBS with growth factor (bFGF, EGF and heparin, similarly to MHM_{w/GF} for proliferation assays) or without (differentiation assays). The monolayer plated cells in PBS were put in a hypoxic incubator with gas composition of 1% O₂, 5% CO₂, 94% N₂ for 30 min. After inducing OGD, the cell medium was changed back into MHM_{w/GF} (proliferation assays) or MHM (differentiation assays). The OGD assays then followed the previous protocols before fixing.

NSPC culture protocol is illustrated on Figure 3.

Immunocytochemistry

Fixing and following immunocytochemistry was performed as described previously (Sakayori et al., 2016). All steps were performed at room temperature unless stated otherwise. After either proliferation or differentiation assays, cells were fixed with 4% PFA for 10 min or 30 min respectively. Fixed cells were rinsed with PBS. Proliferation assays require additional steps of incubation with HCl (2N in pure water) for 5 min and PBS wash. Fixed cells were permeabilized with 0.3% TritonX-100/PBS for 5 min. After rinsing with PBS, cells were incubated for 1 h with a solution of 3% BSA/0.05% TritonX-100/PBS (named blocking buffer). Primary antibodies were added to the blocking buffer and incubated with the cells overnight at 4°C:

- Proliferation : mouse anti-BrdU IgG (1:50) for evaluation of proliferation rates
- Differentiation : mouse anti-Tuj1 IgG (1:1000) and rabbit anti-GFAP IgG (1:1000) for evaluation of neurogenesis and astrogenesis rates or anti-Nrf2 (1:1000) for evaluation of anti-oxidative effect

After PBS wash, secondary antibodies and DAPI (1:1000) were incubated with the cells in blocking buffer for 1 h:

- Proliferation : Alexa 488-conjugated goat anti-mouse (1:400)
- Differentiation : Alexa 488-conjugated goat anti-mouse IgG (1:400) and Cy3-conjugated donkey anti-rabbit IgG (1:400)

Cells were rinsed with PBS and micro cover glasses were mounted with fluoromount. Cells were observed with AxioPlan2 (ZEISS) fluorescence microscope and field pictures for

quantification were taken using AxioVision software (5 fields taken at random on each sample). Detection conditions and exposure times for field pictures were adapted to each filter used and samples from the same experiment were photographed at same exposure times.

IV. LIPID ANALYSIS

Culture media and cells from NSPC culture were separately collected. Culture media were first retrieved and cells were detached from the culture wells by treatment with 0.25% trypsin-EDTA and 5 min incubation at 37°C. They were stored at -20°C for future analysis.

Lipid extraction was performed using liquid/liquid phase separation. To 500 µL of cell medium or lysed cell containing solution, 1.5 mL of MeOH/H₂O (20/80) was added. pH was adjusted to 3 using 80 µL of acetic acid. The solution was then deposited on a C18 solid-phase cartridge previously activated with MeOH and washed with H₂O and hexane. Lipids were retrieved with methyl formate and dried under N₂.

Lipids were analyzed by LC/ESI/MS/MS in the negative ion mode using a Waters 2.1 × 150 mm C18 column and an acetonitrile/water gradient containing 0.1% formic acid on AB SCIEX QTRAP® 4500 system. Lipids were identified and quantified using multiple reaction monitoring. The auxiliary gas pressure was 20 p.s.i., and the sheath gas pressure was 30 p.s.i. The capillary voltage was set to -4500 V. The capillary temperature was 350 °C.

Lipid quantity was expressed as pmoles/µg of proteins. Proteins from culture media and cells were quantified using Bradford assay. Briefly, 10 µL of sample were mixed with 200 µL Bradford reagent and 790 µL distilled water. The mixture was homogenized and left at ambient temperature for 10 min. Optical density was determined on a spectrometer set at $\lambda=595$ nm.

V. STATISTICS

Statistical analysis was performed using Dunnett analysis if applicable or Steel analysis otherwise for multiple comparisons between groups (equivalent to unpaired Student's t-test or unpaired Wilcoxon's test respectively). Data are presented as mean \pm standard error of the mean (SEM). Significance threshold was defined as $p < 0.05$. All statistical analyses were carried out using R software.

RESULTS AND DISCUSSIONS

CHAPTER 1: SYNTHESIS OF AceDoxyPC FROM AceDoPC AND IDENTIFICATION USING LC/ESI/MS

Motivated by the previous study done by Hachem et al. (2016) showing enhanced DHA transport through the BBB when DHA is esterified at the *sn*-2 position of the structured phospholipid AceDoPC, the first objective of this work was to synthesize a readily structured oxidized phospholipid containing PDX, an oxygenated derived mediator of DHA with potent protective properties. By incubating AceDoPC with sLOX, we induced a double enzymatic oxygenation of the molecule of DHA esterified within AceDoPC. Identification of AceDoxyPC was performed using advanced techniques of LC/ESI/MS and is presented in this chapter.

I. ANALYSIS OF AceDoPC BY LC/ESI/MS AND LC/ESI/MS/MS

Analysis by MS

The predicted $[MH]^-$ ion for AceDoPC and AceDoxyPC is m/z 609 and m/z 641, respectively. Analysis of the presence of AceDoPC by LC/ESI/MS revealed molecular ions at m/z 668 $[AceDoPC+CH_3COO]^-$, m/z 594 $[AceDoPC-CH_3]^-$ and m/z 654 $[AceDoPC+HCOOH]^-$ (Figure 4A). The CID (collision-induced dissociation) of $[AceDoPC-CH_3]^-$ produced relevant daughter ions at m/z 327 ($[AceDoPC-CH_3-DHA-H]^-$) and m/z 283 ($[AceDoPC-CH_3-DHA-H-CO_2]^-$) (Figure 4B).

Analysis by MRM

Figure 5 shows the MRM analysis of the transitions of the $[MH]^-$ ions for $[AceDoPC-CH_3]^-$ (m/z 594), $[AceDoPC+HCOOH]^-$ (m/z 654) and $[AceDoPC+CH_3COO]^-$ (m/z 668) to the specific CID ion at m/z 327 $[DHA-H]^-$.

II. EVIDENCE OF AceDoxyPC FORMATION FROM LIPOXYGENATION OF AceDoPC

AceDoxyPC predicted molecular ions

Previously, we have shown that the main dihydroxylated compound issued from the 15-lipoxygenation of DHA by the soybean enzyme is PDX (10(S),17(S)-dihydroxy-docosahexa-4Z,7Z,11E,13Z,15E,19Z-enoic acid) (Chen et al., 2009). To determine whether AceDoxyPC could be formed from AceDoPC, the latter was exposed to sLOX in sodium borate buffer (pH 9.0). The predicted molecular ions of [AceDoxyPC+CH₃COO]⁻, [AceDoxyPC-CH₃]⁻ and [AceDoxyPC+HCOOH]⁻ are *m/z* 700.5, *m/z* 626.5 and *m/z* 686.5, respectively. Corresponding ion masses are shown in Table 2. The predicted [MH]⁻ ion for PDX is *m/z* 359.

Analysis by MRM

Figure 6 shows the MRM analysis of the transitions of the [MH]⁻ ions for [AceDoxyPC-CH₃]⁻ (*m/z* 626), [AceDoxyPC+HCOOH]⁻ (*m/z* 686) and [AceDoxyPC+CH₃COO]⁻ (*m/z* 700) to the specific CID ion at *m/z* 358 [PDX-H]⁻.

Synthesis from a mixture of [¹²C]-labeled-DHA-AceDoPC and [U¹³C]-labeled-DHA-AceDoPC

Additional analyses further supported the identity of the compound as AceDoxyPC. The putative AceDoxyPC was prepared by incubating [¹²C]-AceDoPC and [U¹³C]-labeled-DHA-AceDoPC with sLOX. [U¹³C]-AceDoPC was first synthesized with [U¹³C]-labeled-DHA increasing the mass of AceDoPC by 22 and thus increasing the molecular mass of AceDoxyPC by +22. Therefore, the predicted molecular ions of [[U¹³C]-AceDoxyPC+CH₃COO]⁻, [[U¹³C]-AceDoxyPC-CH₃]⁻ and [[U¹³C]-AceDoPC+HCOOH]⁻ are *m/z* 700.5, *m/z* 626.5 and *m/z* 686.5, respectively. Corresponding ion masses are shown in Table 3.

We first demonstrated that there was no peak present 22 Da above *m/z* 594, *m/z* 654 and *m/z* 668 at *m/z* 616, *m/z* 676 and *m/z* 690, respectively for AceDoPC and that there was no peak present 22 Da above *m/z* 626, *m/z* 686 and *m/z* 700 at *m/z* 648, *m/z* 708 and *m/z* 722, respectively for AceDoxyPC (Figure 7A and Figure 7B). However, following incubation of a mixture of [¹²C]-AceDoPC and [U¹³C]-AceDoPC with sLOX, new peaks appeared at *m/z* 616, *m/z* 676, *m/z* 690, *m/z* 648, *m/z* 708 and *m/z* 722, indicating the presence of [[U¹³C]-AceDoPC-CH₃]⁻; [[U¹³C]-AceDoPC+HCOOH]⁻, and [[U¹³C]-AceDoPC+CH₃COO]⁻, [[U¹³C]-AceDoxyPC-CH₃]⁻; [[U¹³C]-AceDoxyPC+HCOOH]⁻, and [[U¹³C]-AceDoxyPC+CH₃COO]⁻, respectively (Figure 7C and Figure 7D).

Identification of putative esterified PDX in AceDoxyPC

To further substantiate the structural identity of these compounds as AceDoxyPC, the compound was analyzed by LC/ESI/MS³. Specific transition of the [MH]⁻ ion for [AceDoxyPC-CH₃]⁻ (*m/z* 626) to the specific CID ion at *m/z* 359 [PDX-H]⁻ was fragmented upon further collisional activation. The product ion spectra show relevant daughter ions at *m/z* 341, *m/z* 323, *m/z* 297, *m/z* 279, *m/z* 187 and *m/z* 153. The ions at *m/z* 341, *m/z* 323, *m/z* 297, *m/z* 279 represent the loss of one molecule of H₂O [PDX-H₂O]⁻, two molecules of H₂O [PDX-2H₂O]⁻, one molecule of H₂O and one molecule of CO₂ [PDX-H₂O-CO₂]⁻, and two molecules of H₂O and one molecule of CO₂ [PDX-2H₂O-CO₂]⁻, respectively. Other daughter ions present in these CID spectra, *m/z* 187 and *m/z* 153, can be assigned the structures shown in Figure 8.

III. DISCUSSION

This work has identified a novel structured lipid, AceDoxyPC, which is formed from lipoxygenation of DHA esterified in AceDoPC by lipoxygenase. Our motivation for synthesizing a vector of protectin DX stems from the fact that protectins and also oxidized phospholipids have potent anti-inflammatory effects.

Successful double lipoxygenation of DHA esterified within AceDoPC to produce AceDoxyPC

Oxidation of PUFA residues of phospholipids produces compounds named oxidized phospholipids. This oxidation can be the result of either enzymatic reaction by lipoxygenase (Huang et al., 2007; Maccarrone et al., 1994; Murray and Brash, 1988; Takahashi et al., 1993) or lipid peroxidation chain reaction by reactive oxygen species and propagates (Ashraf and Srivastava, 2012; Bochkov et al., 2010). 15-LOX is a lipoxygenase that can accept PUFAs esterified in phospholipids as substrates producing phospholipid-hydroperoxides (Bochkov et al., 2010). Although mono-hydroperoxides are the most prevalent oxidized products, proof of double oxidation of PUFA esterified within phospholipid through lipoxygenation exists (Huang et al., 2007). This is particularly fitting in the case of AceDoxyPC synthesis. DHA conversion to PDX (10(S),17(S)-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid) was previously done by double oxidation with 15-LOX (Chen et al., 2009). Using a similar protocol on AceDoPC, we show that a compound of characteristic molecular ions corresponding to AceDoxyPC and of elution time shorter than AceDoPC on reverse phase chromatography was synthesized. Supplementary synthesis from a mixture of [^{12}C]-AceDoPC and [U^{13}C]-labeled-DHA-AceDoPC resulted in the production of a new compound corresponding to [U^{13}C]-AceDoxyPC.

Synthesis of esterified monohydroxy-DHA as an evidence of the lipoxygenation pathway

Presence of monohydroxy-DHA esterified within 1-acetyl-2-hydroperoxy-DHA-PC was also detected and eluted between AceDoxyPC and AceDoPC elution times (data not shown). According to previous studies, 15-LOX primary oxidizes DHA to 17(S)-hydroperoxy-DHA (Butovich et al., 2006; Huang et al., 2007). Further lipoxygenation of 17(S)-hydroperoxy-DHA by 15-LOX is the expected pathway of conversion into 10(S),17(S)-dihydroxy-DHA or PDX. Thus we can hypothesize that we produced 1-acetyl-2-17(S)-hydroperoxyDHA-PC with our protocol, which was further converted into AceDoxyPC under the double oxygenation by 15-LOX. Conversion rates into 17(S)-hydroperoxy-DHA and PDX were respectively 10% and 3% in Chen et al. study (Chen et al., 2009). In accordance with these rates, the proportion of 1-acetyl-2-17(S)-hydroperoxy-DHA-PC was higher than AceDoxyPC in our reaction products.

Further analysis for the identification of PDX esterified within AceDoxyPC

Using LC/ESI/MS³ mode we could identify the product ion spectra of the fragmented ion [PDX-H]⁻ at m/z 359. The main ions corresponded to PDX with loss of either H₂O or CO₂ molecule(s). Although carbon 10 stereochemistry of PDX was not confirmed, the only possible configuration of the dihydroxy-DHA would be PDX configuration due to the dioxygenation by 15-LOX. PD1 differs from PDX by C10 stereochemistry which is *R* instead of *S*. Since PD1 can only be obtained by lipoxygenation of DHA followed by enzymatic epoxidation and hydrolysis by an epoxide hydrolase, it is very unlikely for our esterified dihydroxy-DHA compound to be PD1 with our current protocol conditions. However, confirmation through hydrolysis and further analysis with nuclear magnetic resonance techniques and UPLC-MS/MS as was done in PDX characterization by Chen et al. (Chen et al., 2009) is to be considered.

On the potent interest of oxidized phospholipid AceDoxyPC for health

Oxidized phospholipids exhibit many health benefits such as anti-inflammatory and anti-oxidative properties (Bochkov et al., 2002; Huang et al., 2010). Expansive review was done by Bochkov et al. (2010) on the diverse potential role of oxidized phospholipids in pathology. Since AceDoPC was shown to be a preferred carrier of DHA through the BBB (Hachem et al., 2016) and lipoxygenase 15-LOX was proven to be able to oxidize DHA contained within AceDoPC to produce the structured phospholipid AceDoxyPC in this study, it is of relevance to investigate the potential neuroprotective effects of such a compound.

We conclude from the present study that using 15-LOX double lipoxygenation on AceDoPC we could synthesize the novel structured phospholipid AceDoxyPC (1-acetyl,2-PDX-glycerophosphorylcholine). AceDoPC being a preferred transporter of DHA to the brain and protectins exhibiting many health benefits notably on neurodegenerative diseases, synthesis of AceDoxyPC and further studies on its biological effects might give better insight on the use of oxidized phospholipids as a treatment to cerebral diseases or stroke.

CHAPTER 2: STUDY OF THE NEUROPROTECTIVE EFFECTS OF DHA, AceDoPC AND PDX, AND ADULT NEUROGENESIS

Neuroprotective effects of DHA are expected to be due to its conversion into derived mediators such as protectins. However AceDoPC, preferential carrier of DHA through the BBB, also showed neuroprotective effects in an *in vivo* model of acute focal cerebral ischemia (Chauveau et al., 2011). Moreover, embryonic NSPCs cultured with DHA *in vitro* showed that DHA promoted neurogenesis and had no effect on astrogenesis (Sakayori et al., 2011). The work presented thereafter aimed at testing the hypotheses that: (1) AceDoPC enhances DHA neuroprotective effects, notably in ischemia and (2) DHA, PDX and AceDoPC promote neurogenesis that could add to DHA neuroprotective effects.

To this intent, we cultured NSPCs derived from the adult mouse brain under physiological conditions or pathological conditions mimicking ischemic conditions *in vitro* with a condition called OGD (Zhang et al., 2014). DHA, AceDoPC or PDX were added to the cultures in the proliferation and differentiation assays.

I. ADULT NSPCs CULTURED UNDER PHYSIOLOGICAL CONDITIONS WITH SUBSTRATE ADDITION

Effects of DHA on proliferation and differentiation

Addition of BrdU in the proliferation assays and labeling BrdU positive cells with immunocytochemistry allowed for the evaluation of DHA effects on proliferation rates of adult NSPCs. Figure 9 shows sample field pictures used for proliferation rates estimation.

