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Application of the adverse outcome pathway to identify molecular changes in prenatal brain programming induced by IUGR: Discoveries after EGCG exposure

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ABSTRACT

Following a multi-disciplinary approach integrating information from several experimental models we have collected new evidence supporting, expanding and redesigning the AOP "Disrupted laminin/int-β1 interaction leading to decreased cognitive function". Investigations *in vitro* in rabbit and rat neurospheres and *in vivo* in mice exposed to EGCG (epigallocatechin-gallate) during neurodevelopment are combined with *in vitro* evaluations in neural progenitor cells overexpressing int-β1 and literature information from int-β1 deficiency models. We have discovered for the first time that neural progenitor cells from intrauterine growth restricted (IUGR) animals overexpress int-β1 at gene and protein level and due to this change in prenatal brain programming they respond differently than control neurospheres to the exposure of EGCG, a compound triggering neural progenitor cell migration alterations. We have also identified that EGCG developmental exposure has deleterious effects on neuronal branching and arborization *in vitro* and *in vivo*. Our results warn that a thorough developmental neurotoxicity characterization of this and other catechin-based food supplements is needed before recommending their consumption during pregnancy.

1. Introduction

Epigallocatechin gallate (EGCG), the most abundant catechin in green tea, is currently being broadly studied as a promising treatment for several diseases including Alzheimer' and Parkinson's disease, cancer, obesity or inflammation (Cano et al., 2019; Pervin et al., 2018). EGCG has even been proposed as a possible treatment during pregnancy to prevent/treat alterations of brain development due to Down's syndrome (Catuara-Solarz et al., 2016; Souchet et al., 2019; Stagni et al., 2016, 2021), or fetal alcoholic syndrome (Almeida-Toledano et al., 2021; Long et al., 2010; Tiwari et al., 2010). However, the safety of EGCG during prenatal development is still unclear because it has not been subject to classical risk assessment evaluations (Abdel-Rahman

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Abbreviations

AOP	Adverse outcome pathway
BMP7	Bone Morphogenetic Protein 7
CA1	Cornu ammonis
CEEA	Ethic Committee for Animal Experimentation
cKE	contrary/compensating key event
CTB	CellTiter-Blue Reagent
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNT	Developmental neurotoxicity
EC50	50% effective concentration
ECG	Epicatechingallate
ECM	extracellular matrix
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
FGF2	Fibroblast growth factor 2
G56	4,4′-bis[(3,4,5-trihydroxybenzoyl)oxy]-1,1′-biphenyl
GD	gestational day
GFAP	glial fibrillary acidic protein
HPLC	High Performance Liquid Chromatography
int-β1	Integrin-β1
int-β4	Integrin-β4
IUGR	Intrauterine growth restriction
KEs	Key events
KNIME	Konstanz Information Miner
MIE	Molecular initiating event
NOAEL	No Observed Adverse Effect Level
NPCs	Neural progenitor cells
OECD	Organisation for Economic Co-operation and Development
OL	Oligodendrocytes
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
PFA	Paraformaldehyde
PND	Postnatal day
poly-HEMA	Polyhydroxyethylmethacrylate
qRT-PCR	Quantitative real-time polymerase chain reaction
ROCK	Rho kinase
SC	Solvent control
SM	Supplementary material
SO	Stratum oriens
SR	Stratum radiatum

et al., 2011) and comprehensive information on specific toxicities concerning subtle endpoints like developmental neurotoxicity (DNT) is missing (Barenys et al., 2016a). Previous studies using the Neurosphere Assay shed light on the potential neurodevelopmental toxic effects of EGCG proving adverse effects on neural progenitor cell' (NPC) migration and unravelling the mechanism behind it: the binding of EGCG to the extracellular matrix protein laminin, which prevents the interaction of the integrin-β1 (int-β1) cell adhesion molecule with laminin (Barenys et al., 2016b, 2017; Kühne et al., 2019; Peter et al., 2017; Suzuki and Isemura, 2001).

However, the dose makes the poison, so in the present study, we investigated in the Neurosphere Assay if 5-, 50- or 500-times lower concentrations of EGCG would no longer have this DNT effect and could be safely used as a neuroprotective prenatal therapy. In parallel we tested the same concentrations of a synthetic analogue of EGCG named 4,4′-bis[(3,4,5-trihydroxybenzoyl)oxy]-1,1′-biphenyl or G56 with the same purpose, because it was previously selected in the Neurosphere Assay as the safest among a group of synthetic analogues regarding NPC migration and it was suggested to be the best candidate for potential future therapeutic or preventive alternatives to EGCG during the prenatal period (Kühne et al., 2019).

For both compounds we didn't only evaluate their safety, but also their efficacy as neuroprotective agents at this lower concentration range. In this case and based on the known antioxidant activity of EGCG we studied their use as neuroprotective prenatal therapies during intrauterine growth restriction (IUGR) (Barenys et al., 2021). IUGR is a widespread disorder during development defined as a significant reduction of fetal growth rate mainly due to placental insufficiency (Kady and Gardosi, 2004). This condition leads to fetal development under chronic hypoxia resulting in white matter injury due to myelination defects and in a significant reduction in oligodendrogenesis (Barenys et al., 2021; Kühne et al., 2022; Reid et al., 2012; Rideau Batista Novais et al., 2016; Tolcos et al., 2011). In the long run, IUGR can manifest in severe neurodevelopmental sequelae like neurocognitive disorders, learning disabilities, attention deficit hyperactivity disorder, or autism spectrum disorder (Abel et al., 2013; Illa et al., 2013; Leitner et al., 2007; Mwaniki et al., 2012). Hence it was reasonable to test the widely used green tea component EGCG and the alternative G56 on the critical neurodevelopmental endpoints of migration and oligodendrocyte (OL) differentiation. To study the neuroprotective potential of EGCG and G56 to rescue IUGR induced oligodendrogenesis defects, we used a novel 3D rabbit neurosphere model, which mimics the basic processes of brain development of control and IUGR rabbit pups including NPC proliferation, migration and differentiation (Barenys et al., 2021; Baumann et al., 2014; Moors et al., 2009). The species rabbit was chosen due to its similarity to human placentation and perinatal brain development (Barenys et al., 2021; Drobyshevsky et al., 2014; Workman et al., 2013) and because it was previously proven to reflect better the neurodevelopmental alterations observed in humans than the rodent species (Batalle et al., 2012, 2014; Eixarch et al., 2012; Illa et al., 2013).

Based on the obtained results with EGCG and G56 in control and IUGR rabbit neurospheres, we further explored the consequences of EGCG exposure to dendrite formation and continued building a previously postulated adverse outcome pathway (AOP) complementing it with a combination of key events (KEs) related with both endpoint, cell migration and dendrite formation.

2. Material and Methods

2.1. IUGR induction

All animal procedures were approved by the Ethics Committee for Animal Experimentation (CEEA) of the University of Barcelona and all protocols were accepted by the Department of Environment and Housing of the 'Generalitat de Catalunya' (license number: 11126, date of approval: 24/5/2021, procedure CEEA number: OB 340/19 SJD). The IUGR induction was performed in pregnant New Zealand rabbit according to the procedure previously described in Eixarch et al. (2009). Briefly, on gestational day (GD) 25, IUGR was induced by surgical ligature of 40–50% of the uterine vessels of each gestational sac of one uterine horn whereas the parallel horn remained as control for normal growth. On GD 30, IUGR and control pups were born under caesarean section and the birth weight was measured. In this study, the inclusion criteria for postnatal day (PND) 0 rabbit pups was the same that was previously used in Barenys et al. (2021): for IUGR pups a birth weight lower and for control pups a birth weight higher than the 25th percentile (39.7 g).

