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# Secretions from placenta, after hypoxia/reoxygenation, can damage developing neurones of brain under experimental conditions ☆

Q1 Daniel J. Curtis [a], Aman Sood [a], Tom J. Phillips [a], Veronica H.L. Leinster [a], Akihiro Nishiguchi [b], Christopher Coyle [a], Lizeth Lacharme-Lora [a], Oliver Beaumont [a], Helena Kemp [c], Roberta Goodall [c], Leila Cornes [c], Michele Giugliano [d], Rocco A. Barone [d], Michiya Matsusaki [b], Mitsuru Akashi [b], Hiroyoshi Y. Tanaka [e], Mitsunobu Kano [e], Jennifer McGarvey [f], Nagaraj D. Halemani [f], Katja Simon [g], Robert Keehan [a], William Ind [a], Tracey Masters [h], Simon Grant [h], Sharan Athwal [h], Gavin Collett [i], Dionne Tannetta [i], Ian L. Sargent [i], Emma Scull-Brown [j], Xun Liu [j], Kristian Aquilina [k], Nicki Cohen [l], Jon D. Lane [f], Marianne Thoresen [j], Jon Hanley [f], Andrew Randall [m], C. Patrick Case [a,*]

[a] Musculoskeletal Research Unit, School of Clinical Sciences, Bristol University, Southmead Hospital, Bristol BS10 5NB, UK
[b] Department of Applied Chemistry, Osaka University, Japan
Q2 [c] Newborn Screening, Southmead Hospital, Bristol, UK
[d] Biomedical Sciences, University of Antwerp, Belgium
[e] Department of Molecular Pathology, University of Tokyo, Japan
[f] School of Biochemistry, University of Bristol, UK
[g] Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, UK
[h] Dept. Obstetrics and Gynaecology, Southmead Hospital, Bristol, UK
[i] Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, UK
[j] Department of Neonatology, University of Bristol, Bristol, UK
[k] Department of Neurosurgery, Frenchay Hospital, Bristol, UK
[l] Department of Neuropathology, University of Bristol, Bristol, UK
[m] School of Physiology and Pharmacology, University of Bristol, Bristol, UK

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ABSTRACT

Q3 Some psychiatric diseases in children and young adults are thought to originate from adverse exposures during foetal life, including hypoxia and hypoxia/reoxygenation. The mechanism is not understood. Several authors have emphasised that the placenta is likely to play an important role as the key interface between mother and foetus. Here we have explored whether a first trimester human placenta or model barrier of primary human cytotrophoblasts might secrete factors, in response to hypoxia or hypoxia/reoxygenation, that could damage neurones. We find that the secretions in conditioned media caused an increase of $[Ca^{2+}]_i$ and mitochondrial free radicals and a decrease of dendritic lengths, branching complexity, spine density and synaptic activity in dissociated neurones from embryonic rat cerebral cortex. There was altered staining of glutamate and GABA receptors. We identify glutamate as an active factor within the conditioned media and demonstrate a specific release of glutamate from the placenta/cytotrophoblast barriers in vitro after hypoxia or hypoxia/reoxygenation. Injection of conditioned media into developing brains of P4 rats reduced the numerical density of parvalbumin-containing neurones in cortex, hippocampus and reticular nucleus, reduced immunostaining of glutamate receptors and altered cellular turnover. These results show that the placenta is able to release factors, in response to altered oxygen, that can damage developing neurones under experimental conditions.



## Introduction

Some human diseases originate from events during foetal life (Barker, 2004). A variety of prenatal insults, including hypoxia, hypoxia/reoxygenation, and infection, as early as late first trimester, are associated with an increased risk of neurodevelopmental disorders including schizophrenia, attention deficit/hyperactivity disorder and autism (Fatemi and Folsom, 2009). In animal models even relatively brief periods of *foetal* hypoxia may lead to the death of susceptible neuronal populations (Rees et al., 2011). The principal pathways for this are initiated by energy depletion followed by an increased neuronal release and a reduced glial uptake of glutamate, an accumulation of cytosolic calcium and a

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* Corresponding author. Fax: +44 117 3238476.
*E-mail address:* c.p.case@bristol.ac.uk (C.P. Case).

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generation of reactive oxygen species (Rees et al., 2011). The death of neurones is thought to occur in three stages. There is an initial period of cell dysfunction and oxidative stress. 8 to 48 h later there is a secondary phase of injury, which results in a neuroinflammatory response, mitochondrial permeabilisation, reperfusion and a loss of cerebral autoregulation. Weeks or months later there may be tertiary injuries as a result of a sensitization to inflammation, persistent gliosis and epigenetic changes (Baburamani et al., 2012; Chicha et al., 2014).

However the precise mechanisms of how transient gestational challenges can lead to neurodevelopmental diseases in later life are largely unknown, and it is thought that the placenta is likely to play a key role (Hsiao and Patterson, 2012; Rapoport et al., 2012). As Hsiao and Patterson emphasise, disruptions to the maternal or intrauterine environment are necessarily conveyed to the developing embryo via the placenta. Where gestational challenges are confined to the uteroplacental compartment, in models of intrauterine infection and intrauterine growth restriction, primary insults to placenta can manifest in perinatal brain damage in the offspring (Hsiao and Patterson, 2012; Mikaelsson et al., 2013).

The past 5 years have seen particular interest in the contribution of placental pathology to neurodevelopmental disorders (Rapoport et al., 2012). Furthermore the placenta actively secretes molecules that are important for infant brain development and which might be affected by gestational challenges (Bonnin et al., 2011; McKay, 2011).