Proliferation rates were expressed as the ratio of BrdU positive cell number over the total cell number, which was computed as the number of DAPI positive cells. All results are expressed as percentages compared to the control group, which corresponds to the addition of vehicle only and was fixed at 100%. Figure 10 shows the proliferation rates and cell numbers of adult NSPCs in our proliferation assays with addition of DHA, from concentration 10^{-9} M to 10^{-5} M. Addition of DHA did not affect proliferation until concentration of 10^{-6} M. The highest concentration of DHA (10^{-5} M) significantly decreased the proliferation rate to 78% of control. Although not significantly, DHA increased the cell number at concentration 10^{-9} M to 127% of control.

In differentiation assays, differentiation was induced by culture media change. Specific markers Tuj1, GFAP, and counterstaining with DAPI allowed us to estimate neurogenesis rates, astrogenesis rates, ratios of cells negative for the markers (later referred as

unmarked cells), and the total cell numbers, respectively. Figure 11 shows sample field pictures used for differentiation rates estimation.

Neurogenesis rates were computed as the ratio of Tuj1 positive cell number over the total cell number. Similarly, astrogenesis rates were computed with the numbers of GFAP positive and negative cells, and the unmarked cell ratios were computed with the number of cells stained with DAPI only. All results are expressed as percentages compared to the control group, which corresponds to the addition of vehicle only and was fixed at 100%. Figure 12 shows the above-mentioned rates in our differentiation assays with addition of DHA, from the same concentration range as the proliferation assays. Addition of DHA did not significantly affect any of the rates or the total cell number. Whereas not significantly, DHA showed slight increase of neurogenesis to 115% of control at 10^{-9} M. Conversely, astrogenesis tended to be decreased at concentrations 10^{-9} M and 10^{-6} M (rates lower than 80% of control group).

Effects of AceDoPC on proliferation and differentiation

Similarly to proliferation assays and differentiation assays with DHA, we added AceDoPC to the cultures to evaluate the effects of esterification of DHA within this acetylated structured phospholipid. We observed that high concentration of AceDoPC (10^{-5} M) increased proliferation rates significantly, to a level of 116% of control (Figure 13). Equivalent high concentration of DHA decreased proliferation.

Results of differentiation assays with AceDoPC addition (10^{-9} to 10^{-5} M) are shown on Figure 14. AceDoPC addition increased neurogenesis to at least 110% compared to control group, and the effect was significant at concentration 10^{-8} M (due to high homogeneity in this group, SEM=1.77). Astrogenesis rates decreased, albeit not significantly, with AceDoPC addition. This tended to increase the unmarked cell ratio of the cultures for concentrations above 10^{-9} M. High concentration of AceDoPC (10^{-5} M) increased cell number (122% of control).

Effects of PDX on proliferation and differentiation

Similarly to the previous assays, we decided to evaluate the effects of DHA oxygenated derivative PDX, which is an isomer of NPD1, a neuroprotective molecule. We added concentrations of half the previous concentrations added (i.e. 0.5×10^{-9} M instead of 10^{-9} M). PDX being a dihydroxylated form of DHA, it should be more biologically active. On Figure 15 are presented results for proliferation assays with PDX addition. Similarly to DHA addition at 10^{-5} M, the addition of 0.5×10^{-5} M PDX significantly decreased proliferation rates (81% of control). Concentrations from 0.5×10^{-8} M to 0.5×10^{-6} M however tended to increase cell number.

Results of differentiation assays with PDX addition are grouped in Figure 16. No significant effect was observed in differentiation rates. However, 0.5×10^{-9} M PDX addition

increased the total cell number significantly. Observation of neurogenesis, astrogenesis and unmarked cell ratio of this group may indicate that neurogenesis might be increased albeit not significantly. PDX tended to decrease astrogenesis rate dose-dependently, which led to an increase of unmarked cell ratio rate with higher concentration of PDX.

II. ADULT NSPCs CULTURED UNDER PATHOLOGICAL CONDITIONS WITH SUBSTRATE ADDITION

Effects of DHA on proliferation and differentiation in OGD condition

To study DHA effects on adult NSPCs cultured under pathological conditions, we induced OGD to the cells and performed the same evaluation of proliferation and cell count as done previously under physiological conditions. Induction of OGD drastically changed the reaction of cells to DHA addition (Figure 17). Whereas not significant, both proliferation rates and cell numbers increased with DHA addition, with 10^{-7} M DHA addition showing the higher increases (143% of control proliferation rate and 151% of control cell number). 10^{-5} M DHA concentration did not impact proliferation negatively as observed under physiological conditions.

Differentiation rates were also greatly impacted by OGD induction (Figure 18). DHA significantly increased neurogenesis at concentrations 10^{-8} M (131% of control) and 10^{-7} M (167% of control). Astrogenesis was not influenced by DHA addition. Total cell number tended to increase (except at the highest concentration, 10^{-5} M, of DHA) (not statistically significant).

Enhanced neurogenesis with AceDoPC addition after OGD induction and effects on proliferation

OGD induction also changed the effects of AceDoPC on adult NSPC culture compared to physiological conditions. Little effect was observed on proliferation and 10^{-5} M AceDoPC addition did not increase proliferation rate. Cell number tended to be increased with AceDoPC. Figure 19 groups results of AceDoPC effects in proliferation assays with OGD induction.

Similarly to DHA addition under pathological conditions of cell culture, AceDoPC showed enhanced effects on differentiation compared to the results observed under physiological condition (Figure 20). 10^{-9} M AceDoPC concentration greatly and significantly increased neurogenesis up to 245% of control. Astrogenesis was also increased at 10^{-8} M AceDoPC concentration albeit not significantly. However, the same concentration increased cell number significantly. Since unmarked cell ratio was not changed at this concentration, both neurogenesis and astrogenesis might be increased at 10^{-8} M.

Effects of PDX on proliferation and differentiation in OGD condition

We applied the same OGD induction to adult NSPCs cultured with PDX addition. PDX effects on proliferation rates observed previously under physiological conditions were increased under pathological conditions of cell culture (Figure 21). Indeed, all concentrations

of PDX addition tended to increased proliferation rate excepted for the highest concentration of PDX (0.5×10^{-5} M). This concentration decreased proliferation rate down to 28% of control (significant) and cell number down to 53% of control cell number.

Figure 22 shows the results of differentiation assays with PDX addition after OGD induction. PDX tended to enhance neurogenesis and astrogenesis rates, notably at concentration 0.5×10^{-8} M (166%) while undifferentiation rate tended to decrease at this concentration, albeit not significantly. We observed that PDX significantly increased total cell count from 0.5×10^{-8} M to 0.5×10^{-6} M (although not significantly, 0.5×10^{-7} M also showed an increase). 0.5×10^{-6} M PDX increased total cell number to 188% of control.

III. STUDY OF THE POTENTIAL MECHANISMS INVOLVED IN THE EFFECTS OF AceDoPC ON NSPCs

Evidence of decrease of oxidation and inflammation with AceDoPC addition after OGD induction

Since OGD mimics ischemic conditions *in vitro*, inducing OGD to our cell cultures should increase oxidative stress and activate pro-inflammatory and/or pro-apoptotic responses from the cells. To evaluate whether DHA and AceDoPC could affect these pathways, we decided to investigate markers of oxidation and inflammation. To this intent, we performed differentiation assays under physiological or pathological conditions. Under physiological conditions, only vehicle was added to the cells. When cells were subjected to OGD, we added either vehicle, 10^{-9} M DHA or 10^{-9} M AceDoPC. We collected either culture media or cells instead of fixing them to extract lipids (liquid/liquid phase extraction) and proteins. The extracted lipids were identified and quantified on LC/ESI/MS. Protein levels were quantified using Bradford assay. Lipid mediators were quantified using available internal standard and their concentrations are expressed as pmoles/ μ g of proteins (as quantified in Bradford assays for each group). Results were presented as the total lipid extracted from both cells and supernatants.

Preliminary results were obtained on a pool of 6 samples for each group. We investigated levels of arachidonic acid (AA, 20:4n-6) derived mediators. The main retrieved lipids are listed in Table 4. Compared to the group cultured under physiological conditions, OGD condition induced the increase of AA derived mediators: prostaglandins PGD₂, PGF₂ and 8-epi-PGF₂ α , leukotriene LTB₄, thromboxane TXB₂ and hydroxylated metabolite 15-HETE. AceDoPC addition at 10^{-9} M reduced all these lipids' levels, to levels even lower than the group cultured under physiological conditions. 10^{-9} M DHA did not show the same decreasing effect except for 15-HETE.

Evidence of activation of anti-oxidative signaling pathway with AceDoPC addition

Since response against oxidative stress may be one involved mechanism of the neuroprotective properties of AceDoPC, we investigated by immunocytochemistry the Nrf2 pathway for two groups of cells cultured under OGD and with or without addition of 10^{-9} M AceDoPC. Using Nrf2-antibody, we could observe as a preliminary result on one sample for each group that AceDoPC activated Nrf2 production in adult NSPCs compared to the cells subjected to OGD and added with vehicle only (Figure 23).

Due to the lack of statistical analysis in this preliminary study, we admit that this is still very early to suggest, but we believe that this tendency could imply the potential mechanisms underlying neuroprotective effects of AceDoPC.

IV. DISCUSSION

This work aimed at studying the effects of esterification of DHA, unesterified or esterified within AceDoPC, and the effects of a derived mediator of DHA, PDX, on the proliferation and differentiation rates of adult NSPCs under physiological or pathological conditions mimicking ischemia *in vitro*. To our knowledge, this study is one of the few available studies on adult neurogenesis and AceDoPC neuroprotective effects.

Esterification of DHA within AceDoPC providing prevention against decreased proliferation of adult NSPCs under physiological conditions

Under physiological conditions, highest concentration of DHA (10^{-5} M) added to the cells in the proliferation assays significantly decreased the proliferation rates of adult NSPCs (Figure 10). Sakayori et al., (2011) previously showed that DHA addition at such concentration range to embryonic NSPCs decreased proliferation rates and also increased proportion of active caspase 3 positive cells, caspase 3 being a marker of cell apoptosis. The authors concluded that high concentration of DHA was toxic for NSPCs due to lipid peroxidation converting DHA to oxidized mediators, thus inducing oxidative stress to the cells. Such results were also observed on cell viability of embryonic NSPCs cultured with 10^{-5} M DHA (Katakura et al., 2009). One study of rats fed with diets containing high levels of DHA showed that lipid peroxidation was higher in plasma, liver and kidney with notably decreased levels of anti-oxidant α -tocopherol (Song et al., 2000).

The same effect is observed in adult NSPCs cultured with PDX at 0.5×10^{-5} M (Figure 15). PDX being an oxygenated mediator of DHA, high levels of PDX might also prove toxic to cell proliferation. NPD1, PDX isomer, was proven to protect human retinal pigment epithelial cells from oxidative stress but tested concentrations were lower than 10^{-5} M (Mukherjee et al., 2004).

Conversely no such effect is observed with addition of 10^{-5} M AceDoPC (Figure 13). This might indicate that esterified DHA is less available for peroxidation induced by oxidative stress, giving AceDoPC a neuroprotective property due to protection against oxidation because of its structure. Protection against lipid peroxidation of DHA esterified within phospholipids was observed in mice fed with DHA containing diets under different forms (Hiratsuka et al., 2008). Same results were obtained in rats (Song and Miyazawa, 2001). High concentration of AceDoPC also showed an increase of proliferation instead. Adding to its putative anti-oxidative property, AceDoPC might also be an inducer of proliferation.

OGD induction as a shifting factor of cell response to DHA

Cell cultures under pathological conditions through OGD induction led to the increase in levels of oxidative markers and pro-inflammatory markers (Table 4). OGD is an *in vitro*

condition mimicking ischemia by reducing oxygen and glucose availability to the cells (Goldberg and Choi, 1993). Cells subjected to OGD showed different responses to DHA, PDX or AceDoPC addition.

Previously described decreased proliferation due to high DHA concentration is not observed after OGD induction (Figure 17). Since DHA showed anti-inflammatory and anti-oxidative properties due to its conversion into derived mediators, notably protectins (Begum et al., 2012; Chen et al., 2011; M. Liu et al., 2014; Lukiw et al., 2005; Marcheselli et al., 2003), DHA added to culture media could have been metabolized by the cells to counteract pathological culture conditions. Preliminary results of lipid identification did show some increase of DHA oxygenated mediators (Table 4). However tested concentration in the latter experiment was 10^{-9} M instead of 10^{-5} M. Therefore, further experiments remain to be done.

There was no change in the effects of high concentration of PDX (0.5×10^{-5} M) on proliferation of adult NSPCs after OGD induction (Figure 21). Proliferation rates are even lower than under physiological conditions. This result could suggest that PDX does involve higher oxidative stress on the cells and that PDX cannot be metabolized into other mediators with beneficial properties, contrary to DHA. However, PDX effects on lipid metabolism were not studied in this work and remain to be investigated.

Neurogenesis rates were greatly influenced by OGD induction. DHA significantly increased neurogenesis of adult NSPCs at concentrations 10^{-8} M and 10^{-7} M (Figure 18). Although small increase of neurogenesis was observed under physiological conditions of culture, the effect is much higher after OGD. Astrogenesis rates were not affected by addition of DHA. Similar results were observed on embryonic NSPCs under physiological conditions (Katakura et al., 2013; Kawakita et al., 2006; Sakayori et al., 2011). Adult NSPCs and embryonic NSPCs differ by the preferred fate of differentiation of the stem cell. Embryonic NSPCs of earlier stage are qualified as neurogenic because of their tendency to become neurons, which is primary for the earliest stages of brain development. Later stage NSPCs tend to become astrocytes, shifting the NSPCs from neurogenic to gliogenic qualification (Okada et al., 2008; Qian et al., 2000). Adult NSPCs could thus be more gliogenic. DHA addition might shift the tendency of differentiation into neurons instead of astrocytes. This is in accordance with the observed high levels of DHA in the earliest stages of brain development (Clandinin et al., 1980; Lauritzen et al., 2001; Martinez, 1992) making DHA a key factor of neurogenesis.

Neurogenesis induction at lower AceDoPC concentration compared to DHA

Addition of AceDoPC also increased neurogenesis rates at concentration of 10^{-9} M and 10^{-8} M (Figure 20). Neurogenesis was increased under physiological conditions but OGD induction showed a greater increase in neurogenesis levels. Since neurogenesis induction happened at lower concentrations of AceDoPC and higher levels compared to DHA addition, we can assume that either AceDoPC allows DHA to be more readily available for use by the cells or that AceDoPC have unique properties allowing for enhancement of neurogenesis.

AceDoPC is a preferred carrier of DHA through the BBB compared to unesterified DHA (Hachem et al., 2016; Lo Van et al., 2016; Nguyen et al., 2014). This could suggest that AceDoPC crosses cell membrane more easily than unesterified DHA and it could explain the observed enhancement at lower concentration. Another suggestion of involved pathway might be that the acetyl group of AceDoPC at *sn*-1 position has beneficial properties on neurogenesis.

Although addition of AceDoPC at 10^{-8} M concentration did not show a statistically significant effect on neurogenesis or astrogenesis, we can note that the total cell number in the differentiation assay was significantly higher at this concentration (Figure 20). Unmarked cell ratio was not affected by AceDoPC addition so this could suggest that increases in both neurogenesis and astrogenesis influenced the total cell number in our cultures. This is of particular interest since no effect of DHA or DHA metabolites on astrogenesis have been reported yet to our knowledge. Moreover, glial cells were shown to be inducers of neurogenesis (Hattiangady et al., 2007; Ma et al., 2005). Therefore AceDoPC putative astrogenesis induction might add to its beneficial effect on neurogenesis induction.

Potential neuroprotective effects of AceDoPC against oxidative damage and inflammation on adult NSPCs

Addition of 10^{-9} M AceDoPC decreased the levels of AA metabolites involved in oxidative stress or inflammation process (8-epi-PGF₂ α , PGD₂, PGF₂, LTB₄, TXB₂ and 15-HETE, Table 4). OGD is a condition mimicking ischemia *in vitro*, and therefore done by inducing hypoxia and ischemia to the cell cultures. Such conditions were shown to activate AA metabolism cascade with upregulation of cyclooxygenase-2 (Sairanen et al., 1998; Tomimoto et al., 2002). 5-LOX expression is also increased and the concentration of leukotrienes is elevated in the brain after cerebral ischemic injury (Ciceri et al., 2001; Ohtsuki et al., 1995; Tomimoto et al., 2002). 15-HETE level, synthesized by 15-lipoxygenase, is also higher in brain of rat exposed to ischemia (Sun et al., 2015; Usui et al., 1987). AA metabolites are considered the main actors of the pro-inflammatory response of cells against damage and are therefore considered as pro-inflammation markers, although recent studies highlighted their potential use as protective agents (Yagami et al., 2016). 8-epi-PGF₂ α , a marker of oxidative stress, is elevated in patients who experienced stroke (Lin et al., 2015) and in rats who were subjected to oxidative stress following traumatic brain injury (Tyurin et al., 2000). Moreover, 8-epi-PGF₂ α induced cell death on cultures of endothelial cells, notably by inducing elevation of TXB₂ levels (Brault et al., 2003).