2.2. Neurosphere preparation

The neurosphere culture was prepared from whole brains of control and IUGR PND 0 rabbit pups. The body weight of the pups was measured after caesarean section. The average birth weight of IUGR rabbit pups was significantly lower than the weight of control pups (control 47.7 ± 19.5 g vs IUGR 28.1 ± 11.4 g, Fig. S1). Neural progenitor cells (NPCs) isolation from PND 0 rabbit brains was performed as previously explained in Barenys et al. (2021), Kühne et al. (2022) and detailed in Pla et al. (2022). In brief, meninges and olfactory bulbs were carefully removed from the brain followed by mechanical cutting and enzymatic digestion with papain 20 U/mL for 20 min at 37 °C. Cell suspension was generated by mechanical homogenization and centrifugation for 10 min at 1200 rpm. Afterwards the cell pellet was resuspended in freezing medium (1:1; volume of pellet: volume of freezing medium [consisting in 70% (v/v) proliferation medium, 20% (v/v) fetal calf serum and 10% (v/v) DMSO]) and immediately stored at −80 °C. A detailed description of the thawing protocol can be found in Pla et al. (2022). After thawing, NPCs were cultured for 11 days in proliferation medium on Petri dishes coated with poly-HEMA at 37 °C and 5% CO_2. Proliferation medium consists of DMEM and Hams F12 3:1 supplemented with 2% B27, and 20 ng/mL epidermal growth factor (EGF) including recombinant human fibroblast growth factor (FGF2), 100 U/mL penicillin, and 100 μg/mL streptomycin, supplemented with 10 μM Rho kinase (ROCK) inhibitor Y–276322. Subsequently, half of the medium was renewed every 2–3 days without ROCK supplements.

2.3. Neurosphere assay

After 11 days of proliferation, neurospheres were mechanically chopped to 0.2 mm size (McIlwain tissue chopper) and incubated at 37 °C and 5% CO_2 for 2–3 days until they reached a size of 0.3 mm. For every endpoint of migration and differentiation, 5 neurospheres per condition were plated as replicates in a dice position in one well of an 8-chamber slide coated with PDL/Laminin. Each chamber contained 500 μL of differentiation medium consisting of DMEM and Hams F12 3:1 supplemented with N2 (Invitrogen), penicillin, and streptomycin (100 U/mL and 100 μg/mL). At least three independent experiments were performed for every endpoint and exposure.

2.3.1. Testing of EGCG and G56

EGCG and G56 were dissolved in DMSO and subsequently in differentiation medium with a maximum solvent concentration of 0.01% DMSO. The highest tested concentration was 1 μM followed by 1:10 dilution. NPCs exposed to the solvent control (SC), or compound were incubated for 72 h or 120 h depending on the endpoint to analyse. After 72 h of differentiation 50 % of the medium was replaced by fresh exposure medium.

2.3.2. Migration distance assay

Radial glial migration was measured as previously described in Baumann et al. (2014). Briefly, after 72 h and 120 h under differentiation conditions, bright-field pictures were taken from each neurosphere [EX–H30 camera (Casio, Japan)]. Migration distance [μm] was measured on four right-angled sides from the neurospheres core until the furthest migrated cell using ImageJ 1.53a software.

2.3.3. Corona formation of migrating cells

After 72 h under differentiation conditions, the angles of cells covering the migration corona around the sphere core was manually measured, being 360° the total corona of migrating cells. The sum of all angles surrounding the sphere core was calculated, as previously described in Kühne et al. (2019).

2.3.4. Viability testing

Cell viability was assessed after 120 h of differentiation using CellTiter-Blue® (CTB) reagent (Promega). In every experiment a lysis control was included by exposing neurospheres to 10% DMSO for 2 h. For a detailed description refer to Pla et al. (2022).

2.3.5. Oligodendrocyte differentiation assay

The OL immunocytostaining was performed after viability assessment as described in (Kühne et al., 2022; Pla et al., 2022). Briefly, after 120 h of exposure neurospheres were fixed with 4% PFA. Slides were washed with PBS and incubated with the 1st antibody solution overnight at 4 °C (1:200 mouse IgM anti-O4 (R&D Systems), 10% goat serum in PBS) and after washing with the 2nd antibody solution for 30 min at 37 °C (1:200 anti-mouse IgG Alexa Fluor® 488 (Invitrogen), 2 % goat serum and 1 % Hoechst 33258 (Sigma Aldrich)). Images from the upper and lower part from each neurosphere were taken with a BX61 microscope (Olympus, Japan). The number of O4+ cells and nuclei were automatically counted using a high-content workflow from KNIME® version 4.4.0 (Berthold et al., 2007), and subsequently normalized by the number of nuclei (KNIME® workflow adapted from the "High content Screening" workflow of KNIME created by Christian Dietz. Our workflow is available in KNIME®-hub: https://hub.knime.com/elisabet_t/spaces/Public/latest/Counting%20OL~fVNKirqqJhzW8qjf. A statistical comparison with manual evaluation can be found in supplementary material SM1 and Fig. S2).

2.3.6. qRT-PCR

RNA isolation and cDNA synthesis was performed from control and IUGR neurospheres after 5 days under differentiation conditions followed by qRT-PCR using self-designed primer sequences for *int-β1* and *int-β4* for the species rabbit (Table S1). A detailed description is described in SM2.

2.3.7. Western blot

NPCs under proliferating conditions obtained from control and IUGR animals were thawed, 2x centrifuged at 163 rcf for 10 min to discard the supernatant and the remaining pellet was homogenized for protein expression analyses of int-β1 and int-β4 by using the Western blot technique. A detailed description of the Western blot method is explained in SM3.

2.4. Neurite length in rat neurosphere assay

For details on the performance of the rat neurosphere assay the reader is referred to Kühne et al. (2019). Briefly, rat neurospheres were cultured under differentiation conditions for 24 h on PDL/Laminin coated 8-chamber slides, and subsequently fixed and immunostained for βIII-tubulin^{+} cells (neurons) and Hoechst 33258 (nuclei). After 24 h exposure to SC (0.01% DMSO) or EGCG [1 μM] the neurite length from 20 neurons (βIII-tubulin^{+} cells) per neurosphere was manually measured using ImageJ 1.53a software.

2.5. Dendrite length after developmental administration of EGCG in vivo

Animal procedures were approved by the Committee of Ethical Animal Experimentation (CEEA-PRBB; Protocol Number: VCU-14-1665) and the Generalitat of Catalonia (Protocol Number: DAAM-8101) in accordance with the guidelines of the European Communities Directive 86/609/EEC. For a description of the *in vivo* study the reader is referred to Ortiz-Romero et al. (2018). Briefly, treatment was started in pregnant female mice (C57BL/6J bred in-house at the University of Barcelona and crossed with mice from Jackson Laboratory) at postcoitum by dissolving EGCG in drinking water freshly prepared from a green tea extract (Mega Green Tea Extract, decaffeinated, from Life Extension®, USA, EGCG content by HPLC 326,25 mg) every 48–60 h. Female mice drank EGCG (2.5–3 mg per day) during the whole pregnancy and breastfeeding period. After weaning (at PND 21) the offspring was maintained with the same EGCG treatment until sacrifice at 8 weeks old. In the control group

mice were drinking water without EGCG. The amount of drink per cage was quantified and normalized to the number of animals per cage and to the number of hours that passed between each change. Consumption of water with or without EGCG was not significantly different between groups. At 8 weeks old, after cardiac perfusion with 1x PBS followed by 4% PFA, brains were removed and postfixed in 4% PFA for 24 h at 4 °C, in PBS for 24 h at 4 °C and, afterwards, cryoprotected in 30 % sucrose for 24 h at 4 °C. Finally, serial coronal sections (150 μm) of brain were collected on a glass slide and directly mounted with Mowiol. For morphological analysis of the stratum radiatum (SR) and stratum oriens (SO), 1360 × 1024 images of CA1 hippocampus were obtained with an Olympus DP71 camera attached to an Olympus BX51 microscopy with an Olympus U-RFL-T source of fluorescence at 4× magnification. Measures from six hippocampal sections per animal were averaged. For spine density and spine length analyses we obtained 1024 × 1024 pixel confocal fluorescent image stacks from coronal tissue sections of using a TCS SP2 LEICA confocal microscope. Measurements were performed according to a previously published method in Borralleras et al. (2016). The number of animals included for the dendrite length and spine evaluations was 12 in the control group and 12 in the EGCG exposed group.

