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The blood-brain barrier; protecting the developing fetal brain

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1 **Review**

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3 **The blood-brain barrier; protecting the developing fetal brain**

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Abstract

While placental function is fundamental to normal fetal development, the blood-brain barrier provides a second checkpoint critical to protecting the fetal brain and ensuring healthy brain development. The placenta is considered the key barrier between the mother and fetus, regulating delivery of essential nutrients, removing waste as well as protecting the fetus from potentially noxious substances. However, disturbances to the maternal environment and subsequent adaptations to placental function may render the placenta ineffective for providing a suitable environment for the developing fetus and to providing sufficient protection from harmful substances. The developing brain is particularly vulnerable to changes in the maternal/fetal environment. Development of the blood-brain barrier and maturation of barrier transporter systems work to protect the fetal brain from exposure to drugs, excluding them from the fetal CNS. This review will focus on the role of the 'other' key barrier during gestation – the blood-brain barrier – which has been shown to be functional as early as 8 weeks' gestation.

47

48 **Introduction**

49 There are a number of physiological barriers present in the body throughout life. Arguably

50 the most important during pregnancy and the development of the fetus is the blood-placental

51 barrier. The placenta forms the primary barrier between the maternal environment and the

52 fetus regulating a wide variety of functions required for healthy development including gas

53 exchange, hormone production and secretion and transfer of nutrients and waste [1, 2]. The

54 placenta is essential for survival; it is responsible for stimulating the maternal endocrine

55 system to release hormones necessary for the continuation of pregnancy but also functions to

56 provide protection of the fetus from potentially harmful agents. The placenta plays a key role

57 in adaption during pregnancy, responding and adjusting throughout to signals from both the

58 mother and the fetus to ensure optimal growth and is essential to the development of the fetal

59 brain [3]. Both intrinsic and extrinsic factors from the maternal environment result in

60 modulation of intra-uterine development. In addition to the placenta however, other barrier

61 systems exist in the fetus. The blood-brain barrier (BBB) is vital for protection of the brain

62 and fundamental for the effective function of the central nervous system (CNS).

63

64 The BBB is the interface between the systemic circulation and brain parenchyma. It is

65 responsible for the regulation of movement between the two compartments and is essential

66 for maintaining homeostasis in the CNS. The brain requires a carefully maintained

67 microenvironment and protection from toxic endogenous and exogenous substances for

68 normal function. While the placenta provides the first regulatory barrier between the mother

69 and fetus, the BBB serves as a highly specific safeguard of the developing fetal brain. The

70 placenta and BBB work to protect the fetus from potentially toxic substances which can have

71 long term pathological consequences. To date, our understanding of the development and

72 functionality of this barrier has been poor, to the point that many believed that the fetal BBB

73 is immature if not altogether absent. Across time this belief has been perpetuated, and it was

74 believed protection of the growing fetus was provided solely by the placenta [4]. However,

75 experiments performed nearly a century ago as well as several recent studies shows not only

76 the presence of the BBB in the developing fetus but that it is functionally capable, possessing

77 many of the barrier properties observed in the fully developed BBB of the adult brain [5]. In

78 this review we will explore the development of the BBB, its function in the growing fetus

79 and, how such changes in the maternal environment may impact the developing brain.

80

81 **Development of the blood-brain barrier**

82 The BBB is primarily a diffusional barrier between the systemic vascular system and the

83 brain. Like the placenta, the BBB is responsible for maintaining an optimal environment for

84 development. It does this through the complex cellular structure that makes up the BBB as

85 well via a number of transport mechanisms responsible for molecule transfer and protection

86 of the brain from toxic substances. The BBB is made up of endothelial cells of the

87 vasculature forming cell-to-cell tight and adherens junctions to limit transcellular/paracellular

88 movement between the two compartments. These endothelial cells lack fenestrations, have

89 low turnover and proliferation rates, and have high electrical resistance [6]. Under normal

90 conditions these properties limit the free movement of ions, large proteins, and water

91 allowing tight control of concentration gradients between the blood and brain. This results in

92 the protection of the brain from vasogenic edema, other toxic effects, and regulation of

93 neuronal excitability. However, a fully functional BBB requires the support of a range of cell

94 types including neurons, pericytes, astrocytes, and microglia, all of which contribute to

95 barrier integrity; together these are known as the neurovascular unit (NVU).