Analysis of expression of Nrf2 by immunocytochemistry showed that Nrf2 pathway was better activated with addition of AceDoPC (Figure 23). Nrf2 is involved in the anti-oxidative response of cells against oxidative damage by upregulation of enzymes such as heme oxygenase 1 (Zhang et al., 2014). Addition of DHA also showed the same activation of Nrf2 (not shown) but no decrease of levels of markers of oxidation. Whereas these results are preliminary, we suggest that AceDoPC presents neuroprotective effects on cells in part by

enhancing the anti-oxidative capacity of cells to respond to oxidation. AceDoPC effects might be due to either DHA esterified at the *sn*-2 position of the phospholipid or the acetyl group esterified at the *sn*-1 position. This neuroprotective effect might affect cell condition and allow for maintenance of newly differentiated neurons, thus enhancing neurogenesis in our experiments. Although still preliminary, these results are promising and will help us identify which pathways to investigate in future studies.

PDX promotes maintenance of cells in differentiation assay

Previous observations suggest that high concentration of PDX decreases proliferation rates of NSPCs due to induction of oxidative stress. However, we could observe that concentration of 10^{-9} M increased the total cell number in the differentiation assays under physiological conditions (Figure 16). Although seemingly contradictory, this effect was not observable in the proliferation assays. This could be explained by the composition of culture media in each assay type. Proliferation assay culture medium contains growth factors that maintain the cells in a proliferative state while culture medium of differentiation assay does not contain such factors. This effect is still observed in cell culture under pathological conditions but at higher concentrations of PDX. Since no significant effect was observed in differentiation rates, PDX influence on adult NSPCs remains unclear and to be investigated.

Another possibility is that PDX promotes survival of newly differentiated cells. Yokose et al. previously showed that important cell death rate occurs during a certain critical period following initial proliferation of cells in hippocampal slice culture from rat brain (Yokose et al., 2011). Such results are in accordance with previous studies (Dayer et al., 2003; Sasaki et al., 2006). PDX might play a role in the maintenance of newly generated neurons facing this critical period of postmitotic cell death. DHA, PDX precursor, and NPD1, PDX isomer, notably proved to be anti-apoptotic in ischemic brain tissues from rats (Pan et al., 2009) and *in vitro* studies of human neurons and glia cultures (Lukiw et al., 2005) respectively. Therefore, PDX might also have anti-apoptotic effects on adult NSPCs cultured under pathological conditions.

Possible action of DHA, AceDoPC and PDX on oligodendrogenesis

Although not quantified in our study, NSPCs can also differentiate into a third cell type that is oligodendrocytes (Teh et al., 2014; Waly et al., 2014). Effects observed on unmarked cell ratio might include influence on oligodendrogenesis, which should be more studied in further experiments. Since cerebral infarction induces demyelination followed by increase of oligodendrogenesis, several considered therapies are about acting on the increase of oligodendrogenesis after ischemia to induce regeneration of damaged tissues (Zhang et al., 2013).

We conclude from this work that DHA and AceDoPC are effective neurogenesis inducers on adult NSPCs, notably when the cells are subjected to pathological conditions. Moreover, AceDoPC effects were induced at lower concentration compared to unesterified DHA addition to the cells, hinting at either a better incorporation of DHA inside the cell or a specific effect of AceDoPC due to its structure and composition. This induction of neurogenesis might be paralleled with neuroprotection due to putative anti-oxidative effects of AceDoPC. These results lead toward a better understanding of the effects of AceDoPC on the brain after the passage through the BBB.

CONCLUSION AND PERSPECTIVES

With increasing number of patients suffering from neurodegenerative diseases, the research for efficient treatment or prevention of such pathology is a priority. DHA is an omega-3 PUFA uniquely enriched in the brain and it was proved essential for proper brain development and function. DHA and its many oxygenated derivatives have been numerously studied as putative treatments to many health conditions including neurodegenerative and cardiovascular diseases. However limitation in *de novo* biosynthesis of DHA makes it necessary to be incorporated in our metabolism through dietary intake. Moreover, with change of eating habits in Western countries, the ratio of omega-3 and omega-6 PUFA, competitors of omega-3 PUFA for the same enzymes of elongation of PUFAs and oxygenation into derived mediators, is becoming lesser with time. Since the human brain needs a large amount of DHA for proper behavior, it is therefore particularly relevant to work on efficient transport and incorporation of DHA into the brain. Phospholipids such as lysoPC-DHA and AceDoPC, a structured phospholipid containing DHA at the *sn*-2 position and an acetyl group at the *sn*-1 position, were shown to be efficient transporters of DHA through the BBB.

The aim of this work was to study the effects of brain targeting with structured phospholipids containing either DHA or its derived mediators, namely PDX in this thesis, on a model of neurogenesis and ischemia *in vitro*. The two main objectives were to synthesize a protein-containing structured phospholipid similar to AceDoPC and to study the effects of AceDoPC, DHA and PDX on adult NSPC cultures under physiological or pathological conditions, as a model of neurogenesis and ischemia *in vitro*.

We enzymatically synthesized the structured phospholipid named AceDoxyPC, with PDX esterified at the *sn*-2 position. Successful identification of AceDoxyPC was performed using advanced techniques of mass spectrometry and reported here. Cultures of adult NSPCs showed that AceDoPC and DHA could enhance neurogenesis of adult NSPCs especially when cells were subjected to pathological stress, induced with OGD here. AceDoPC proved to be a better neurogenesis inducer. This enhanced effect is assumed to be due to either better transport of DHA through cell membrane with AceDoPC compared to unesterified DHA, or to specific function of AceDoPC giving its peculiar structure. AceDoPC also exhibited neuroprotective properties through putative anti-oxidative pathway induction. This neuroprotection could also add to the observed neurogenesis enhancement by allowing the newly differentiated neurons to survive through pathological conditions.

With this work, we were able to observe the potential of AceDoPC and DHA as neuroprotective agents and neurogenesis inducers on adult NSPCs. These preliminary results hint at the possible use of AceDoPC and DHA as a treatment or prevention to stroke to allow for preservation and regeneration of stressed cells. Effects of DHA derived mediator PDX remain however unclear. Future studies to compare the effects of both PDX and newly synthesized and identified AceDoxyPC will be investigated.

It will be also relevant to reproduce the same experiments on adult NSPCs to account for the great heterogeneity in our data. Comparative study of AceDoxyPC and unesterified PDX might also give better insight into the putative neuroprotective effects of this DHA

derived mediator. It is necessary to confirm the preliminary results obtained with lipid analysis by reproducing the same experiments and by investigating inflammation, oxidation or pro-apoptotic markers with other techniques such as ELISA or Western Blot analysis. Since experiments on Nrf2 seem promising but are still lacking, it might prove interesting to study instead the expression of downstream genes of Nrf2 activation such as HO-1 gene or NQO1 gene.

To investigate the effects of such molecules on inflammation, co-cultures of adult NSPCs with astrocytes might also be considered as astrocytes are the main initiator of anti-inflammatory response against pathological stress. Co-cultures with neurons might also prove relevant since ischemic neurons induce the overload of extracellular calcium and glutamate, which are pathogenic factors that could be influenced by DHA neuroprotective effects.

We used OGD to induce ischemia *in vitro*. However, other cellular models of neurodegenerative diseases *in vitro* exist and might be interesting to study. We recently started a collaborative study with another group culturing cortical neurons with soluble amyloid- β oligomers as a model of precocious stages of Alzheimer's disease (Florent et al., 2006). Preliminary observations showed neuroprotective effects of AceDoPC. Other considered ways to induce pathological conditions experiments include cultures with addition of lipopolysaccharides or neuro-inflammatory mediators, such as interleukin-6 or tumor necrosis factor alpha.

In vivo studies in humans are also planned as the aim of the study is to investigate the potential use of DHA, PDX, AceDoPC and AceDoxyPC as treatment or prevention to neurodegenerative diseases. One clinical trial on the biodisponibility in healthy patient body of radiolabeled [^{13}C]-DHA incorporated into either triacylglycerol or PC or AceDoPC has been initiated in 2014. Outcomes of this study will allow us to evaluate DHA incorporation in the brain of healthy subjects notably. This trial will be followed by another one on patients who had experienced stroke in order to investigate the beneficial effects of these DHA metabolites or DHA preferential carriers on stroke.

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FIGURE LEGENDS

Figure 1. *AceDoPC synthesis*

Synthesis of AceDoPC. Two-steps reaction: first, alpha-glycerophosphocholine (1) is acylated through an enzymatic reaction into 1-acetyl-glycerophosphocholine (2). Thereafter DHA is esterified on the *sn*-2 position of 1-acetyl-glycerophosphocholine (2) with DCC/DMAP, resulting in the synthesis of 1-acetyl,2-docosaheptaenoyl-glycerophosphocholine (3). Modified from Hachem et al., 2016.

Figure 2. *AceDoxyPC synthesis*

1-acetyl,2-docosaheptaenoyl-glycerophosphocholine or AceDoPC (1) is incubated with sLOX to induce double lipooxygenation of DHA esterified at the *sn*-2 position. Intermediate dihydroperoxy compound obtained is then reduced to 1-acetyl, 2-PDX-glycerophosphocholine or AceDoxyPC (2).

Figure 3. *NSPC culture protocol*

“Substrate addition” refers to either DHA, AceDoPC or PDX addition here.

Figure 4. *Characterization of AceDoPC using LC/ESI/MS and LC/ESI/MS/MS*

(A) Identification of major molecular ions of AceDoPC using LC/ESI/MS in the negative mode. (B) LC/ESI/MS/MS analysis of $[\text{AceDoPC-CH}_3]^-$. The $[\text{MH}]^-$ ion m/z 594 of $[\text{AceDoPC-CH}_3]^-$ was subjected to CID and daughter ions were scanned from m/z 50 to m/z 600.

Figure 5. *LC/ESI/MS/MS analysis of the major molecular ions of AceDoPC to daughter ion [DHA-H]*

Selected reaction monitoring of the transitions m/z 594, m/z 654, m/z 668 to m/z 327 for $[\text{AceDoPC-CH}_3]^-$, $[\text{AceDoPC+HCOOH}]^-$ and $[\text{AceDoPC+CH}_3\text{COO}]^-$, respectively, was performed.

Figure 6. *LC/ESI/MS/MS analysis of the major molecular ions of AceDoxyPC to daughter ion [PDX-H]*

Selected reaction monitoring of the transitions m/z 626, m/z 686, m/z 700 to m/z 359 for $[\text{AceDoxyPC-CH}_3]^-$, $[\text{AceDoxyPC+HCOOH}]^-$ and $[\text{AceDoxyPC+CH}_3\text{COO}]^-$, respectively, was performed.

Figure 7. *LC/ESI/MS analysis of AceDoPC and AceDoxyPC*

(A) LC/ESI/MS analysis of ^{12}C -AceDoPC. (B) LC/ESI/MS analysis of ^{12}C -AceDoxyPC. (C) LC/ESI/MS analysis of $^{12}\text{C}/^{13}\text{C}$ -AceDoPC. (D) LC/ESI/MS analysis of $^{12}\text{C}/^{13}\text{C}$ -AceDoxyPC formed from mixture of $^{12}\text{C}/^{13}\text{C}$ -AceDoPC.

Figure 8. *Identification of PDX as daughter ion of [AceDoxyPC-CH₃]*

LC/ESI/MS³ analysis of $[\text{AceDoxyPC-CH}_3]^-$. The specific transition of the $[\text{MH}]^-$ ion for $[\text{AceDoxyPC-CH}_3]^-$ to the specific CID ion $[\text{PDX-H}]^-$ was further subjected to CID and daughter ions were scanned from m/z 50 to m/z 360.

Figure 9. Immunocytochemistry following proliferation assays with DHA

Samples from proliferation assays under physiological conditions with DHA addition (10^{-9} to 10^{-5} M). “Vehicle” refers to control condition with no DHA added to the cells. Marker used was BrdU. Cell nuclei were counterstained with DAPI. An enlargement of some part of one field is provided. Scale bars correspond to 50 μ m.

Figure 10. Proliferation rates and cell number of proliferation assays with DHA

The total cell number is calculated as the number of DAPI positive cell counted (right panel). Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (left panel). Adult NSPCs cultured with DHA do not exhibit higher proliferation rates nor enhanced cell number. Highest concentration of DHA added (10^{-5} M) induced a decrease in proliferation. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 4$ for each group. * $p < 0.05$.

Figure 11. Immunocytochemistry following differentiation assays with DHA

Samples from differentiation assays under physiological conditions with DHA addition (10^{-9} to 10^{-5} M). “Vehicle” refers to control condition with no DHA added to the cells. Markers used were Tuj1 and GFAP. Cell nuclei were counterstained with DAPI. An enlargement of some part of one field is provided. Scale bars correspond to 50 μ m.

Figure 12. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number in differentiation assays with DHA

The total cell number is calculated as the number of DAPI positive cell counted (bottom right). Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (left top). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (right top). Unmarked cell ratios are evaluated as the number of DAPI only stained cells over the total cell number (left bottom). No significant effect of DHA on adult NSPCs differentiation was observed. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 5$ for each group.

Figure 13. Proliferation rates and cell number of proliferation assays with AceDoPC

The total cell number is calculated as the number of DAPI positive cell counted (right). Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (left). AceDoPC increased cell proliferation at 10^{-5} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n = 6$ for each group. * $p < 0.05$.

Figure 14. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with AceDoPC

The total cell number is calculated as the number of DAPI positive cell counted (bottom right). Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (left top). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (right top). Unmarked cell ratios are evaluated as the number of DAPI only stained cells over the total cell number (left bottom). A significant effect of AceDoPC on adult NSPCs neurogenesis was observed at 10^{-8} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n = 6$ for each group. $*p < 0.05$.

Figure 15. Proliferation rates and cell number of proliferation assays with PDX

The total cell number is calculated as the number of DAPI positive cell counted (right). Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (left). PDX decreased cell proliferation at 10^{-5} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 4$ for each group. $*p < 0.05$.

Figure 16. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with PDX

The total cell number is calculated as the number of DAPI positive cell counted (bottom right). Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (left top). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (right top). Unmarked cell ratios are evaluated as the number of DAPI only stained cells over the total cell number (left bottom). A significant effect of PDX on total cell number was observed at 0.5×10^{-9} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 6$ for each group. $*p < 0.05$.

Figure 17. Proliferation rates and cell number of proliferation assays with DHA under OGD condition

The total cell number is calculated as the number of DAPI positive cell counted (right). Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (left). DHA did not decrease proliferation at 10^{-5} M as observed under physiological condition. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 7$ for each group. $*p < 0.05$.

Figure 18. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with DHA under OGD condition

The total cell number is calculated as the number of DAPI positive cell counted (bottom right). Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (left top). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (right top). Unmarked cell ratios are evaluated as the number of DAPI only stained cells over the total cell number (left bottom). DHA increased neurogenesis rates at 10^{-8} M and 10^{-7} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 10$ for each group. * $p < 0.05$.

Figure 19. Proliferation rates and cell number of proliferation assays with AceDoPC under OGD condition

The total cell number is calculated as the number of DAPI positive cell counted (right). Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (left). No significant effect was observed. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 5$ for each group.

Figure 20. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with AceDoPC under OGD condition

The total cell number is calculated as the number of DAPI positive cell counted (bottom right). Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (left top). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (right top). Unmarked cell ratios are evaluated as the number of DAPI only stained cells over the total cell number (left bottom). AceDoPC increased neurogenesis at 10^{-9} M and cell number at 10^{-8} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 6$ for each group. * $p < 0.05$.

Figure 21. Proliferation rates and cell number of proliferation assays with PDX under OGD condition

The total cell number is calculated as the number of DAPI positive cell counted (right). Proliferation rates are evaluated as the ratio of BrdU positive cell number over the total cell number (left). PDX decreased proliferation even more than under physiological conditions at 0.5×10^{-5} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 4$ for each group. * $p < 0.05$.