2.6. Behavioural tests

All experiments were performed during the light phase of the dark/light cycle by researchers unaware of the different experimental groups. At the level of the task apparatus there was a constant illumination of about <10 lux. In all tests each apparatus was cleaned with a diluted ethanol solution after each mouse.

2.6.1. Social interaction test

We used the same previously described test in Borralleras et al. (2016), conducted in an open field at postnatal week 7. First, an empty wire cup-container was placed in the centre of the arena. A male mouse was allowed to explore the arena, and the amount of time sniffing the empty container was measured for 5 min. Next, an intruder mouse was hold in the container and, again, the amount of time nose to nose sniffing was measured for 5 min. 12 animals of the control group and 11 animals of the EGCG exposed group were evaluated. The aim of the test was to evaluate whether EGCG had any effect on sociability of the offspring.

2.6.2. Marble-burying test

At postnatal week 7, the day before the social interaction test, the marble-burying test was conducted in a polycarbonate rat cage filled with bedding to a depth of 5 cm and lightly tamped down. A regular pattern of 20 glass marbles (five rows of four marbles) was placed on the surface of the bedding prior to each test. An individual male mouse was placed in each cage. The number of buried marbles (>2/3 marble covered) was counted every 5 min for 20 min. 10 animals per experimental group were evaluated. The aim of the test was to identify if EGCG induced anxiety-like/compulsive behaviour in the offspring.

2.7. Statistics

Statistical analysis was performed using GraphPad Prism v9. Two groups were compared with two-way ANOVA followed by post-hoc Tukey's multiple comparison test. The difference between two groups was calculated with a two-tailed Student's *t*-test. The significance threshold was established at $p < 0.05$.

3. Results

3.1. Safety and efficacy testing of EGCG and G56 in the neurosphere assay

3.1.1. Safety testing

A fundamental neurodevelopmental key event previously found to be affected by EGCG exposure at 5 and 10 μM is NPC radial migration, including the migration distance and its corona formation (Barenys et al., 2017). Therefore, to test the safety of lower concentrations, we analysed the effects of EGCG and G56 on neurospheres derived from control and IUGR brains. After 3 days *in vitro* exposure to the highest tested concentration of EGCG [1 μM], control neurospheres had a significantly reduced migration distance (62.4 ± 4.9% of SC), whereas migration was not disturbed in IUGR neurospheres (102.9 ± 6.8% of SC; Fig. 1A and B). This result remained the same after 5 days *in vitro* (Suppl. Fig. S3). Thus, after exposure to 1 μM of EGCG, the migration distance of control and IUGR neurospheres was significantly different (Fig. 1A) because the controls were affected but IUGR neurospheres were not. Similarly, EGCG [1 μM] significantly reduced the corona formation surrounding the sphere core in control neurospheres (313° ± 12.9° vs 360° of SC), but not in IUGR neurospheres (347° ± 13.0° vs 360° in SC; Fig. 1B). G56 did not significantly interfere with the migration distance or corona formation at any tested concentration in both groups, control or IUGR (Fig. 1C and D), which shows, as described before, that this EGCG derivative is safer than the parent compound (Kühne et al., 2019). After exposure to EGCG or G56, cell viability determined by metabolic activity was not significantly affected (Fig. 1D).

3.1.2. Efficacy testing

Within the migration area, NPCs differentiated under control conditions to 7.9 ± 0.8% oligodendrocytes (OLs). NPCs obtained from IUGR brains had a significantly reduced ability to differentiate to OLs (4.6 ± 0.5% IUGR) compared to control (Fig. 2A–C), reproducing previous findings of our group (Kühne et al., 2022). We observed that none of the tested concentrations of EGCG or G56 could significantly increase OL differentiation, neither in control nor in IUGR neurospheres. The endpoint-specific positive control BMP7 significantly reduced OL differentiation to 1.3 ± 0.6% (Suppl. Fig. S4). With these results it was clear that neither EGCG nor G56 is a good candidate to be used as a neuroprotective therapy to prevent or treat white matter alterations induced by IUGR.

3.2. IUGR neural/progenitor cells overexpress int-β1

Since we identified a significant difference in susceptibility to migration effects between control and IUGR neurospheres, being the IUGR less sensitive, we analysed the gene expression of subunit int-β1 in control and IUGR neurospheres, because previous studies revealed that EGCG interferes with the laminin/int-β1 interaction leading to a disturbed migration pattern (Barenys et al., 2017). We also analysed the expression of int-β4 since this is another integrin subunit known to bind to laminin (Barczyk et al., 2010; Humphries et al., 2006) but it is not involved in the mechanism of action of EGCG disturbed migration (Barenys et al., 2017). Our qRT-PCR results confirmed that in the IUGR group, the expression of int-β1 was significantly increased to a 10-fold higher amount than in the control (control 1.14 vs IUGR 10.97, $p = 0.047$, Fig. 3A). No significant difference was observed in the fold-change expression of int-β4 between control and IUGR (control 1.09 vs IUGR 1.08, $p = 0.964$, Fig. 3A). In line with these results, a significant increase in int-β1 protein to a 3.5-fold higher amount than in control was observed in IUGR NPCs (control 1.00 vs IUGR 3.49, $p = 0.0465$, Fig. 3B) and no significant difference was observed in the fold-change expression of int-β4 protein between control and IUGR (control 1.00 vs IUGR 1.05, $p = 0.8116$ Fig. 3B).