Previously we showed that the placenta or a model placental barrier responds to toxins or altered oxygen by secreting factors that cause genetic damage in fibroblasts or human embryonic stem cells (Bhabra et al., 2009; Sood et al., 2011). Therefore we explored here whether it would also respond to altered oxygen by secreting factors that could damage developing neurones under experimental conditions.

We show that the placenta responds in vitro to hypoxia or hypoxia/reoxygenation by secreting factors that increase calcium and mitochondrial free radicals in embryonic cortical neurones in vitro and reduce synaptic activity, dendritic length, branching complexity and spine density. Exposure of a developing brain to media conditioned by placenta under hypoxia and hypoxia reoxygenation results in decreased density of parvalbumin containing neurones in cortex hippocampus and reticular nucleus. We identify glutamate as an active factor mediating these changes.

## Methods

### *Preparation of barriers/explants*

Bilayered BeWo cell barriers were prepared on transwell inserts at 2% or 21% oxygen according to our previous protocols (Sood et al., 2011). Human placenta from 1st and 3rd trimester was obtained with ethical approval and patient consent from patients with normal pregnancies at voluntary termination of pregnancy or elective caesarean section.

Primary villous cytotrophoblasts, were extracted from 3rd trimester placenta according to our previous protocols (Tannetta et al., 2008). They were used to create barriers using a modification of the cell-accumulation technique (Nishiguchi et al., 2011). This allows cell barriers to be made in 1 day by coating the cells with extracellular matrix nanofilms approximately 5 nm in thickness.

The primary cytotrophoblasts were alternately incubated nine times with 0.04 mg/ml type IV-collagen (Sigma) and laminin (Invitrogen) in 50 mM Tris–HCl (pH = 7.4) for 1 min each. The collagen laminin coated cells were seeded into transwell inserts and cultured under 2% and 21% oxygen conditions. After 1 day of incubation, the barriers were obtained and subsequently cultured for 1 day with or without a change of the oxygen conditions from 2% to 8% and 21%. The media beneath the barriers were collected and exposed to neurones for 6 days. All barriers were assessed with confocal imaging of E cadherin (Cell Signaling) immunocytochemistry or vital dye staining.

1st trimester placenta (mean 9 weeks 4 days gestation n = 20) was transferred to the laboratory under atmospheric conditions and dissected into 2.5 × 2.5 mm pieces to cut across and expose placental villi.

### *Preparation of conditioned media*

Neurobasal media or HEPES buffered saline were conditioned by placing placental explants within them or prepared barriers above them at 37 °C in a Ruskin 5ive hypoxic chamber under 4 different oxygen conditions; 21% or 2% for 48 h, or transfer from 2% (24 h) to either 8% or 21% (24 h) (Hung et al., 2004) (Fig. 1).

### *Neurone exposures*

Neuronal cultures were prepared from the cerebral cortex of embryonic day 18 Wistar rat embryos as previously described (Gray et al., 2011). Following enzymatic and mechanical dissociation, cells were counted and plated onto poly L-lysine-coated 13-mm coverslips at 50,000–100,000 cells/coverslip, and cultured in DMEM supplemented with 2% B27 (Gibco, Paisley, UK) and 1% penicillin/streptomycin. Neurones were left to settle out overnight in plating medium before being transferred to neurobasal feeding medium. They were grown for either 8 or 12 days, in case of critical periods of sensitivity (Metzger, 2010), and exposed to filtered conditioned media for up to 6 days.

### *$Ca^{2+}$-imaging*

Changes in $[Ca^{2+}]_i$ after immediate exposure to conditioned media were recorded with Fura 2 imaging according to our previous protocols (Sood et al., 2011).

### *Flow cytometry*

Mitochondrial stains were performed after 24 h of exposure by incubating at 37 °C for 30 min with either 100 nM NaO or 5 μM MitoSOX Red (all from Molecular Probes, Invitrogen) and directly analysed without fixing by flow cytometry using a FACScalibur (BD). Annexin V-PE (BD Pharmingen) staining was performed according to the manufacturer's instructions followed by analysis on FACScalibur (BD).

### *Primary neuronal cultures on MEAs*

Neurones dissociated from P0 Wistar rat cortices were plated (at 3000 cells/$mm^2$) and cultured on glass-substrate arrays of microelectrodes (MEAs; Multichannel Systems, Germany) (Potter and DeMarse, 2001). Prior to seeding, 12 MEAs were treated by polyethylenimine-laminin (10–0.02 mg/ml, respectively) and sister cultures were prepared and maintained on each of them at 37 °C, 5% $CO_2$. During the first 8–12 days in vitro (DIVs), the culture medium contained neurobasal, 2% B-27 supplement, 1% L-glutamine, 1% penicillin–streptomycin, and 10% horse serum (Gibco, Invitrogen Corporation), and was replaced three times per week. The 12 cultures were then divided into two groups: one exposed to the medium conditioned at 2–8%, the other to a control conditioned at 21%. Each group was further divided in two: one where exposure occurred at 8 DIVs, the other at 12 DIVs.

### *Electrophysiological recordings*

Spontaneous electrophysiological (multiunit) activity was monitored immediately after the media change, as well as one, four, and eight days after. Extracellular electric fields were monitored from up to 59 independent electrodes in each MEAs, sampled at 25 kHz/channel, 1200× amplified, bandpass-filtered (200–3000 Hz), and digitally recorded for 20 min per session. Simple spike-sorting, based on spike-unit polarity, was performed. Epochs of spontaneous synchronized firing across the MEA electrodes were identified over 25 ms bins by



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