Figure 22. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with PDX under OGD condition

The total cell number is calculated as the number of DAPI positive cell counted (bottom right). Neurogenesis rates are evaluated as the ratio of Tuj1 positive cell number over the total cell number (left top). Astrogenesis rates are evaluated as the ratio of the number of GFAP positive and negative cell number over the total cell number (right top). Unmarked cell ratios are evaluated as the number of DAPI only stained cells over the total cell number (left bottom). PDX significantly increased cell number at 0.5×10^{-8} M and 0.5×10^{-6} M. Quantitative data were calculated over five fields taken at random on each sample and were expressed as percentages compared to control, which was fixed at 100%. Data present means \pm SEM. $n \geq 6$ for each group. * $p < 0.05$.

Figure 23. Immunocytochemistry with Nrf2 following differentiation assays under varying conditions

Samples from differentiation assays under pathological conditions with vehicle or 10^{-9} M AceDoPC addition. “Vehicle” refers to control condition with vehicle only added to the cells. Marker used was Nrf2-antibody and cells were counterstained with DAPI. Scale bars correspond to 50 μ m. $n=1$ for each group.

FIGURES

Figure 1. AceDoPC synthesis

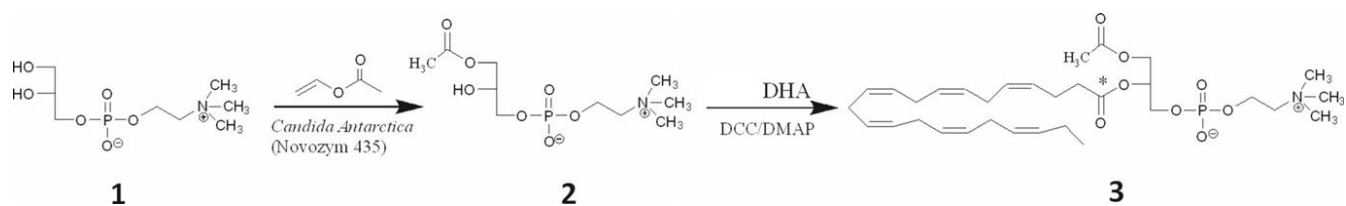


Figure 2. *AceDoxyPC* synthesis

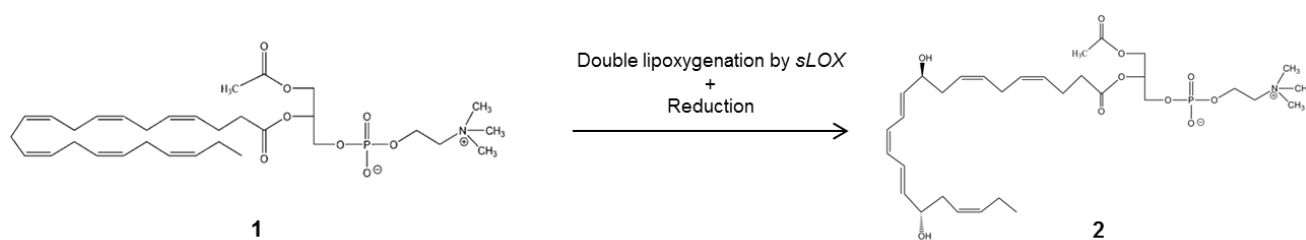


Figure 3. NSPC culture protocol

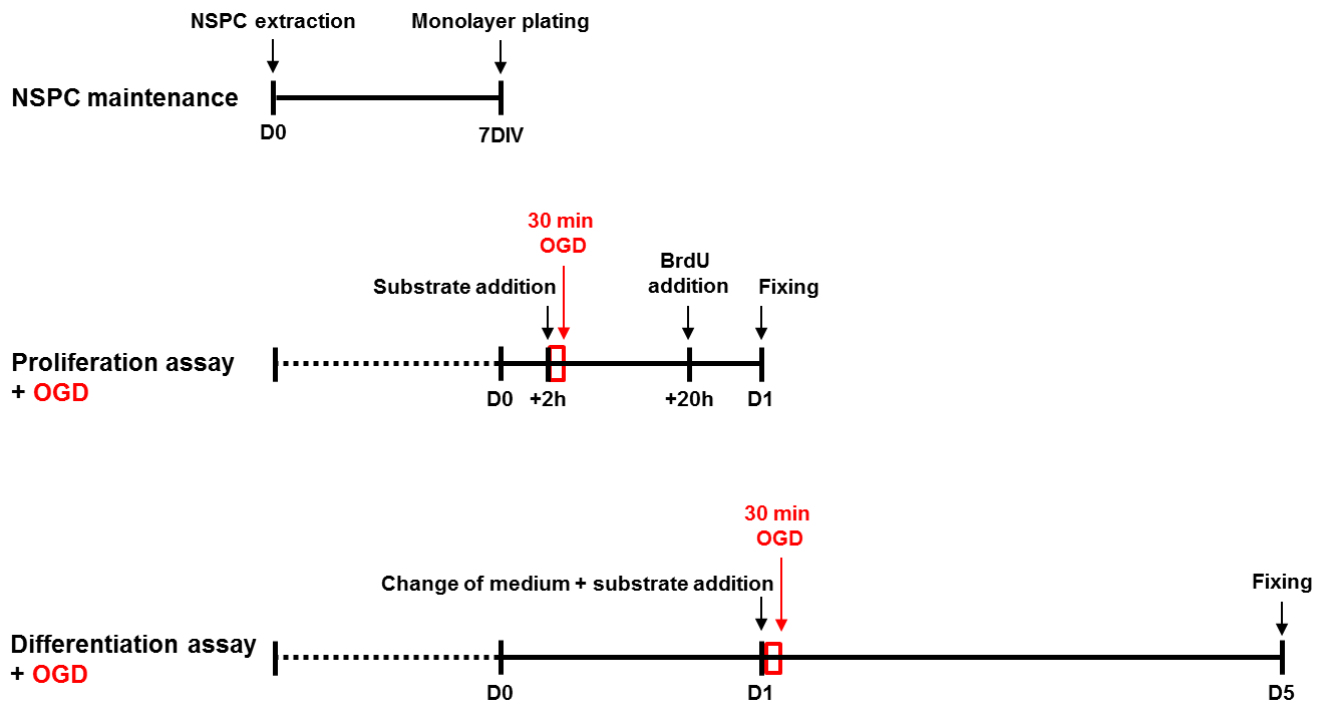


Figure 4. Characterization of AceDoPC using LC/ESI/MS and LC/ESI/MS/MS

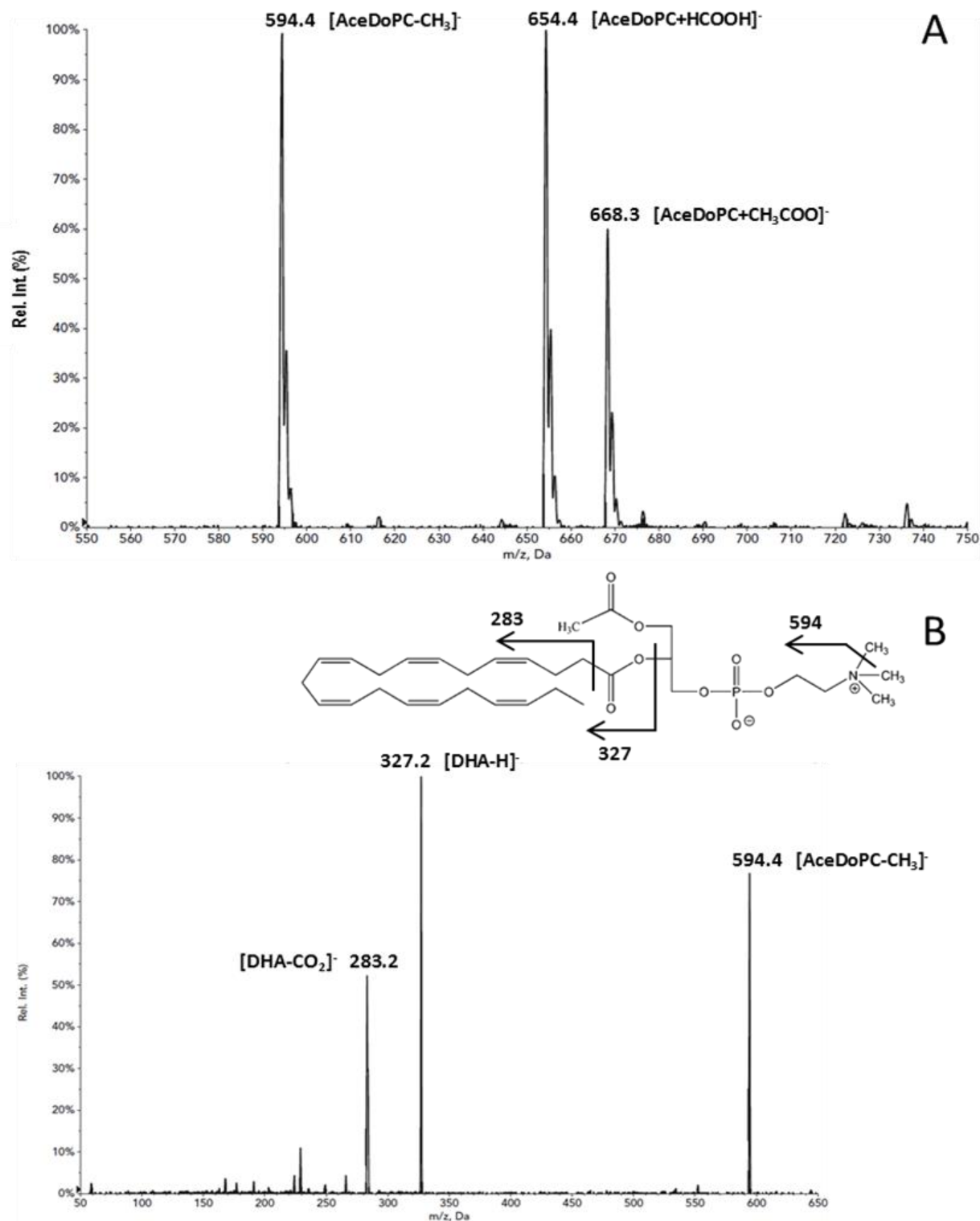


Figure 5. LC/ESI/MS/MS analysis of the major molecular ions of AceDoPC to daughter ion [DHA-H]⁻

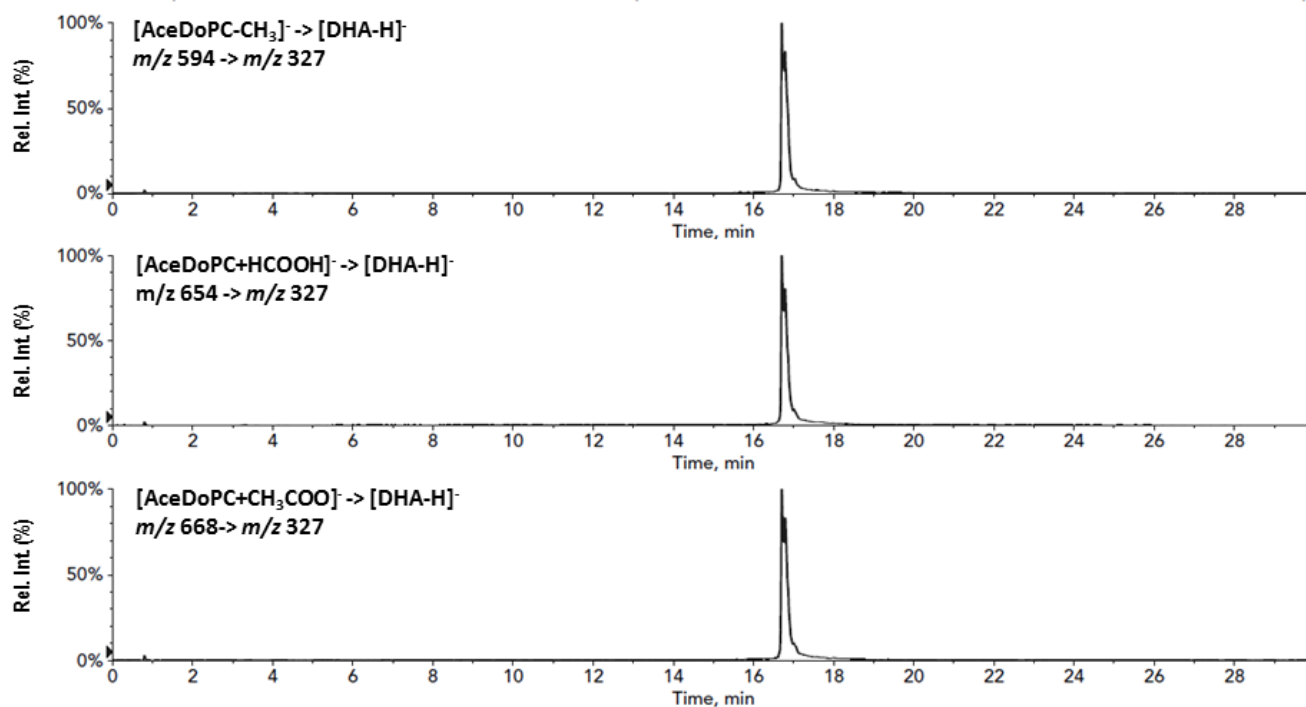


Figure 6. LC/ESI/MS/MS analysis of the major molecular ions of AceDoxyPC to daughter ion [PDX-H]⁻