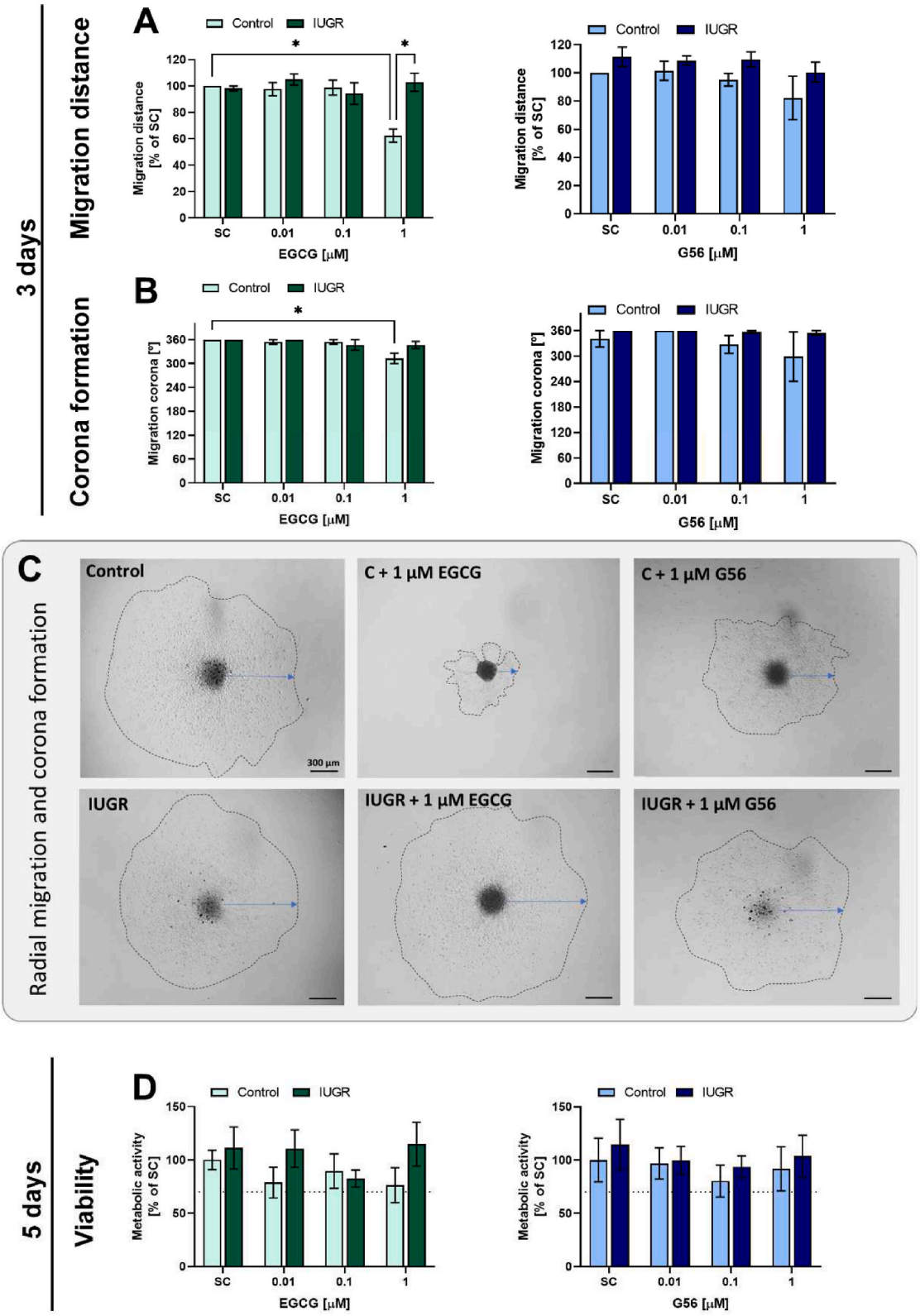

Fig. 1. EGCG disturbs migration in control but not in IUGR neurospheres. Rabbit neurospheres obtained from Control and IUGR pups were comparatively analysed for each endpoint. Migration pattern was analysed after 3 days under differentiation conditions. (A) Migration distance [µm] after exposure to EGCG (green) and G56 (blue). (B) Formation of migration corona [°] after exposure to EGCG (green) and G56 (blue). (C) Representative pictures of migrated neural progenitor cells (NPCs) after 3 days *in vitro* of control and IUGR neurospheres with and without exposure to 1 µM EGCG or G56, arrow: migration distance, dotted line: corona formation. (D) Cell viability determined by metabolic activity after exposure to EGCG (green) and G56 (blue). Analysis was evaluated in five neurospheres/condition in at least three independent experiments. Mean ± SEM, *: $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

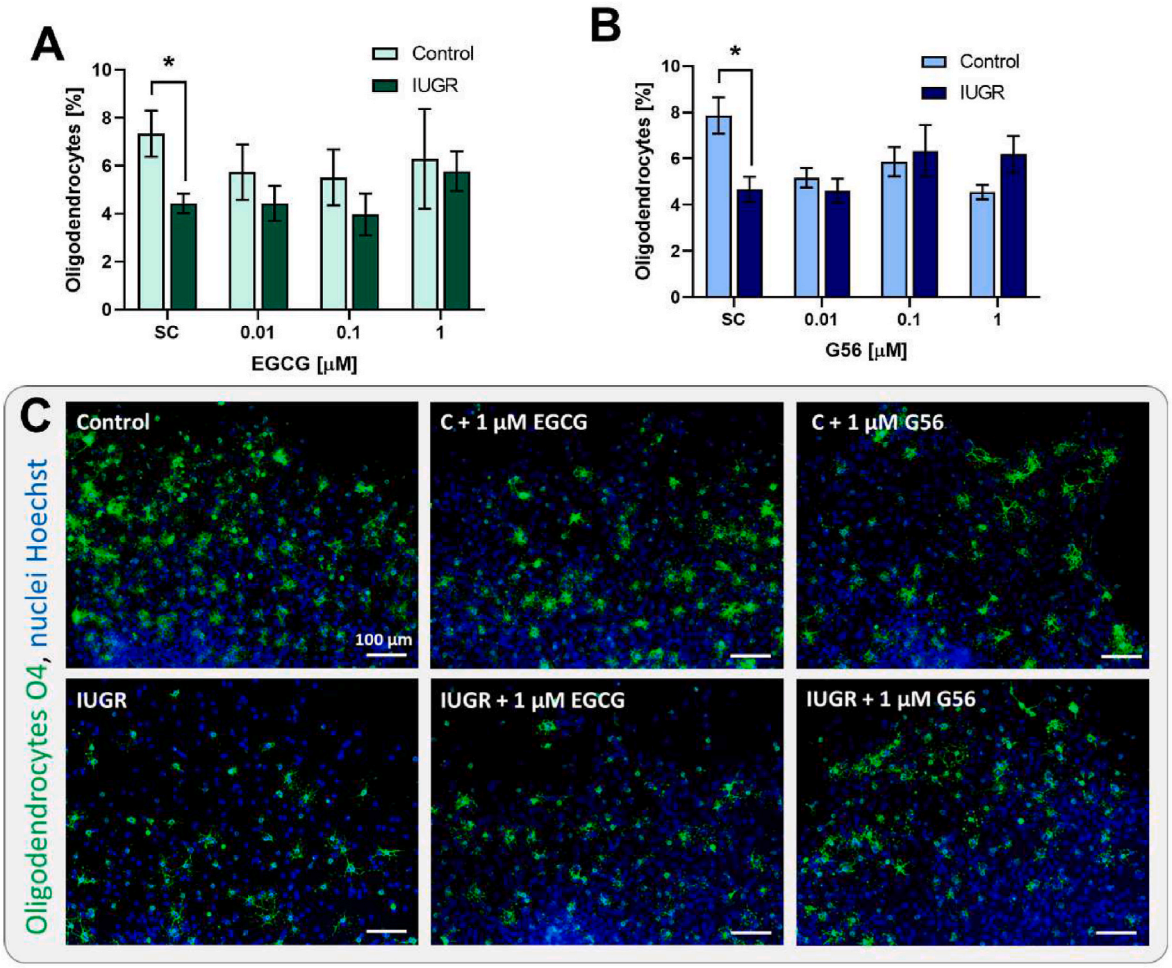

Fig. 2. Lack of efficacy of EGCG or G56 upon decreased oligodendrocyte differentiation in IUGR neurospheres. Rabbit neurospheres obtained from control and IUGR pups were comparatively analysed for each endpoint. Oligodendrocyte differentiation after 5 days exposed to (A) EGCG and (B) G56. (C) Representative pictures of control and IUGR neurospheres exposed to the 1 µM EGCG or G56, oligodendrocyte marker O4 (green) and Hoechst 33258 (blue), Scale bar = 100 µm. Analysis was evaluated in five neurospheres/condition in at least three independent experiments. Mean ± SEM, *: $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Neurodevelopmental EGCG exposure has deleterious effects on neurites