96

4

97　Historically it was proposed that the BBB was not mature during early stages of development

98　and that the vulnerable developing brain was fully protected by the barrier properties of the

99　placenta. Early studies using vascularly injected dyes such as trypan blue in animal embryos

100　and fetuses showed permeation into all tissues including the brain, perpetuating the idea that

101　in the immature animal the BBB was undeveloped, leaky, or lacking altogether (for a

102　comprehensive review see Saunders et al. 2014 [5]). However, several studies such as those

103　by Weed (embryonic pig), Cohen and Davies (embryonic guinea pig) and Grazer (rat E10-

104　birth) did not show any evidence of staining in the brain [7-9]. In human, post-mortem tissue

105　from fetuses and neonates showed that from as early as 12 weeks gestation trypan blue did

106　not cross the BBB [10]. Overloading the binding capacity of plasma albumin, to which many

107　dyes such as trypan blue bind, results in excess dye that can easily penetrate into the brain; in

108　many of the early studies such toxic levels of dye were used that many animals died [5, 11].

109

110　**Structural development of the blood-brain barrier**

111　The precise structure of the BBB is key to its functional ability to protect and maintain the

112　brain microenvironment. Recent studies have demonstrated that humans, rats, and sheep have

113　a number of functional barrier mechanisms in place from early gestational time-points [12,

114　13]. These include tight junction proteins and several transporters at the cerebral vasculature.

115

116　The development of the BBB is a multistep process. Initial vascularisation is followed by

117　tight junction protein and nutrient transporter expression. The BBB then matures further with

118　contact of pericytes and astrocytes of the NVU [14]. Development continues with the

119　increased expression of efflux transporters, decreased levels of transcytosis, and sealing of

120　the inter-endothelial cleft. Across BBB development there are changes to the NVU, electrical

121　resistance of the endothelial cells themselves, tight junction proteins, and influx and efflux

122 transport which may alter the permeability of the BBB to different substances such as water,

123 proteins, or ions. Alterations to barrier structure and function address specific needs of the

124 brain at various developmental stages.

125

126 Vascularisation of the human telencephalon begins at approximately week 8 of gestation, by

127 the 12th week tight junction proteins occludin and claudin-5 are expressed in the primary

128 vessels [15]. The appearance of tight junction proteins at this time appears sufficient to

129 prevent endogenous albumin from entering the brain, providing evidence of early

130 functionality of the barrier [15]. By the 18th week of gestation, these tight junction proteins

131 demonstrate similar staining patterns to the tight junctions of the adult BBB [15]. Freeze

132 fracture and thin section electron microscopy in neonatal human tissue demonstrates that tight

133 junctions are organised in complex, linear, near-continuous tracts between endothelial cells of

134 the microvasculature [16]. Similar detailed findings have been reported in rat BBB tight

135 junction development, where tight junctions are abundantly present in late gestation and

136 undergo increases in complexity in terms of integrity and length throughout gestation and

137 after birth [17, 18].

138

139 There are several studies demonstrating *in vitro* that astrocytes are essential for tight junction

140 formation [19-21]. However, it has been demonstrated in rodents that tight junctions are

141 present and functional at birth [18], whereas the primary period of astrocytic differentiation

142 and vessel encirclement does not occur until the third postnatal week [22]. It is unclear how

143 astrocytes contribute to the development of the barrier phenotype, however there is evidence

144 to support a role in the progressive tightening of tight junctions after birth [23]. Another cell

145 type critical for development and maintenance of the BBB is the pericyte [24]. While

146 pericytes are expressed throughout the systemic vasculature, the highest density of pericytes

147 is found in the brain [24]. Pericytes have also been shown to be essential for the formation of

148 tight junctions [24, 25]. Generation of pericytes and their effects on tight junction formation

149 occur as early as P13 in the rodent preceding those of the astrocyte [24]. The process of

150 pericyte encirclement of cerebral vessels is associated with a decrease in BBB solute

151 permeability – demonstrating their functional importance in CNS protection [26].