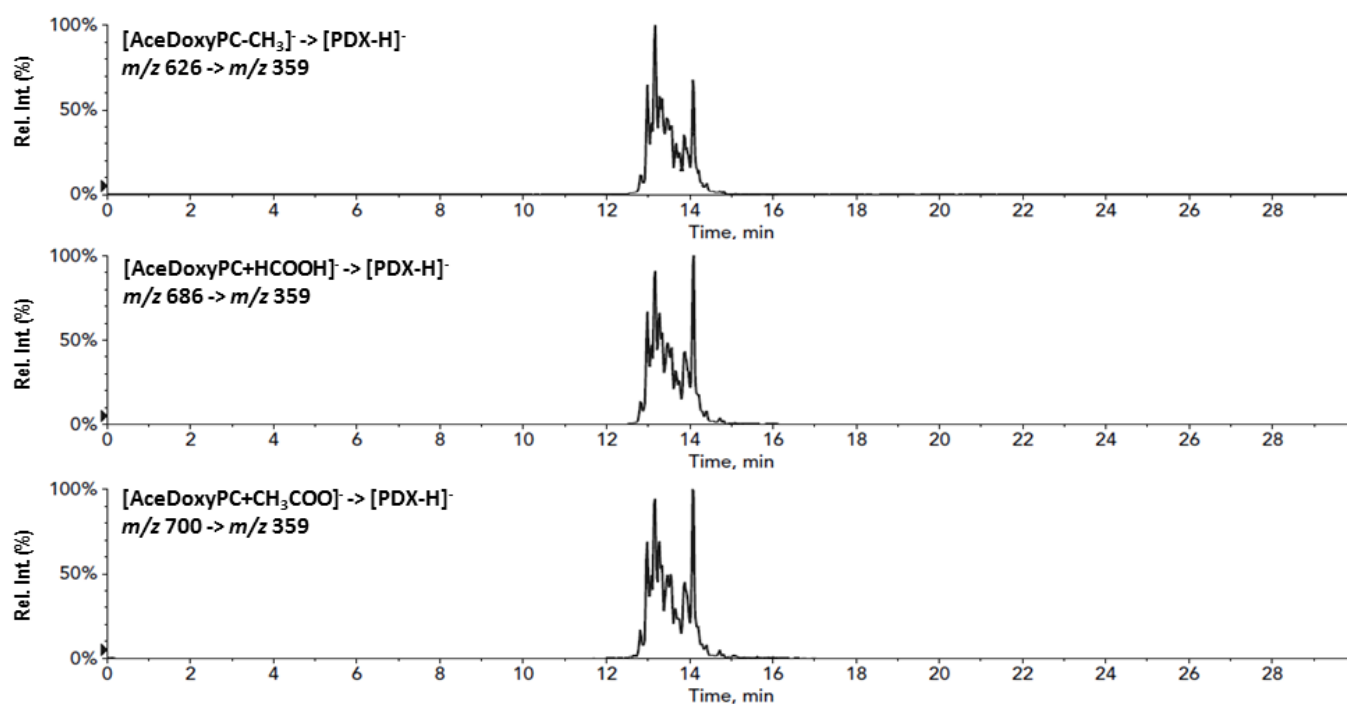


Figure 7. LC/ESI/MS analysis of AceDoPC and AceDoxyPC

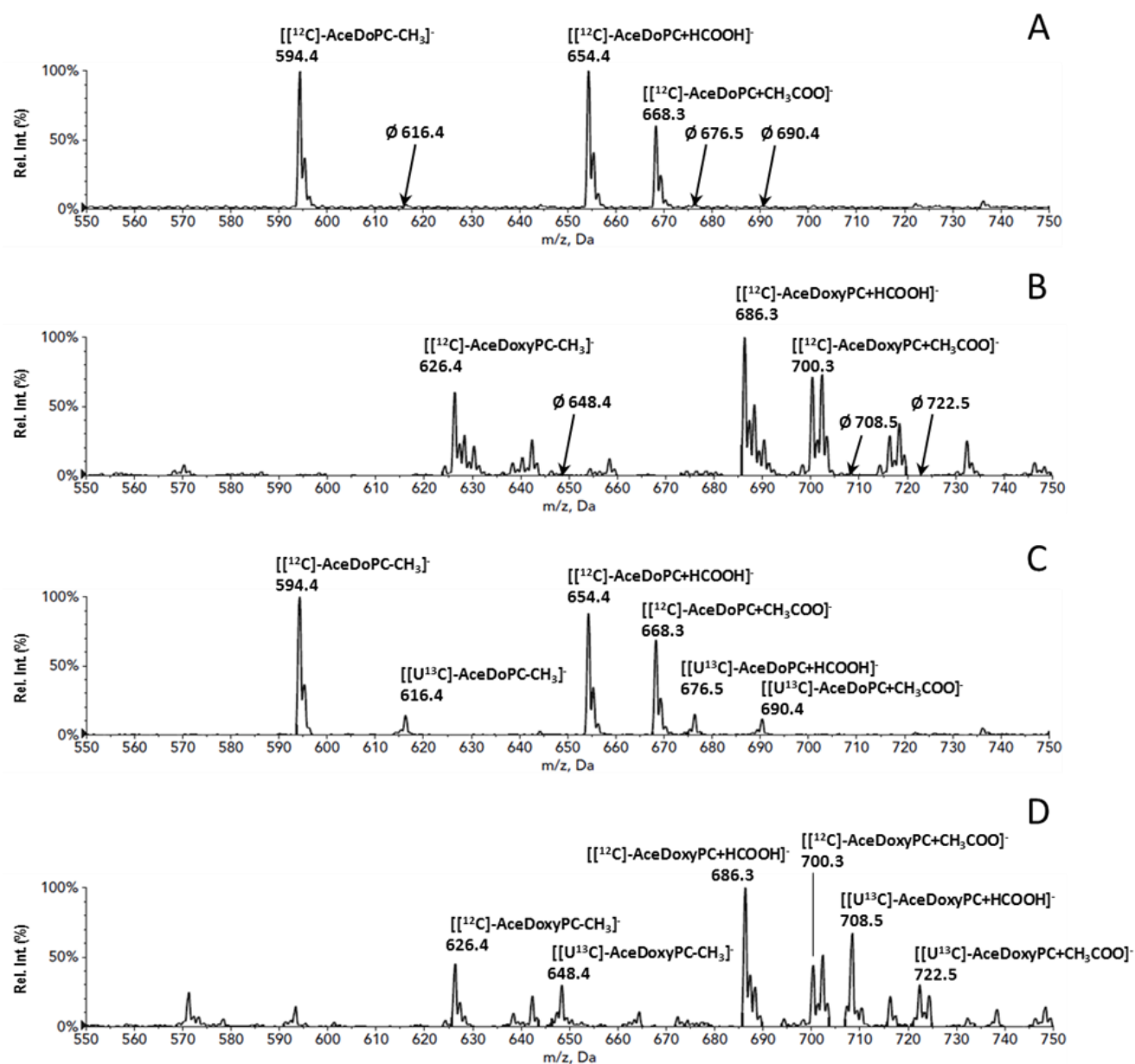


Figure 8. Identification of PDX as daughter ion of [AceDoxyPC-CH₃]

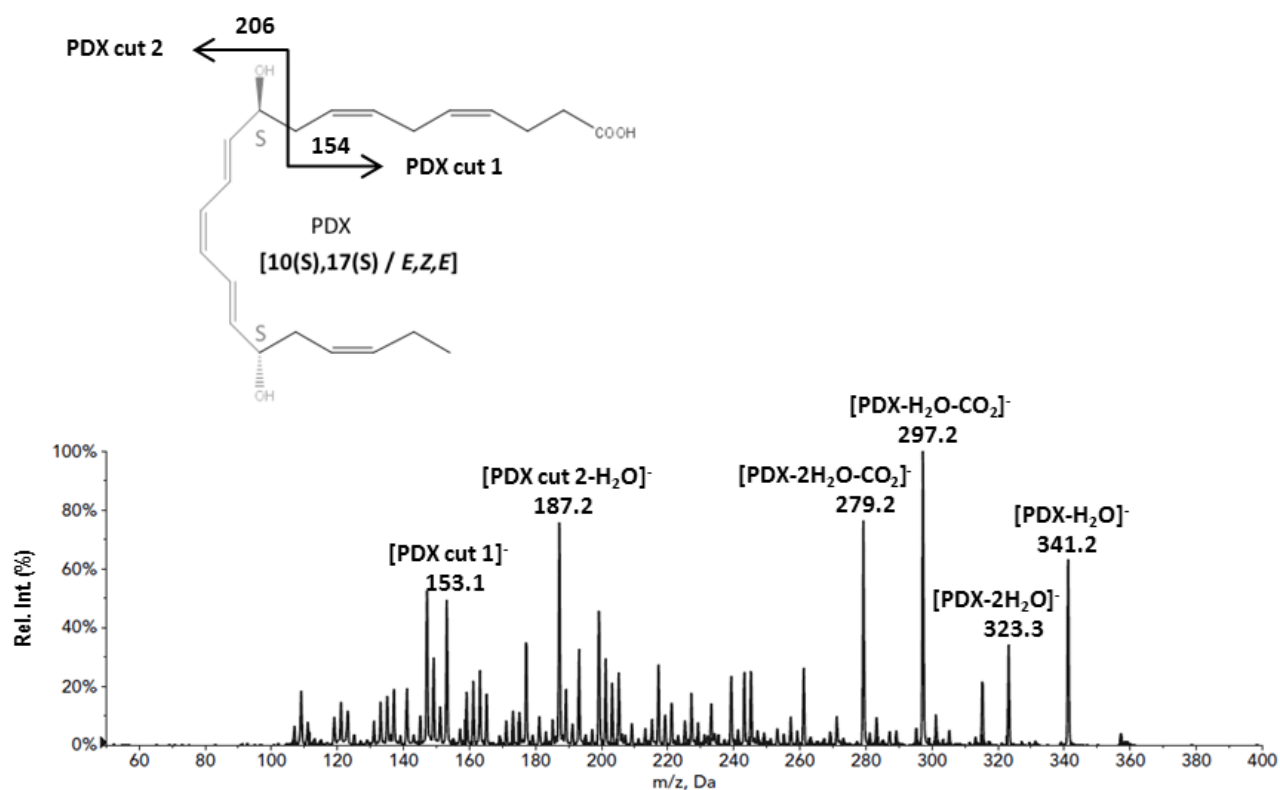


Figure 9. Immunocytochemistry following proliferation assays with DHA

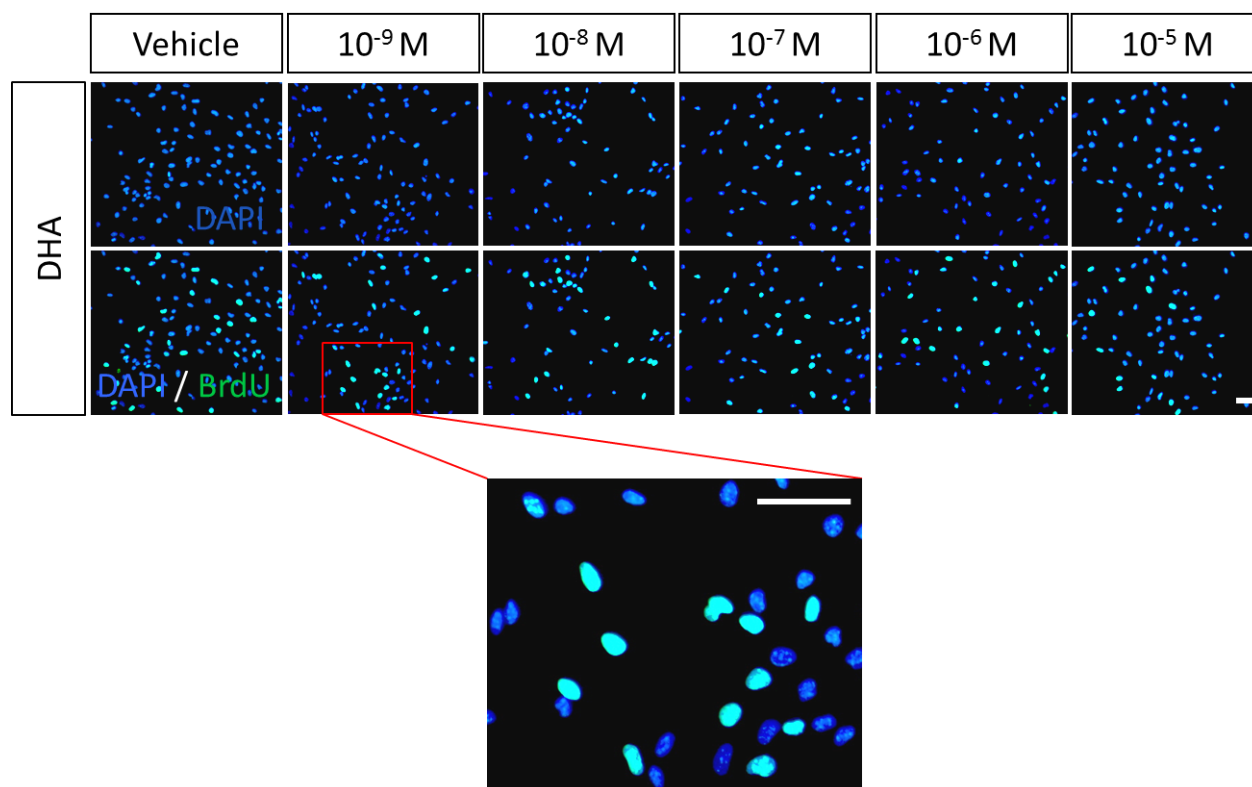


Figure 10. Proliferation rates and cell number of proliferation assays with DHA

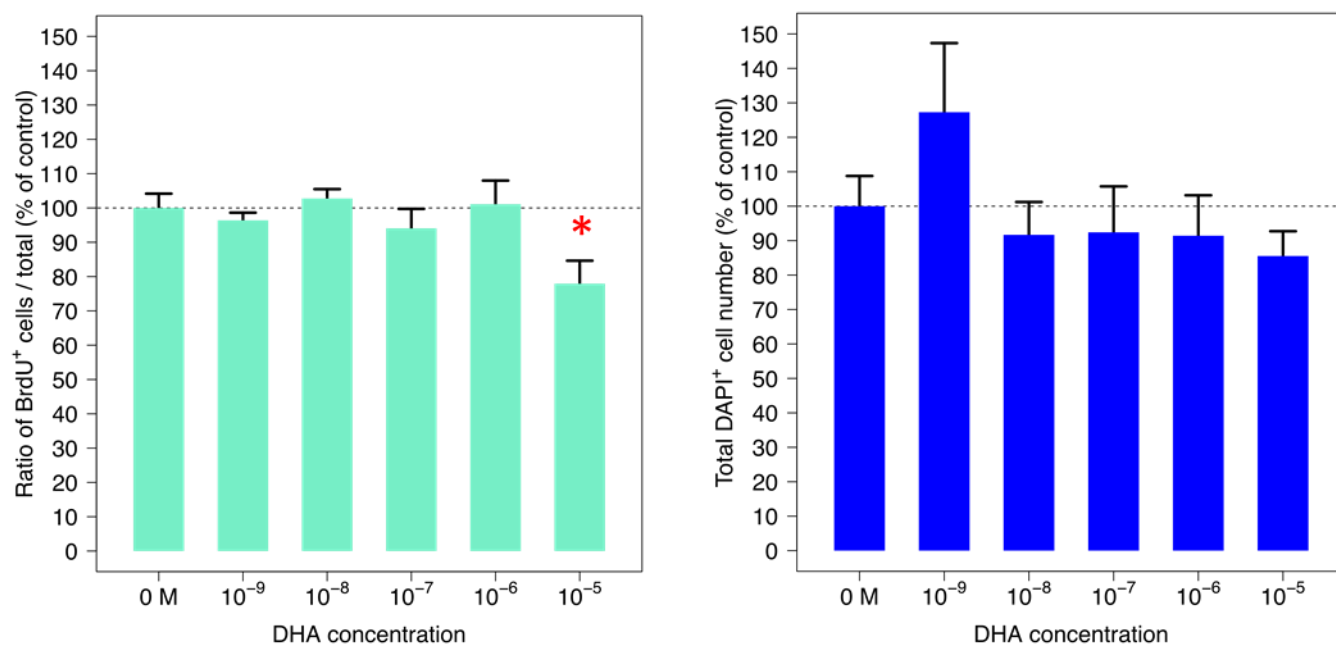


Figure 11. Immunocytochemistry following differentiation assays with DHA

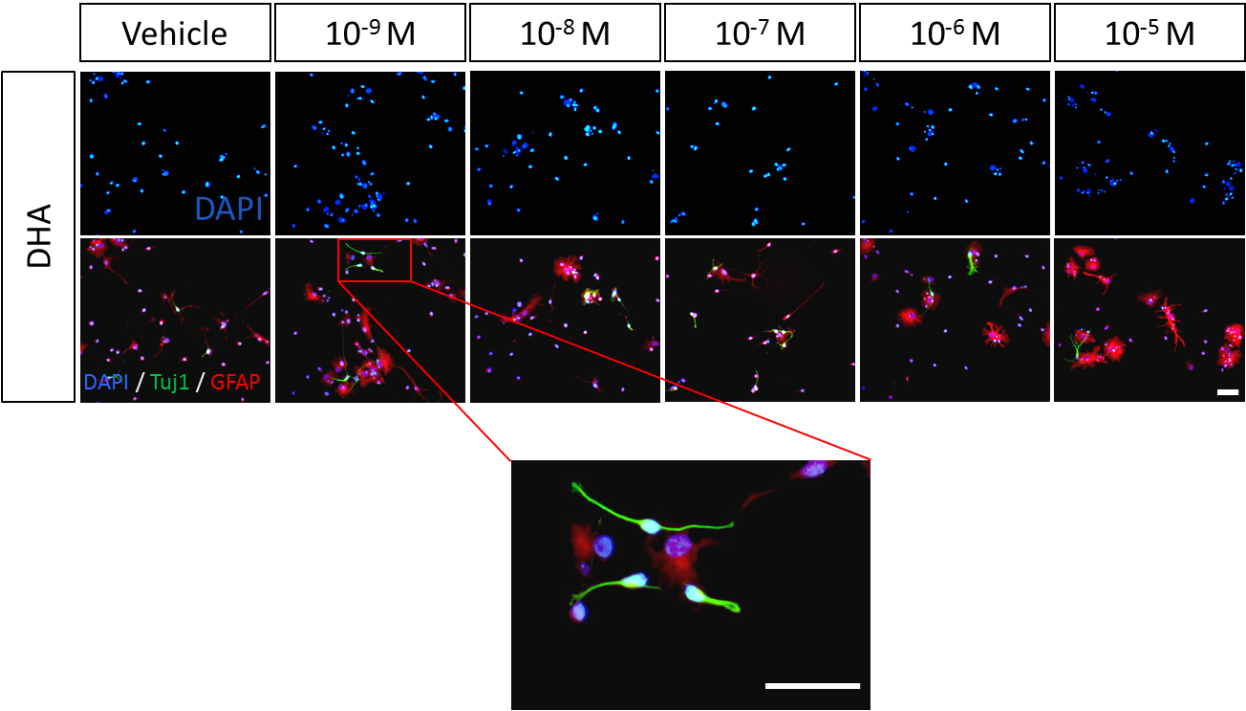


Figure 12. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number in differentiation assays with DHA