Besides being involved in cell adhesion and migration, int-β1 is crucial for other neurodevelopmental processes like neurite length and arborization (Belvindrah et al., 2007; Marrs et al., 2006). Knowing that, and linking this information with the fact that IUGR animals present a higher arborization of neurons (Pla et al., 2020), we wondered if EGCG could also affect neurite length and arborization during neurodevelopment. We first analysed *in vitro* the neurite length of neurons exposed for 24 h to EGCG [1 µM] in a neurosphere culture using the pictures of a previous study (Kühne et al., 2019). Indeed, the neurite length *in vitro* was significantly reduced after EGCG exposure compared to the solvent control (51.0 ± 2.7 µm SC vs 42.0 ± 3.9 µm EGCG, $p = 0.0138$, Fig. 4A–B). In a next step we searched for evidence of the relevance of this effect *in vivo* in the literature and we found an article performed in mice indicating that one of the experimental groups was exposed to EGCG during gestation and the postnatal period (Ortiz-Romero et al., 2018). Since the results of control animals exposed to EGCG on dendritic length for this specific group were not included in that publication (the publication was focusing on the effects of EGCG in a Williams-Beuren syndrome mouse model), we contacted the authors obtained the raw data of controls and controls exposed to EGCG and we present the results here. In this case the dendritic length was analysed at postnatal week 8 after administration of EGCG from postcoitum until sacrifice. Indeed, the dendritic length of CA1 cortical neurons in the stratum radiatum (SR) and stratum oriens (SO) decreased significantly in the EGCG group compared to the untreated group (SR: 225.9 ± 4.0 vs 197.2 ± 2.5; SO: 130.1 ± 1.6 vs 100.1 ± 1.4 Fig. 4C–D), without affecting birth weight or brain weight (Suppl. Fig. S5). Contrarily, the dendritic spine density and the two behavioural tests performed (social interaction test and marble-burying test) were not affected by EGCG exposure.

3.4. Integration in an adverse outcome pathway (AOP) concept

By combining previous results from the literature with our novel investigations, we bring new evidence supporting, expanding and redesigning the previously postulated AOP "Binding to the extracellular matrix protein laminin leading to decreased cognitive function." (Klose et al., 2022) which is now entitled: "Disrupted laminin/int-β1 interaction leading to decreased cognitive function" (Fig. 5). Our new design includes information from three kind of experimental data obtained 1) after exposure to EGCG (in green), 2) from an int-β1 deficiency in partial int-β1 knock-out animals or via blocking experiments with int-β1 antibody (in blue), and 3) from an overexpression of int-β1 due to IUGR (in yellow). Moreover, we bring together evidence at both levels, *in vivo* (blank box) and *in vitro* (dotted box).

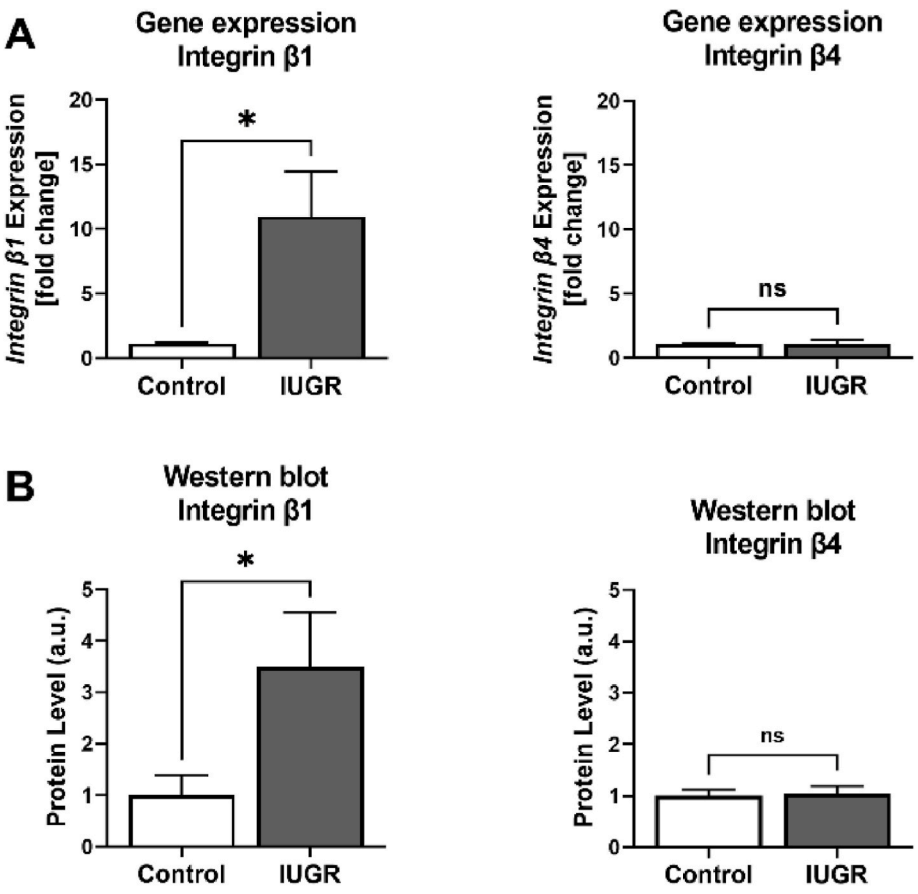

Fig. 3. Overexpression of int-β1 in IUGR neurospheres on gene and protein level. (A) qRT-PCR from int-β1 and int-β4 expression in control and IUGR neurospheres after 5 days of differentiation (five neurospheres/condition). (B) Western blot analysis from control and IUGR proliferating NPCs. Analysis was evaluated in at least three independent experiments. Mean ± SEM, *: $p < 0.05$, ns: not significant.

The AOP begins with a disrupted laminin–int-β1 interaction (MIE (Barenys et al., 2017; Lo et al., 2007; Melgarejo et al., 2009; Park et al., 2010)) due to a chemical, in this case EGCG, binding to laminin (Barenys et al., 2017; Lo et al., 2007; Suzuki and Isemura, 2001). As previously described, this interference causes decreased adhesion of NPC's *in vitro* (KE1 (Barenys et al., 2017; Graus-Porta et al., 2001)) and GFAP + processes alterations (KE2 (Barenys et al., 2017; Belvindrah et al., 2007)), which leads to a reduced migration distance (KE3 (Barenys et al., 2017; Chen et al., 2003; Kühne et al., 2019)) and a reduced corona formation with a lower density of migrating cells (KE4 (Barenys et al., 2017; Kühne et al., 2019)). Our new results in IUGR neurospheres exposed to EGCG support this previously established chain of KEs, because KE3 and KE4 are not present if cells overexpress int-β1 (cKE3 and cKE4; Fig. 1A and B). The altered GFAP + processes (KE2) are an *in vitro* observation after EGCG exposure but also an effect described in cell cultures from mice lacking the expression of int-β1 subunit in radial glial cells, which was linked with the same effect (disturbed glial cell alignment in the developing brain cortex) in brain sections of these mice (KE5 (Belvindrah et al., 2007; Graus-Porta et al., 2001)). As a result of this alteration in GFAP + processes outgrowth, the same main effect has been described *in vitro* and *in vivo*: a lower cell density in the migration area (*in vitro*; KE4) or in cortical layers (KE6 (Belvindrah et al., 2007; El-Borm and Abd El-Gaber, 2021; Graus-Porta et al., 2001)). A preliminary indication that EGCG could also produce this effect is given by the fact that prenatal exposure to green tea extract leads to a decreased number of cells in the cerebellar cortex and cerebellum (El-Borm and Abd El-Gaber, 2021), but a study with only EGCG exposure at doses not producing significant decreases in body weight of the pups should be performed to confirm these results. As a consequence, another key event at organ level, already described after int-β1 knock-out induction in glial cells, was included: cerebellar hypoplasia (KE7 (Frick et al., 2012; Robel et al., 2009)), but the behavioural consequences of this group of KEs, are to the best of our knowledge, not explored after int-β1/laminin disruption. The behavioural consequence of cerebellar hypoplasia described in the literature is "cerebellar cognitive affective syndrome" (KE8 (Basson and Wingate, 2013)), but this remains as a hypothetical linkage in our AOP.