152

153 How the development of the BBB across time affects barrier functionality is not well

154 understood. It is clear that each of these changes is necessary for the tight control at the BBB

155 demonstrable in adults. However, differences in permeability to drugs of differing size or

156 pharmacological properties across development are unknown and likely to be species-

157 dependant. This presents challenges when targeting treatments towards the mother or fetus in

158 the prediction of efficacy and toxic effects. These factors need to be kept in mind when

159 studying the effects of the maternal environment on placental function and impacts on the

160 CNS.

161

162 **Transport across the blood-brain barrier**

163 There are numerous transport mechanisms present at the BBB to provide the brain with

164 essential nutrients and to provide protection from toxic substances. Transport at the BBB

165 occurs via free-diffusion of small lipophilic substances or via catalysed transport processes

166 such as carrier mediated transport, receptor-mediated transport, and active efflux transport.

167 Efflux transport is essential for the protection of the brain from endogenous substances such

168 as the excitatory neurotransmitter glutamate (although crucial for neuronal signalling,

169 excessive levels are neurotoxic) and is key in regulating drug entry to the fetal brain [27, 28].

170 Present at high concentrations in the fetal circulation, glutamate levels in the brain are

171 regulated at the BBB by the excitatory amino acid transporters (EAATs 1-4) [27].

7

172

173   There are a number of transporter systems expressed throughout the body several of which

174   are common to both the placenta and the BBB. Active efflux transporters come from two

175   major classes of transporters which extrude metabolic waste, xenobiotics and a large number

176   of drugs from the brain back into the blood. The first superfamily that has BBB efflux

177   transporter members is the solute carrier proteins (SLC) superfamily. Transporters from three

178   SLC subfamilies comprise the majority of known SLC efflux transporters. At the BBB these

179   include SLC22 and SLCO (SLC21). The second, and most comprehensively studied of the

180   efflux transporters at the BBB is the ATP-binding cassette (ABC) efflux transporter family.

181   Permeability glycoprotein (P-gp) is an important member of this super family along with

182   breast cancer resistance protein (BCRP) and the multidrug resistance associated proteins

183   (MRPs) [29].

184

185   **Expression and Function of SLC efflux transporters**

186   SLC efflux transporters play an important role in elimination of organic compounds,

187   especially organic anions, from the brain. Efflux members of this family are bi-directional

188   transporters and commonly localise to the basolateral membrane of cerebral endothelial cells

189   to remove toxic compounds from the brain extracellular space [30]. Unlike the ABC

190   transporters much less is known about developmental expression changes to SLC efflux

191   transporters at the BBB. Evidence from rodent models suggest that expression of Oat3

192   (Slc22a8), which is present on endothelial cells of the BBB and at the choroid plexus,

193   remains mostly unchanged across development [31-34]. Of the SLCO family Oatp1a4

194   (Slco1a4) and Oatp1a5 (Slco1a5) are present on the rodent BBB with expression of

195   Oatp1a4/Slco1a4 demonstrated to increase from P2 to P84 postnatal days, equivalent to that

196   of human brain development from the preterm to the adult [34]. Oatp1a4 is located at both

197 apical and basolateral membranes in the rat brain, whereas Oatp1a5 membrane localisation is

198 yet to be elucidated [35, 36]. In humans, OATP1A2 is the only isoform that shows relatively

199 high homology with rodent Oatp1a4 and Oatp1a5. OATP1A2 is predominantly expressed in

200 the brain at cerebral vasculature endothelial cells, however the membrane localisation is not

201 yet clear [37] (Figure 1).