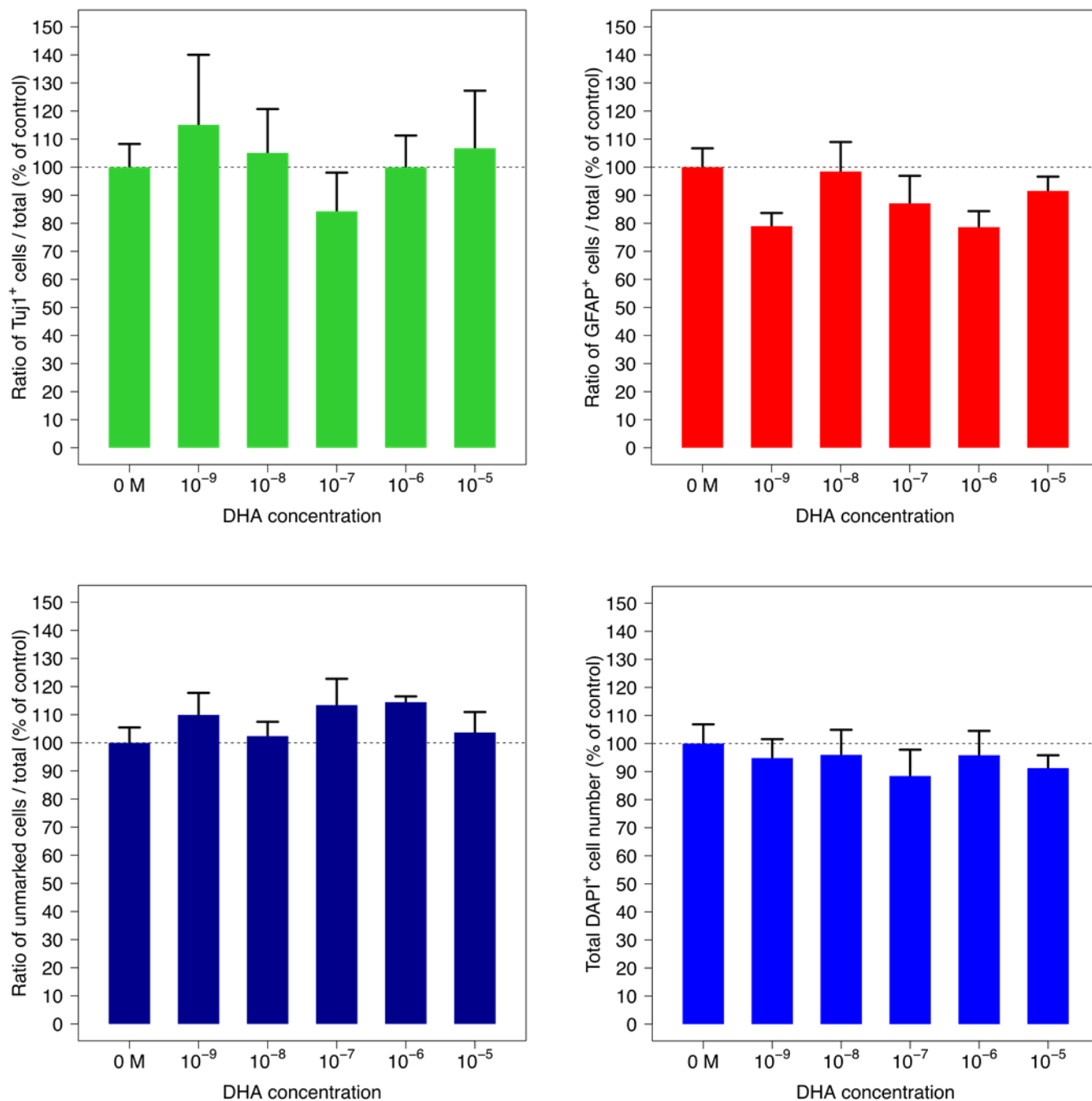


Figure 13. Proliferation rates and cell number of proliferation assays with AceDoPC

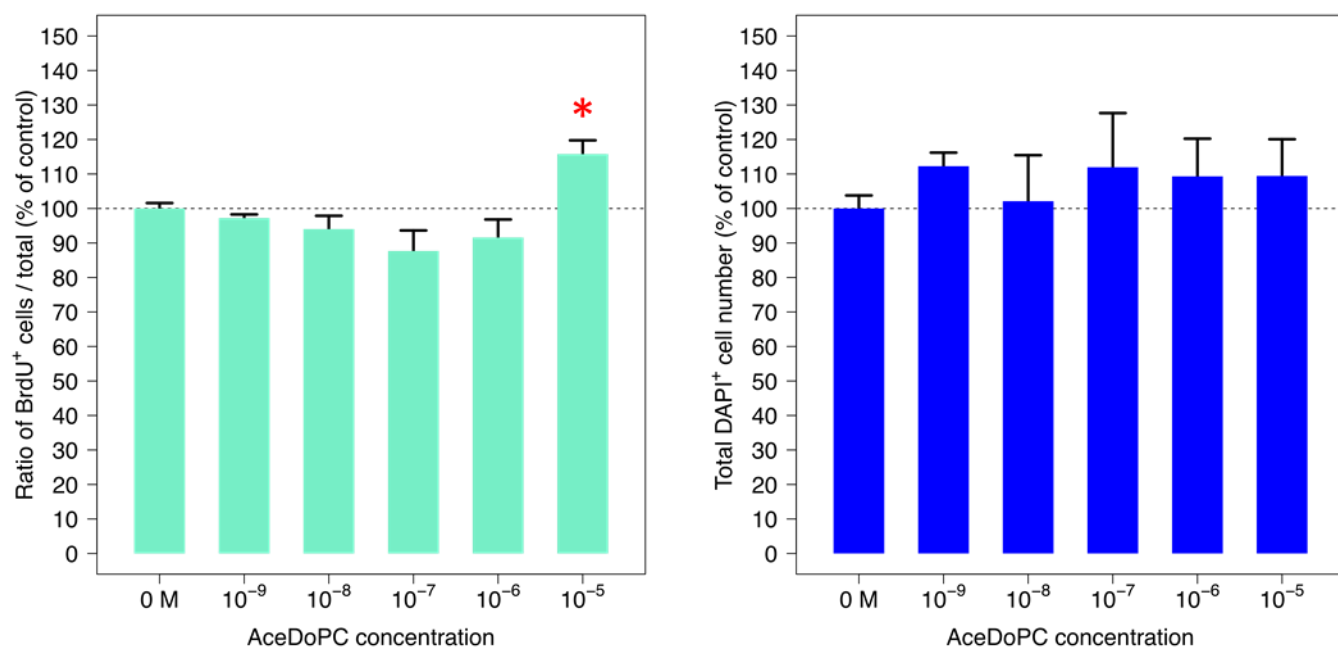


Figure 14. *Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with AceDoPC*

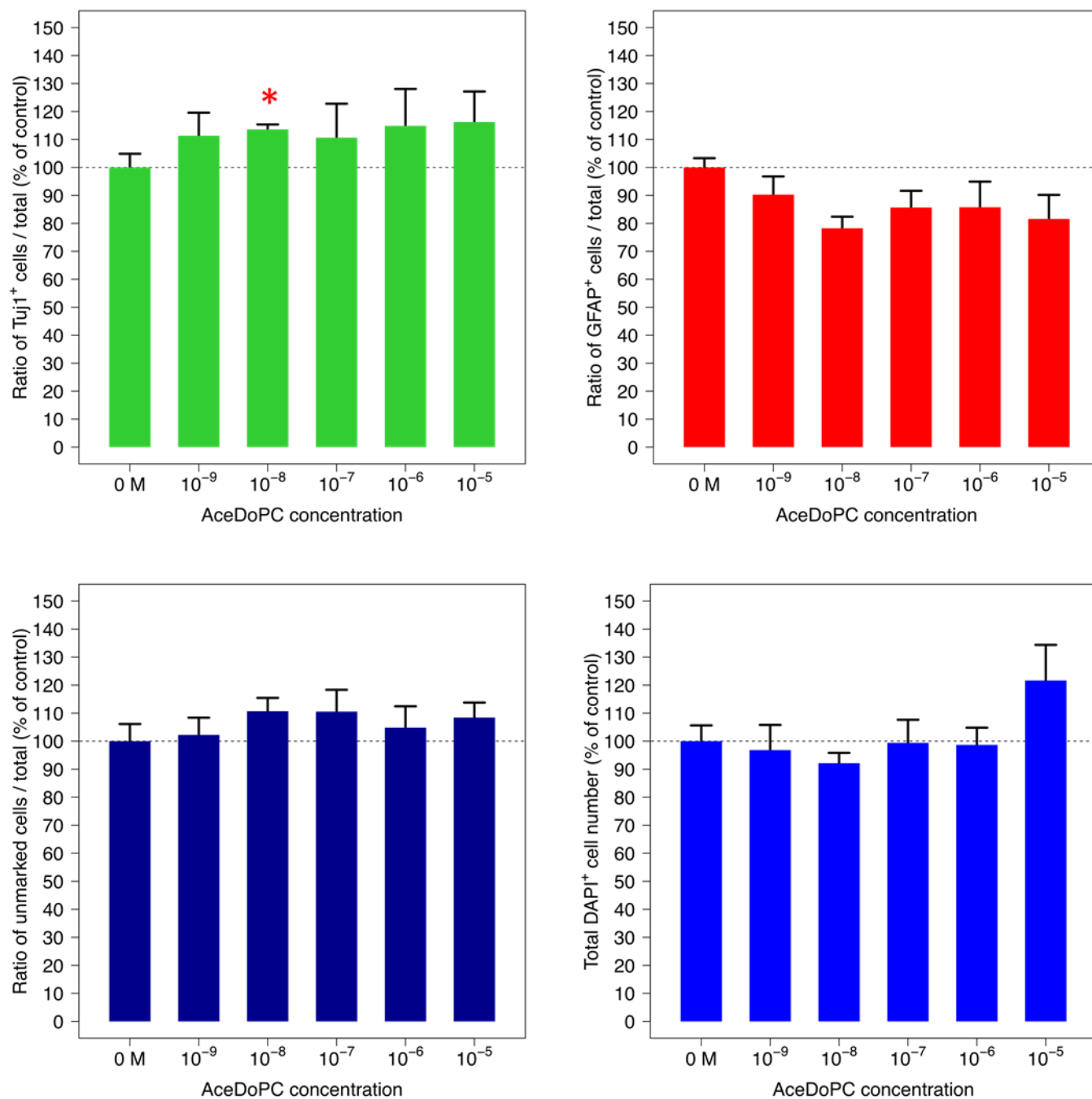


Figure 15. Proliferation rates and cell number of proliferation assays with PDX

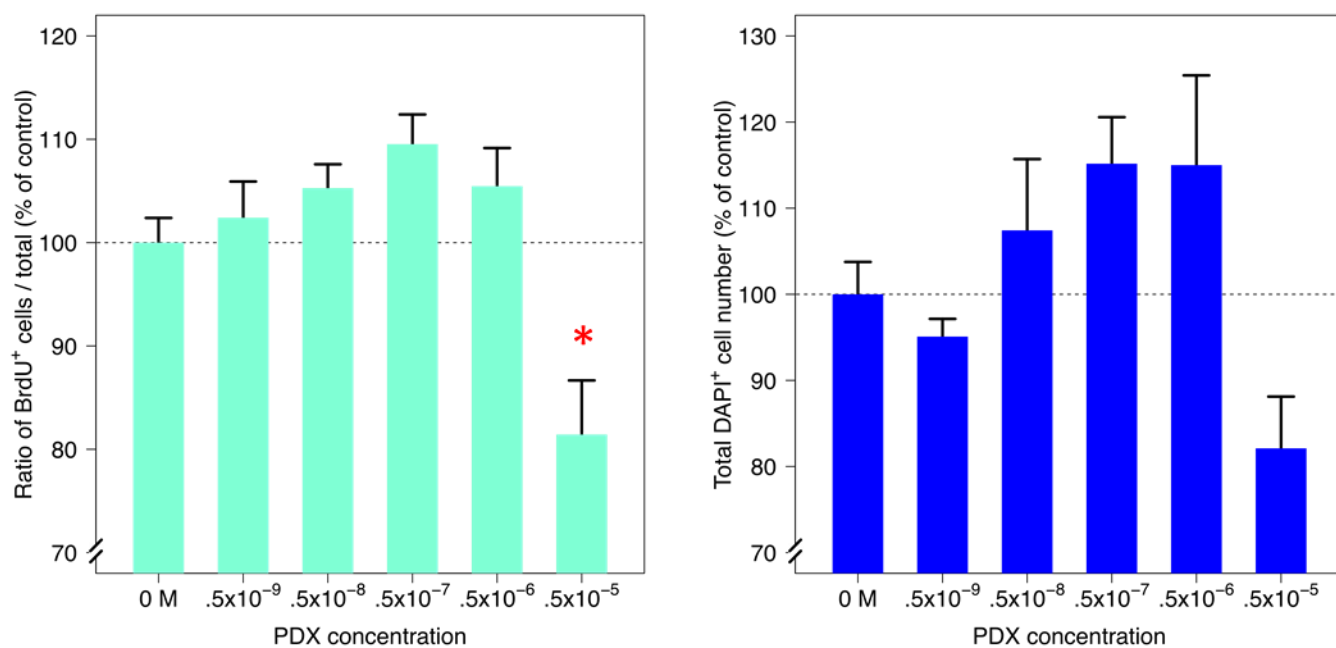


Figure 16. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with PDX

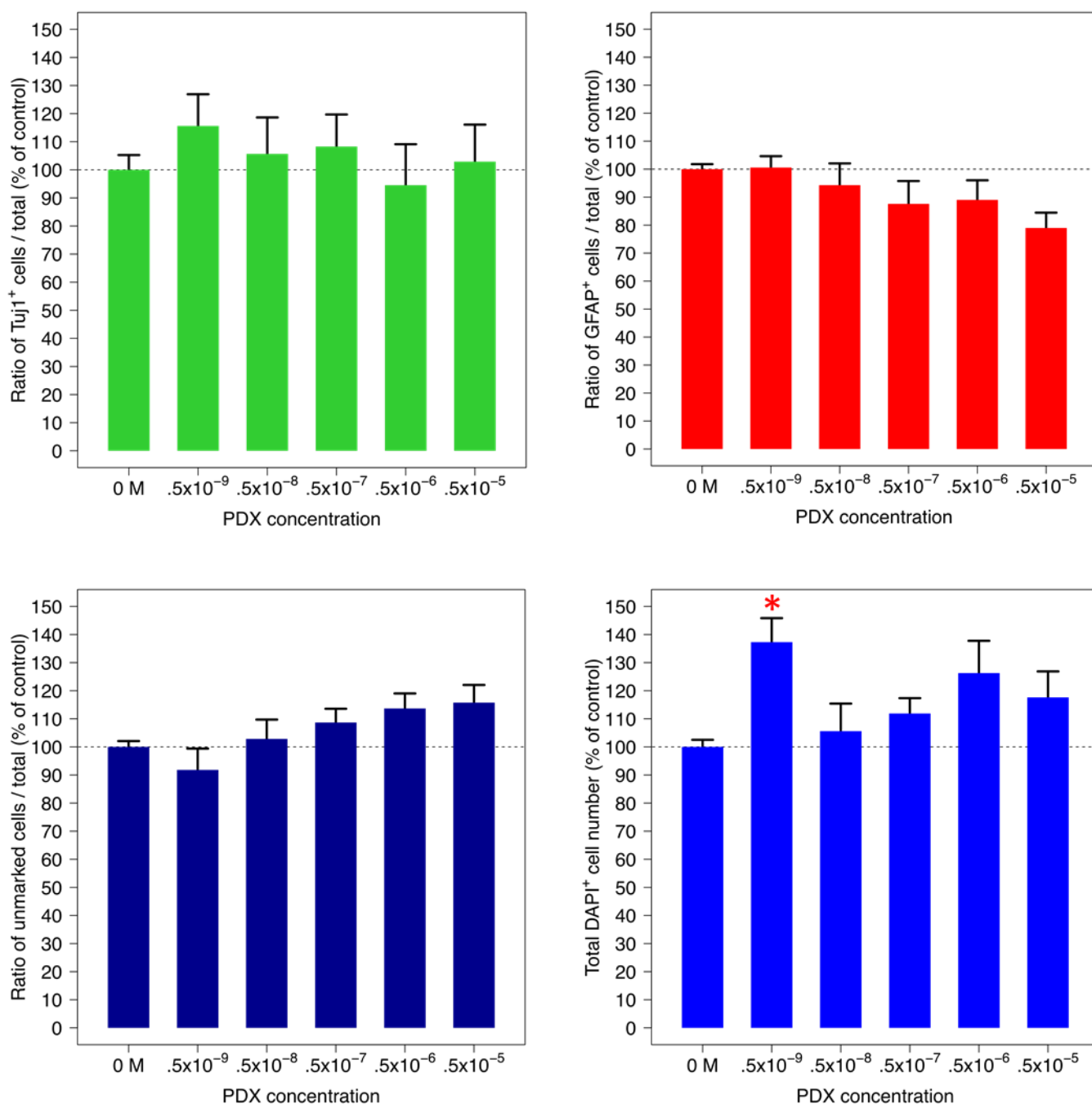


Figure 17. Proliferation rates and cell number of proliferation assays with DHA under OGD condition