Our new results have opened a second branch in this AOP related to adverse effects on neurite growth due to the same MIE: disrupted interaction between ECM (laminin) and β1-integrin. At the *in vitro* level there were already several evidence: 1) int-β1 deficiency in cultures of partial knock-out mice cells decreases neurite length (KE9 (Belvindrah et al., 2007; Marrs et al., 2006)), 2) int-β1 blocking peptide β1P reduces the length of neurites on laminin (KE10 (Belvindrah et al., 2007; Marrs et al., 2006)) and, 3) int-β1 inhibitor toxin echistatin eliminates neurite branching and elongation of cortical neurons on laminin (Moresco et al., 2005). Again, our investigations in IUGR neurospheres support this link because neurons obtained from IUGR neurospheres, overexpressing int-β1, present longer neurites (cKE9; Kühne et al., in preparation). Moreover, the *in vivo* relevance of this AOP part was already demonstrated in mice lacking int-β1 in excitatory forebrain neurons and failing to elaborate dendritic arborization (KE10 (Ortiz-Romero et al., 2018; Warren et al., 2012)), while IUGR animals overexpressing int-β1 present more complex dendrite arborization than controls (cKE10). Thanks to this AOP approach, we could build all these connections between the KEs and finally demonstrate for the first time that EGCG *in vitro* exposure decreases neurite length (Fig. 4A and B) and EGCG *in vivo* developmental exposure impairs dendritic arborization and decreases dendrite length in

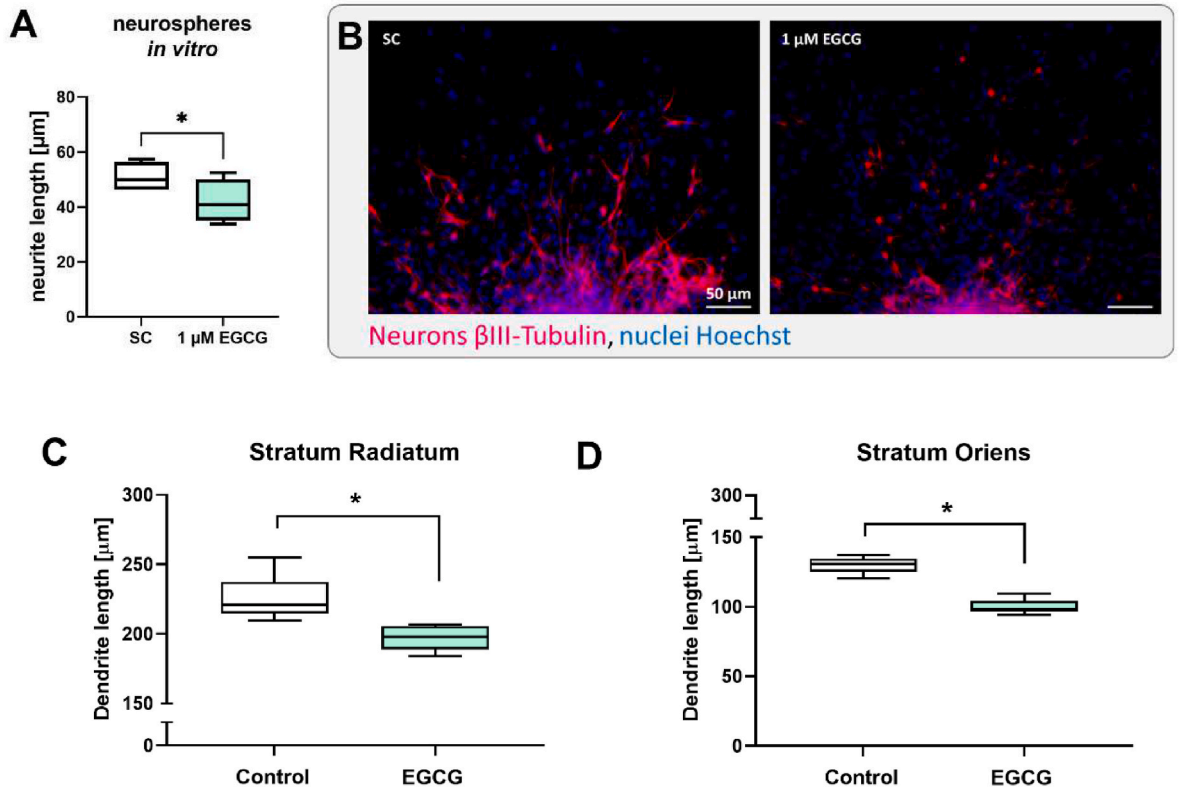

Fig. 4. Neurodevelopmental exposure to EGCG decreases neurite length *in vivo* and *in vitro*. (A) *In vitro* neurite length measurement in rat neurospheres exposed for 24h to solvent control (SC) or 1 µM EGCG; (B) representative images of rat neurospheres stained for neurons with βIII-tubulin (red) and nuclei with Hoechst 33258 (blue), Scale bar = 50 µm. (C) I*n vivo* dendrite length measurement of CA1 cortical neurons in stratum radiatum and (D) stratum oriens with (n = 12) and without (n = 12) developmental administration of EGCG to mice; boxplot represented in Tukey's style (Median, and 25th and 75th percentiles), *: $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the hippocampus (Fig. 4C and D). The AO described in mice lacking int-β1 in excitatory forebrain neurons was cognitive deficits, specifically impaired hippocampus-dependent learning measured by the novel object recognition test (KE11 (Warren et al., 2012)), so the expected AO would be "decreased cognitive function" after developmental exposure to EGCG.

4. Discussion

We have discovered a significant increase in the expression of *int-β1* in IUGR neurospheres, at both gene expression and protein expression level, which enables a better adhesion of NPCs to the ECM and an appropriate migration phenotype under exposure to EGCG at concentrations disturbing migration in control neurospheres. This finding unravels one of the probably several molecular adaptations that brains suffer when developing under chronic mild hypoxic conditions. To the best of our knowledge, this is the first time that this molecular change is identified in IUGR brains, and the implications of this finding are very relevant because it can explain some of the neurodevelopmental alterations described after IUGR *in vivo*, as for example the changes in neuronal arborization. Other authors have described a marked upregulation of integrin α6β1 or α5β1 in brain endothelial cells (Halder et al., 2018; Li et al., 2010) or increases in astrocyte end-feet adhesion molecules such as integrin α6β4 in adult brains after chronic mild hypoxia (Li et al., 2010) or increases in the expression of laminin-associated integrins α6β1 or α7β1 in mature neurons after nerve injury, which promote axonal regeneration in a mature nerve system (Nieuwenhuis et al., 2018), so it is not surprising that changes in adhesion molecules also occur in developing brains under IUGR induced by chronic mild hypoxic conditions.