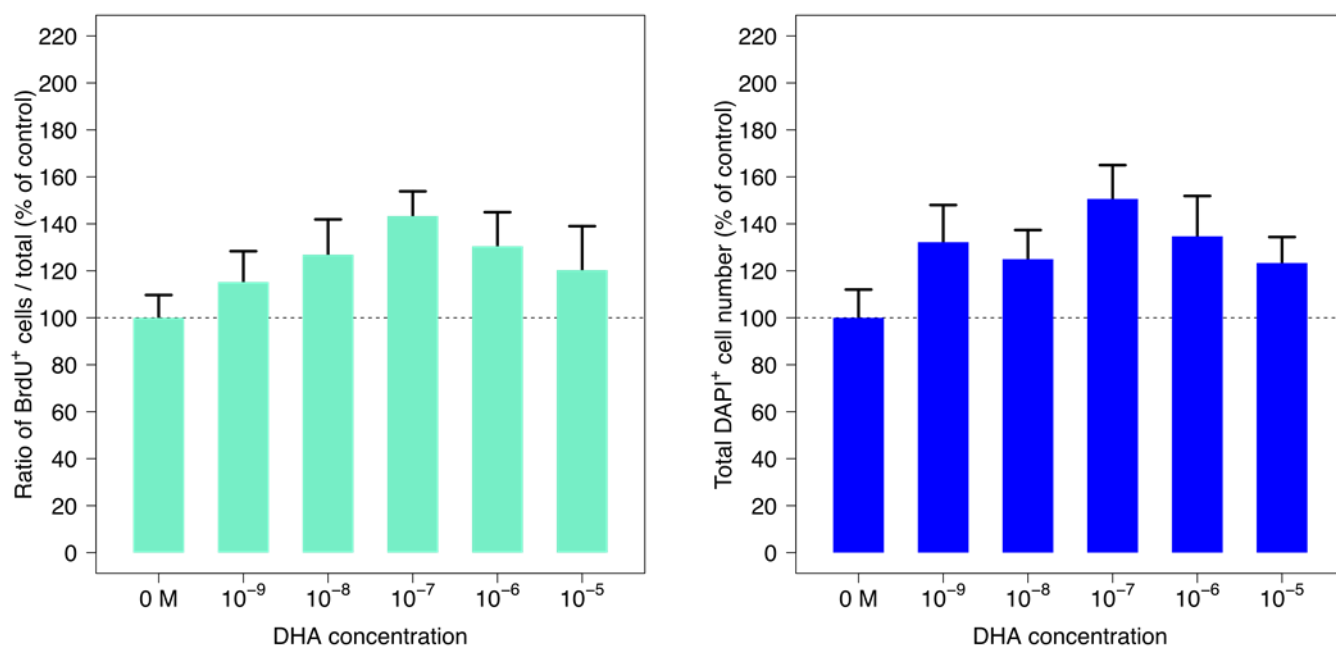


Figure 18. *Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with DHA under OGD condition*

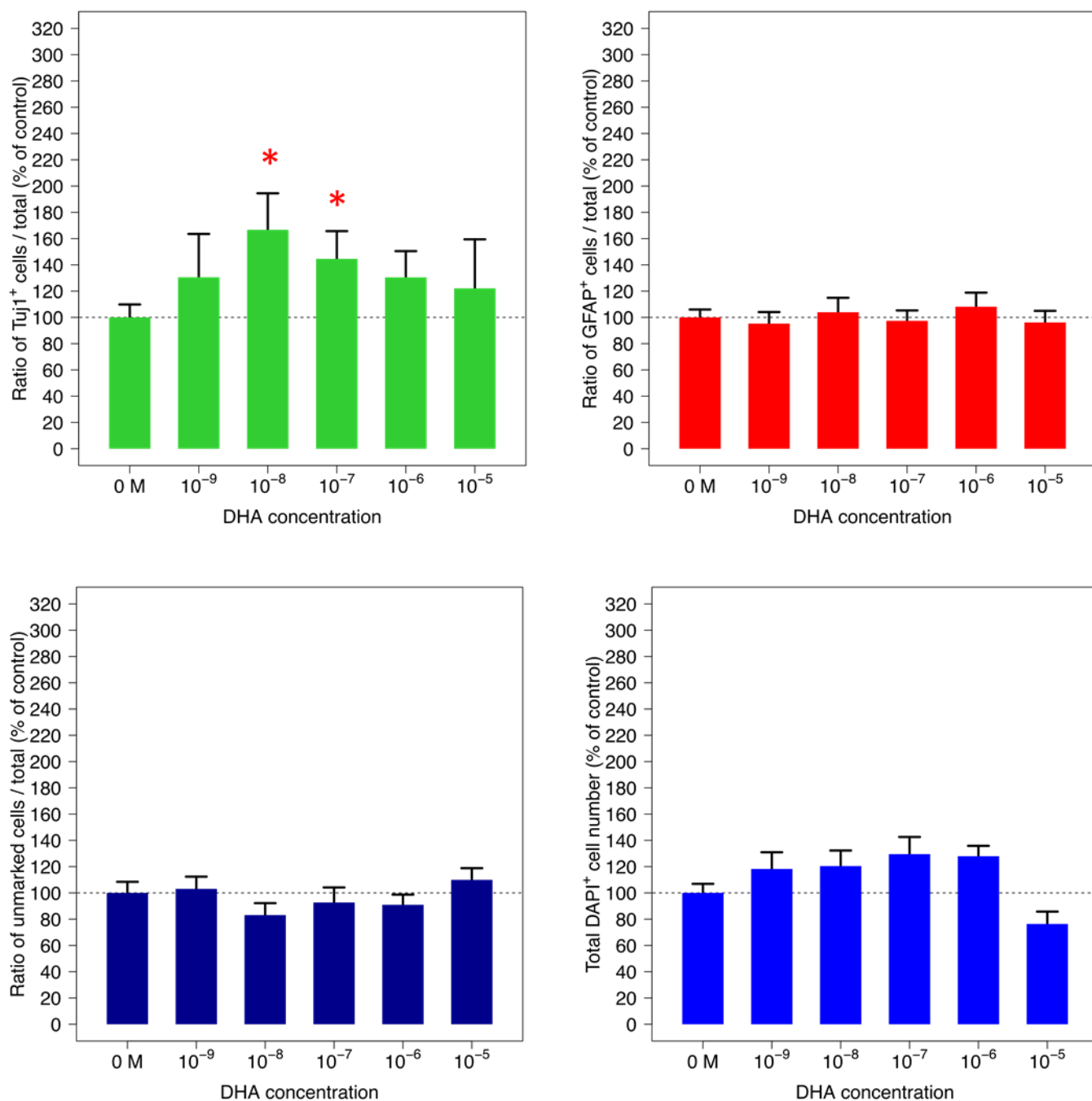


Figure 19. Proliferation rates and cell number of proliferation assays with AceDoPC under OGD condition

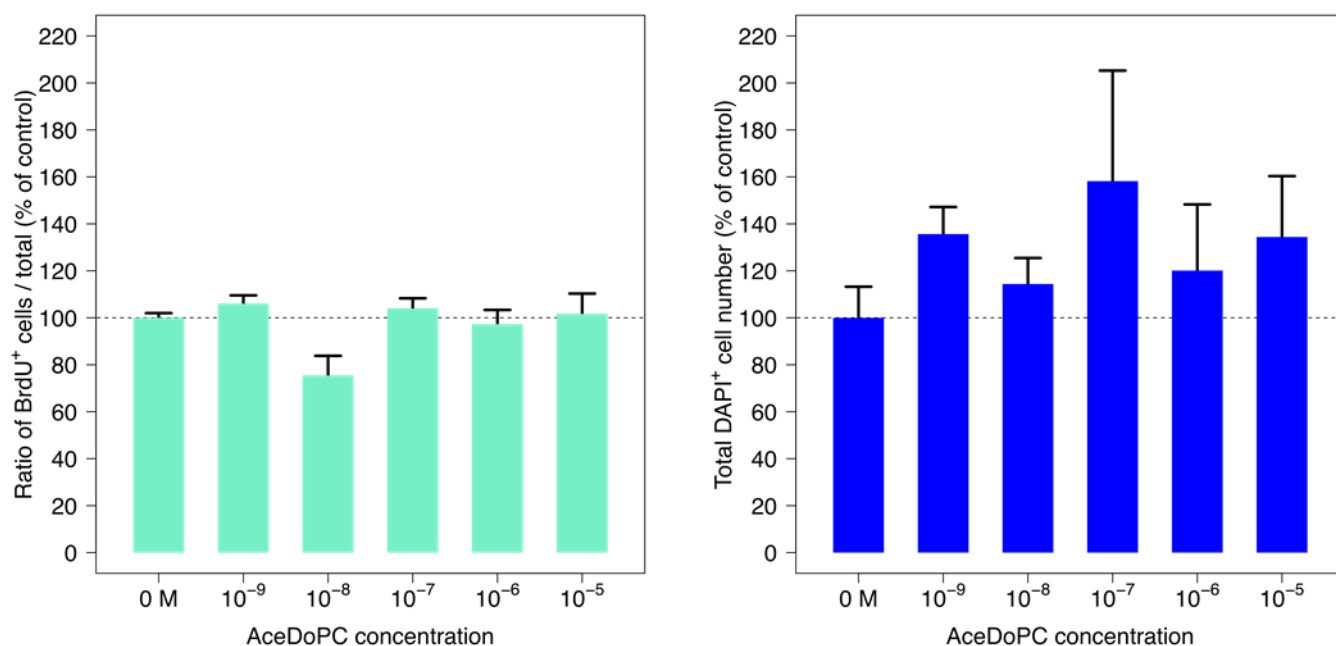


Figure 20. *Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with AceDoPC under OGD condition*

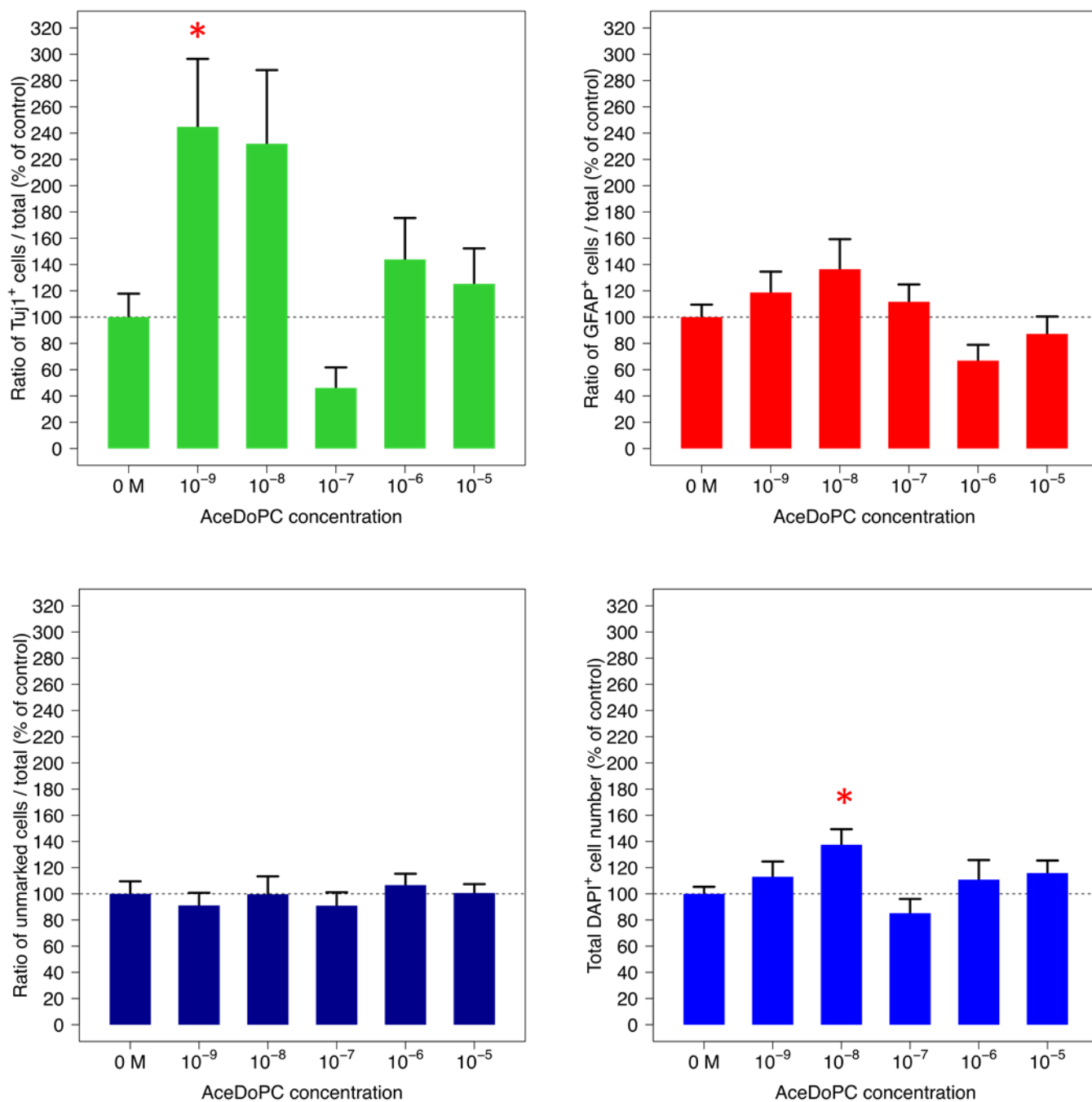


Figure 21. Proliferation rates and cell number of proliferation assays with PDX under OGD condition

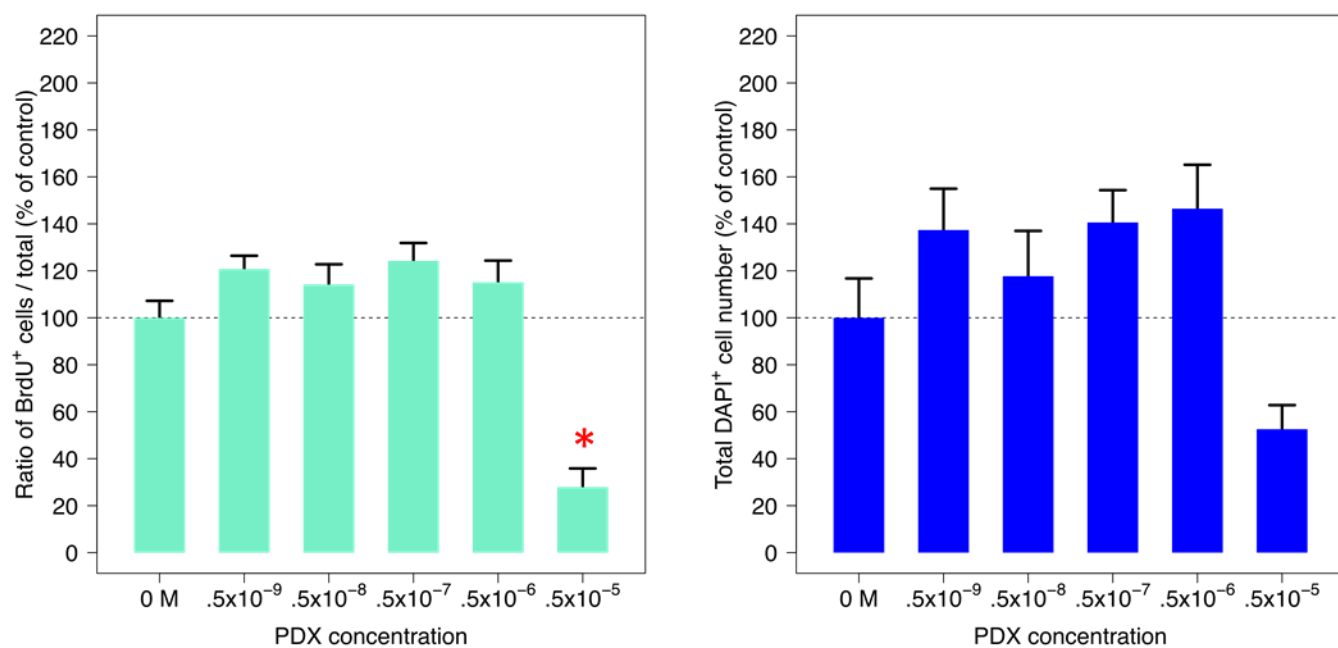


Figure 22. Neurogenesis rates, astrogenesis rates, unidentified cell number and total cell number of differentiation assays with PDX under OGD condition

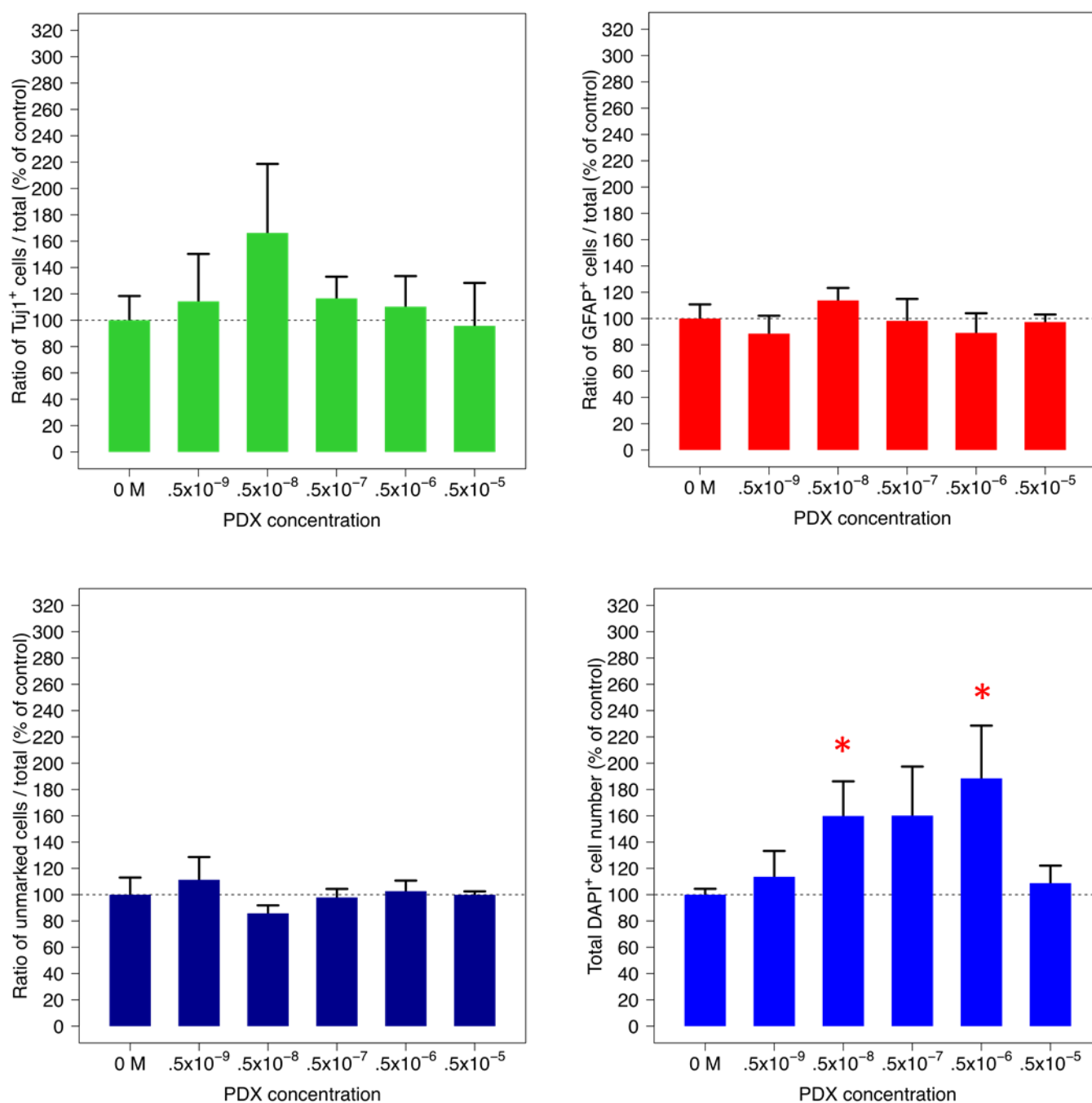


Figure 23. *Immunocytochemistry with Nrf2 following differentiation assays under varying conditions*

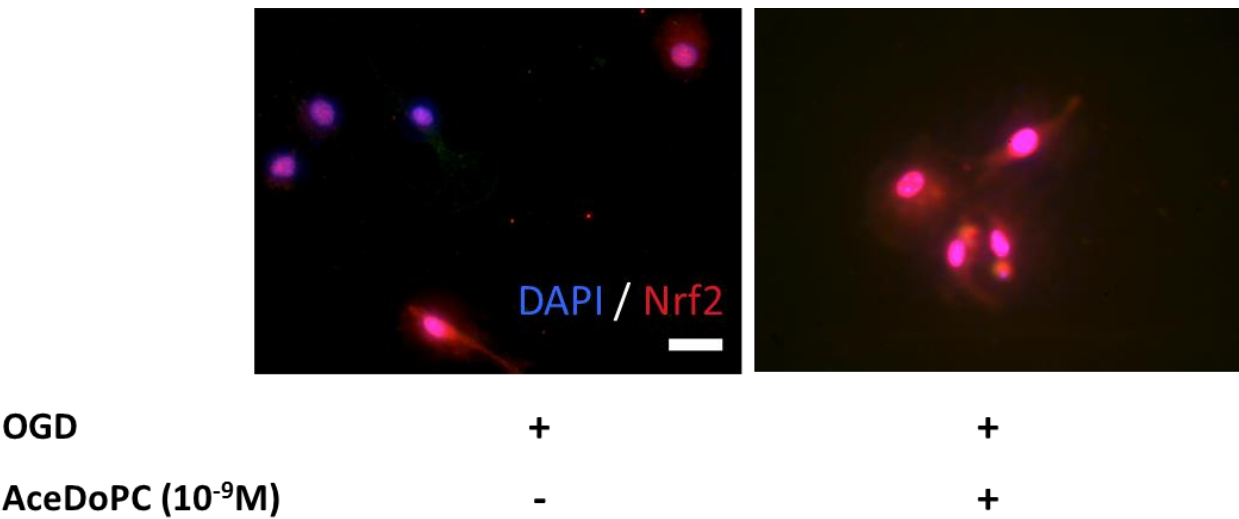


Table 1. Material list of components used for cell culture and origins

Origin	Components
Sigma-Aldrich (St. Louis, MO, U.S.A.).	D-glucose NaHCO ₃ progesterone heparin HEPES solution putrescine selenite apo-transferrin NaCl KCl MgCl ₂ CaCl ₂ trypsin hyaluronidase kynurenic acid poly-L-ornithine TritonX bovine serum albumin (BSA) fatty acid-free BSA DAPI Bradford reagent paraformaldehyde (PFA) insulin HCl α -tocopherol recombinant DNase 1 bromodeoxyuridine (BrdU) rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) IgG micro cover glass
Thermo Fisher Scientific (Waltham, MA, U.S.A.)	10x Dulbecco's modified Eagle Medium/Nutrient mixture F12 (DMEM/F12) Hanks' balanced salt solution (HBSS) L-glutamine penicillin/streptomycin laminin 0.25% trypsin-EDTA Alexa 488-conjugated goat anti-mouse IgG phosphate buffered saline (PBS)
Wako Chemicals (Osaka, Japan)	insulin HCl α -tocopherol
Peprtech (Offenbach, Germany)	human fibroblast growth factor-basic (bFGF) human epidermal growth factor (EGF)
Takara (Kusatsu, Japan)	recombinant DNase 1

Roche (Bale, Switzerland)	BrdU
BD Biosciences (Franklin Lakes, NJ, U.S.A.)	mouse anti-BrdU IgG
Covance (Princeton, NJ, U.S.A.)	mouse anti- β III tubulin (Tuj1) IgG
Jackson ImmunoResearch (West Grove, PA, U.S.A)	Cy3-conjugated donkey anti-rabbit IgG
Dako (Glostrup, Denmark)	rabbit polyclonal anti-GFAP IgG
Enzo Life Science (Farmingdale, NY, U.S.A)	rabbit polyclonal anti-nuclear factor erythroid 2-related factor 2 (Nrf2) antibody
Diagnostic Biosystems (Pleasanton, CA, U.S.A.)	fluoromount
BD Falcon (Corning, Tewksbury, MA, U.S.A.)	petri dish cell strainer (40 μ m nylon mesh)
Matsunami (Kishiwada, Japan)	micro cover glass
Cayman Chemicals (Ann Arbor, MI, U.S.A.)	DHA internal standards for lipid quantification
CarMen laboratory (Lyon, France)	AceDoPC internal standards for lipid quantification

Table 2. Ion mass of major molecular ions of AceDoPC and corresponding mass for AceDoxyPC ions

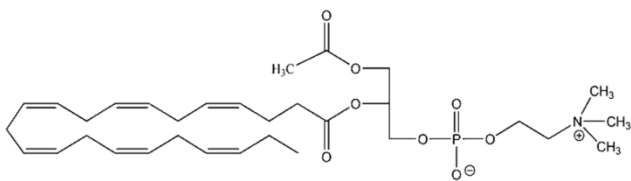
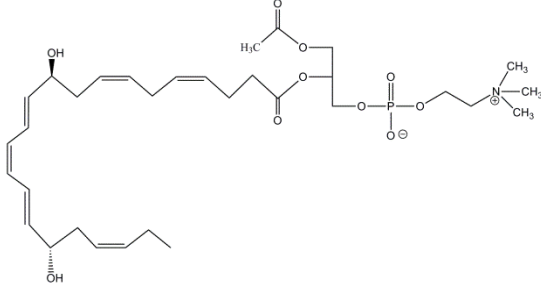
MRM negative mode			
AceDoPC (<i>m/z</i> 609.5)		AceDoxyPC (<i>m/z</i> 641.5)	
			
Parent ions (<i>m/z</i>)	Daughter ion (<i>m/z</i>)	Parent ions (<i>m/z</i>)	Daughter ion (<i>m/z</i>)
[AceDoPC-CH ₃] ⁻ (594.5)	[DHA-H] ⁻ (327.5)	[AceDoxyPC-CH ₃] ⁻ (626.5)	[PDX-H] ⁻ (359.5)
[AceDoPC+HCOOH] ⁻ (654.5)		[AceDoxyPC+HCOOH] ⁻ (686.5)	
[AceDoPC+CH ₃ COO] ⁻ (668.5)		[AceDoxyPC+CH ₃ COO] ⁻ (700.5)	

Table 3. Ion mass of major molecular ions of AceDoPC ($[^{12}\text{C}]$ and $[^{13}\text{C}]$) and corresponding mass for AceDoxyPC ions ($[^{12}\text{C}]$ and $[^{13}\text{C}]$)

Masses in red color represent masses of $[^{13}\text{C}]$ -labeled molecular ions.

MRM negative mode			
AceDoPC (m/z 609.5 ; 631.5)		AceDoxyPC (m/z 641.5 ; 663.5)	
Parent ions (m/z)	Daughter ion (m/z)	Parent ions (m/z)	Daughter ion (m/z)
[AceDoPC-CH ₃] ⁻ (594.5 ; 616.5)	[DHA-H] ⁻ (327.5 ; 349.5)	[AceDoxyPC-CH ₃] ⁻ (626.5 ; 648.5)	[PDX-H] ⁻ (359.5 ; 381.5)
[AceDoPC+HCOOH] ⁻ (654.5 ; 676.5)		[AceDoxyPC+HCOOH] ⁻ (686.5 ; 708.5)	
[AceDoPC+CH ₃ COO] ⁻ (668.5 ; 690.5)		[AceDoxyPC+CH ₃ COO] ⁻ (700.5 ; 722.5)	

Table 4. Identification and quantification of lipids extracted from adult NSPC culture under different conditions of differentiation assays

Lipid extraction and quantification from differentiation assays under physiological conditions with vehicle addition (no OGD) or pathological conditions (OGD) with vehicle, 10^{-9} M DHA or 10^{-9} M AceDoPC addition. “Vehicle” refers to control condition with vehicle only added to the cells. Quantification was computed with internal standards and expressed as pmoles/ μ g of proteins. Pool of n=6 for each group.

Quantification (pmoles/μg proteins)	NO OGD + Vehicle	OGD + Vehicle	OGD + DHA 10^{-9} M	OGD + AceDoPC 10^{-9} M
PGD2	40.19	85.86	91.13	11.71
PGF2a	N.D.	467.28	507.64	82.37
8-epi-PGF2a	203.14	334.85	362.38	55.19
LTB4	1.42	5.01	8.17	0.37
TXB2	15.85	21.05	23.45	3.49
15-HETE	48.89	171.30	42.72	6.59



FOLIO ADMINISTRATIF

THESE DE L'UNIVERSITE DE LYON OPEREE AU SEIN DE L'INSA LYON

NOM : LO VAN

DATE de SOUTENANCE : 12/01/2017

Prénoms : Amanda

TITRE : Study of the effects of docosahexaenoic acid (DHA) and a structured phospholipid containing DHA on physiological and pathological conditions of neurogenesis *in vitro*

NATURE : Doctorat

Numéro d'ordre : 2017LYSEI005

Ecole doctorale : ED205 Ecole Doctorale Interdisciplinaire Sciences Santé

Spécialité : Biochimie

RESUME :

Docosahexaenoic acid (DHA, 22:6n-3) is an essential omega-3 polyunsaturated fatty acid (PUFA). It is specifically enriched in the brain and the retina and it is required for visual acuity, proper brain development and cerebral functions. While DHA deficiency in the brain was shown to be linked to the emergence of cerebral diseases (i.e. Alzheimer's disease or Parkinson's disease), studies showed that a dietary intake of omega-3 PUFA could prevent or attenuate neurologic disturbances linked with ageing or neurodegenerative diseases. In this context, it is primary to deliver DHA efficiently to the brain. Targeting the brain with DHA might offer great promise in developing new therapeutics for neurodegenerative diseases. The French host laboratory previously synthesized a stabilized form of lysophosphatidylcholine-DHA, which is main vector of DHA transportation to the brain, of structure 1-acetyl,2-docosahexaenoyl-glycerophosphocholine, patented and named AceDoPC®. Injection of AceDoPC or DHA after experimental ischemic stroke showed that both molecules also had neuroprotective effects. These potential neuroprotective effects are expected to be due, in part, to DHA conversion into oxygenated metabolites. This study aims to investigate the beneficial effects of DHA and its derived metabolites either unesterified or esterified within structured phospholipids on a model of neurogenesis *in vitro* under physiological or pathological conditions.

The first objective of this work was then to synthesize the DHA-containing structured phospholipid AceDoPC®, DHA oxygenated derivative protectin DX (PDX) and a novel structured phospholipid of protectin: 1-acetyl,2-protectinDX-glycerophosphocholine (AceDoxyPC). The second objective was to investigate the effects of DHA, AceDoPC and PDX on neurogenesis using an *in vitro* model of neurogenesis, namely cultures of neural stem progenitor cells (NSPCs) derived from the adult mouse brain under physiological or pathological conditions (ischemic conditions). Following this, the third objective of this work was to identify the mechanisms involved in such response to stress induced under pathological conditions.

Synthesis of the novel structured phospholipid AceDoxyPC was successfully performed by double enzymatic lipoxygenation of AceDoPC and identification of the product was possible using advanced techniques of liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS). Future studies on this potential neuroprotective molecule transporter are to be investigated in the near future. Neurogenesis study of cell cultures with AceDoPC showed enhanced neurogenesis compared to addition of unesterified DHA or vehicle control, especially under pathological conditions. Preliminary studies of the potential mechanisms involved in neuroprotection hinted that AceDoPC neuroprotective and regenerative effects might be due in part to its anti-oxidative effects.

MOTS-CLÉS : docosahexaenoic acid, 1-acetyl,2-docosahexaenoyl-glycerophosphocholine, protectin, phospholipid, neuroprotection, neurogenesis, stroke, oxidation, brain

Laboratoire (s) de recherche : Laboratoire en Cardiovasculaire, Métabolisme, Diabétologie et Nutrition (CarMeN) et Department of Developmental Neuroscience, Center for Neuroscience and Translational Research

Directeur de thèse: Pr. Nathalie BERNOUD-HUBAC et Pr. Noriko Osumi

Président de jury : Pr. Hiromu YAWO

Composition du jury :

Pr. Hiromu YAWO

Pr. Nathalie BERNOUD-HUBAC

Pr. Toru NAKAZAWA

Dr. Ichiko NISHIJIMA

Dr. Tetsuya YAMADA