During brain development integrins are involved in laminin adhesion and in axonal growth and pathfinding (Myers et al., 2011). Several studies clearly support that int-β1 increases extension and complexity of dendritic branching of cortical neurons (Moresco et al., 2005; Warren et al., 2012) and previous studies of our group have discovered that IUGR rabbit pups present a significantly higher complexity of dendritic branches in the frontal cortex *in vivo* (Pla et al., 2020). Therefore, it is plausible to postulate that the increased *int-β1* expression in rabbit IUGR NPCs might underlie this increased dendritic arborization in IUGR brains. Although further studies need to be done to unequivocally link this mechanism with the histological outcome observed in IUGR pups, our discovery gives insights of an int-β1 mediated mechanism in neurogenesis and opens the door for a better characterization of changes in prenatal brain programming induced by IUGR.

This discovery was possible thanks to the previous existence of a putative AOP: "Binding to the extracellular matrix protein laminin leading to decreased cognitive function" (Klose et al., 2022), which was submitted to the OECD in 2019. Creating AOP-structured collections of molecular/cellular/organ/organism-event networks underlying human biology responding to toxic insults (named as toxicological ontology (Baker et al., 2018; Desprez et al., 2019; Heusinkveld et al., 2021)) is an extremely powerful strategy because it allows to cross information from different fields, in this case developmental neurotoxicity, developmental biology, and developmental physiopathology, to identify the molecular events behind specific cellular effects and finally design experiments which are directly aimed to evaluate the relevant KE or AO while saving animals, time and resources because the search is more directed.

Our results support and expand the previously submitted AOP, and after combining them with the literature results, we can say that compared to the previous one, the new version of the AOP: 1) is more chemically agnostic, 2) includes a new branch of key events, and 3) is

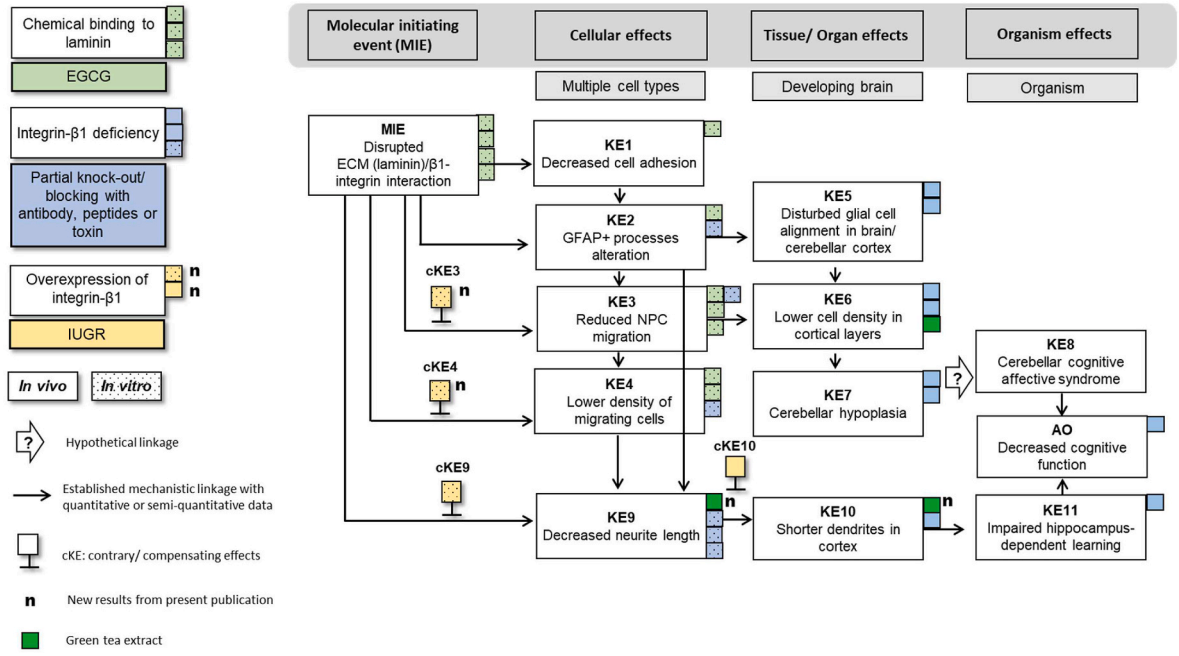

Fig. 5. AOP "Disrupted laminin/int-β1 interaction leading to decreased cognitive function". The AOP frame begins with a MIE leading to a cascade of KEs on cellular and tissue/organ level and results ultimately in an AO at the organism level. Every little box refers to a publication including the MIE, interference with a KE or AO respectively, either *in vivo* (blank box) or *in vitro* (dotted box). The interference of KEs was due to EGCG exposure or administration (green) or int-β1 deficiency due to partial knock-out or blocking experiments with int-β1 antibody, specific peptides, or toxins (blue). The yellow boxes present a contrary/compensating effect in IUGR models exhibiting an overexpression of int-β1 (yellow). n: new results including in the present paper; AOP: adverse outcome pathway; MIE: molecular initiating event; KE: key event; AO: adverse outcome.
References Legend: Chemical binding to laminin: (Barenys et al., 2017; Lo et al., 2007; Suzuki and Isemura, 2001). Int-β1 deficiency: (Barenys et al., 2017; Belvindrah et al., 2007; Graus-Porta et al., 2001). Overexpression of int-β1: Fig. 3 A & B. References KEs. MIE (Barenys et al., 2017; Lo et al., 2007; Melgarejo et al., 2009; Park et al., 2010). KE1 (Barenys et al., 2017). KE2 (Barenys et al., 2017; Belvindrah et al., 2007). KE3 (Barenys et al., 2017 (green and blue); Chen et al., 2003; Kühne et al., 2019). KE4 (Barenys et al., 2017; Kühne et al., 2019). KE5 (Belvindrah et al., 2007; Graus-Porta et al., 2001). KE6 (Belvindrah et al., 2007; El-Borm and Abd El-Gaber, 2021; Graus-Porta et al., 2001). KE7 (Frick et al., 2012; Robel et al., 2009). KE8 (hypothetical). KE9 green (n): Fig. 4A and B; (Belvindrah et al., 2007; Marrs et al., 2006; Moresco et al., 2005). KE10 green (n): Fig. 4C and D; (Warren et al., 2012). KE11 (Warren et al., 2012). AO (Warren et al., 2012). References contrary/compensating effects (cKE): cKE3: Fig. 1A cKE4: Fig. 1B cKE9: (Kühne et al., in preparation: IUGR increased neurite length). cKE10: (Pla et al., 2020). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

more specific at the organ and organism level. Since we have found evidence of other compounds following the same chain of KEs (mainly blocking peptides or spider toxins) but not having the same MIE (which was previously defined as "binding compound to laminin" (Bal-Price et al., 2016)) to make the AOP more chemically agnostic, as required per definition in AOPs (Bal-Price et al., 2017), we have changed the MIE to a broader definition: "disrupted laminin/integrin beta-1 interaction", which encompasses both situations: those were the interaction is disrupted due to lower laminin availability and those due to lower int-β1 availability. As a consequence, the title of the AOP has also changed to: "Disrupted laminin/integrin beta-1 interaction leading to decreased cognitive function". Thanks to the cross-linked information in this AOP we could also identify a new adverse effect of developmental exposure to EGCG, and we have therefore included a new branch of events related to adverse effects on neurite outgrowth, which could be independent or related to the glial effects described in the other branch of the AOP. We have characterized for the first time adverse effects on dendritic length of CA1 neurons in the stratum radiatum and stratum oriens in mouse brains after developmental exposure to EGCG *in vivo*. In accordance, EGCG reduced the neurite length in a neurosphere culture after 24h of exposure, so both *in vitro* and *in vivo* evidence are added to this new branch of the AOP. The interrelationship between both branches is suspected because Belvindrah et al., discovered that "the defect in neurite outgrowth of int-β1-deficient neurons is rescued when glial cells express β1 integrins and undergo normal morphological differentiation, suggesting that the perturbations in neurite outgrowth are a secondary consequence of defects in glial cells" (Belvindrah et al., 2007), however, after *in vitro* or *in vivo* exposure to EGCG it is not possible to distinguish if the effect is direct or secondary. By including this information together with new evidence supporting the glial branch, we have made the AOP more specific at the organ and organism level: we have collected evidence *in vivo* for both branches, we have identified a more specific and more probable organ effect for the glial branch "cerebellar hypoplasia" (based on int-β1 knock-out induction in glial cells, leading to cerebellar hypoplasia in mice (Robel et al., 2009 and Frick et al., 2012), and identified which behavioural alteration is probably disturbed due to the neurite outgrowth effects, impaired hippocampus-dependent learning (based on previous publications revealing that a selective loss of int-β1 in excitatory neurons leads to these effects measured with the Novel Object Recognition test (Warren et al., 2012)). The final AO of both branches would still be "decreased cognitive function", as previously described in the AOP (Klose et al., 2022). It is true that the evidence *in vivo* is based on green tea extract exposure studies instead of only EGCG exposure studies, so there is still the need to confirm this relationship, but one important aspect is that the new branch could have even more relevance *in vivo* than the previous one because adverse effects in animals appear at lower doses of green tea extract, and of EGCG. In the lower case the dose is 2.5–3 mg/animal/day, which is a dose within the range of the recommended doses in pre-clinical and clinical studies (Almeida-Toledano et al., 2021; Souchet et al., 2019; www.clinicaltrials.gov, 2022). As an orientation, according to the EFSA calculations (EFSA, 2018) the daily intake of EGCG from food supplements ("calculated multiplying the dose unit of EGCG for the daily number of recommended doses of the product") ranges from 5 to 1000 mg/day for the

adult population. Kinetic studies in humans have reported that a single 800 mg dose leads to maximum plasma concentrations of 3.5 μM (calculated from Ullmann et al., 2003). Therefore, it is reasonable to state that the doses and concentrations of EGCG used in our study are relevant when considering an exposure to EGCG as a food supplement.

Altogether, our investigations strongly suggest that exposure to EGCG food supplements can produce developmental neurotoxicity and they are the basis for future studies required to confirm the postulated AO of this pathway. From our work, it is clear that there is the need to do an *in vivo* study in mice exposed to EGCG, to green tea extract (with an equivalent dose of EGCG) or to water where behavioural alterations in the Novel Object Recognition test and cerebellar hypoplasia and "Cerebellar cognitive affective syndrome" are evaluated. Performing behavioural tests, such as anxiety-like behaviour tests or sociability tests has not shown any effects in the present study (see Suppl. Fig. S6 including non-significant results of the marble-burying test and social interaction test), but the dose used was low within the range of relevant food supplement exposure and these effects could still appear at higher doses. Given the high importance EGCG prenatal exposure is acquiring in the last years in the preclinical field for the treatment of several diseases (Almeida-Toledano et al., 2021; Ortiz-Romero et al., 2018; Souchet et al., 2019), it is of utmost importance to clarify its possible neurodevelopmental toxicity and to perform a correct risk assessment evaluation depending on the dose of exposure. Since the quantitative aspect of the proposed AOP is still missing, the inclusion of different doses of EGCG would be required to identify a NOAEL. The idea of including two groups, one exposed to EGCG and one to the green tea extract relies on the already mentioned fact that all results *in vivo* included in the current version of the AOP are based on studies were EGCG was administered as a green tea extract and because in these extracts several catechins are present. Other catechins like EGC (epigallocatechin) or ECG (epicatechingallate) have been shown to produce the same KEs than EGCG *in vitro* (Barenys et al., 2017), so an additive effect among them can be expected and would be important to be identified.

Our work also contributes to future evaluation of chemicals triggering the same KEs, which would be expected to have the same MIE and induce the same AO. So far, several compounds or plant extracts have been proved to potently trigger migration disturbance (KE3 – KE4) with the same specific phenotype than EGCG: Lei Gong Teng extract (another Chinese herbal medicine) (Klose et al., 2022), and two synthetic analogues of EGCG including G37 (1,4-bis[(3,4,5-trihydroxybenzoyl)oxy] naphthalene) and M2 (3-hydroxy-1-naphthyl 3,4,5-trihydroxybenzoate) (Kühne et al., 2019). For future substances suspected to be candidates to follow this AOP, a simultaneous *in vitro* evaluation of KE3, KE4 and KE9 in a neurosphere model could be enough to evaluate the potency of the compound in comparison to EGCG and to assess the suitability of its use during pregnancy, avoiding the unnecessary use of animal experiments. Using this strategy, we give evidence here that another analogue of EGCG named G56 does not adversely affect these key events and can therefore be considered as a safer alternative to EGCG during pregnancy, in this specific DNT aspect. However, neither G56 nor EGCG could rescue the decrease in oligodendrocyte differentiation induced by IUGR, so despite being safer, this compound would still not be a promising neuroprotective treatment in this condition. The oligodendrocyte differentiation evaluation was performed for the first time using a high-content workflow of KNIME® adapted from the "High content Screening" workflow of KNIME created by Christian Dietz for oligodendrocyte automatic counting (see link in Material and Method 2.3.5). We have proved that our workflow results are statistically comparable to those obtained with our manual counting (see Suppl. Fig. S4), and we contribute to the field by sharing this workflow in open access to make the evaluation of this endpoint faster, easier because it can be used in manually taken fluorescence microscope pictures, and cheaper because no expensive evaluation software is needed (see link in Material and Method 2.3.5).

5. Conclusions

We have discovered for the first time that IUGR neurospheres overexpress int-β1 and they respond differently than control neurospheres to the exposure of a compound triggering migration alterations. Because int-β1 is involved in NPC migration but also in axonal growth and neuronal branching, and IUGR animals have branching alterations, we strongly think that our discovery is bringing relevant new insights to the characterization of IUGR-induced neurodevelopmental alterations.

On top of that, we have also discovered that EGCG developmental exposure has deleterious effects on neuronal branching and arborization. This important finding warns for a further DNT thorough characterization of this and other food supplement compounds before recommending them as prenatal treatments.

Finally, our study contributes to demonstrate that the neurosphere assay is well suited for hazard assessment of DNT, that the results obtained with this technique correlate well with *in vivo* evidence and that when they are combined with gene/protein expression analyses they are powerful tools for building AOPs which ultimately allow a better comprehension of the DNT effect or related neurodevelopmental diseases.

CRediT authorship contribution statement

Britta Anna Kühne: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Validation, Writing – original draft. **Elisabet Teixidó:** Data curation, Formal analysis, Software. **Miren Ettcheto:** Methodology, Formal analysis, Investigation. **Teresa Puig:** Resources. **Marta Planas:** Resources. **Lidia Feliu:** Resources. **Laura Pla:** Methodology. **Victoria Campuzano:** Data curation, Formal analysis, Investigation, Methodology, Validation. **Eduard Gratacós:** Resources, Funding acquisition. **Ellen Fritsche:** Validation, Writing – review & editing. **Miriam Illa:** Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Marta Barenys:** Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113506.

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