202

203 **Expression and function of ABC efflux transporters**

204 An important member of the ABC efflux transporter family, the P-gp transporter has a

205 significant role in regulating drug transfer across both the placenta and BBB and a crucial

206 role in neuroprotection [38, 39]. In the CNS, P-gp is primarily expressed on the luminal

207 surface of endothelial cells of the cerebral vasculature as early as 8 weeks gestation [29, 40,

208 41]. In contrast with placental P-gp expression which is maximal at the beginning of

209 pregnancy and declines with increasing gestation, BBB protein and mRNA levels of P-gp are

210 low and dramatically increase with advancing gestation in rodents and humans [42-45]. This

211 suggests a compensatory mechanism that ensures continual protection of the brain from

212 xenobiotics that are no longer sufficiently repelled by the placenta [46]. The mechanism of

213 this action is unclear, however it is known that P-gp expression at the BBB appears to be

214 regulated by glucocorticoids and, that BBB P-gp levels rise with a simultaneous increase in

215 maternal and fetal cortisol levels [47, 48].

216

217 P-gp is involved in the transport of several prescription medications commonly administered

218 to women during pregnancy to treat various conditions including asthma, hypertension,

219 diabetes and epilepsy [49, 50]. Considering the highly lipophilic nature of such drugs the role

220 of P-gp in excluding them from the fetal circulation is critical to protection of the developing

221 fetus .However, P-gp may be altered by exposure to some drugs, and maternal factors such as

9

222 undernutrition have also been reported to impair P-gp expression both in the placenta and the

223 brain potentially compromising placental function and may contribute to fetal exposure to

224 potentially teratogenic drugs [39, 51].

225

226 Other efflux transporter membranes of the ABC superfamily are MRPs, specifically 1, 2, 4,

227 and 5 which have definitive localisation on cerebral vessels [44, 52-54]. MRP homologs have

228 a substantial degree of substrate overlap, and transport drugs conjugated to glutathione,

229 sulphate, or glucoronate. Selected members may also transport endogenous substances such

230 as leukotriene, bilirubin glucuronides and prostaglandins [53]. Expression of the MRPs at the

231 BBB varies with MRP1, 2, and 5 located on the apical membrane of cerebral endothelial cells

232 whereas MRP4 is equally distributed on apical and basolateral membranes (Figure 1) [54,

233 55]. In rat forebrain it has been demonstrated that Mrp1 and 4 mRNA increase from

234 embryonic day 13 through to postnatal time-points (P1 and P7 respectively) [44]. To date,

235 changes to the developmental expression of MRP2 and 5 is unknown.

236 BCRP removes a wide range of substances including chemotherapeutic agents, antiviral

237 drugs, and carcinogens from the brain into the blood [56]. It is expressed on the apical

238 membrane of the cerebral endothelial cells from early developmental ages and in rodents has

239 been shown to be expressed from embryonic day 12.5 – approximately week 22 of human

240 gestation [42, 44, 57, 58]. BCRP expression remains largely unchanged across development,

241 with a moderate increase in mRNA found between postnatal ages and adult in the mouse and

242 rat [33, 34].

243

244 **Exploiting the fetal blood-brain barrier**

245 P-gp transporters repel many classes of prescription and illicit drugs from the brain. These

246 drugs include calcium channel blockers, statins, opioids, chemotherapies, HIV protease

247 inhibitors, and anti-epileptic drugs [59]. Many women require prescription drugs that are

248 substrates of the P-gp transporter throughout pregnancy, either for acute or chronic conditions

249 such as asthma, epilepsy, or cancer. In the early 2000's, the percentage of pregnant women

250 who used prescription drugs ranged from 27 to 93% [60-62].

251

252 Improved understanding of drug transport systems at the BBB during development could

253 allow exploitation of endogenous systems for the protection of the fetal brain. Exogenous

254 glucocorticoids such as those given to pregnant women at risk of premature birth can result in

255 premature maturation of P-gp expression and function at the BBB as demonstrated in *in vitro*

256 experiments [46, 51]. This effect may be particularly useful in babies at risk of brain damage

257 due to prescription or illicit drug use in the mother. It must be noted, however, that P-gp also

258 regulates entry of substrates such as cortisol and aldosterone that are required for normal

259 brain development [46].

260

261 Conversely, a reduction in P-gp function is of interest clinically to enhance the delivery of

262 drugs to specifically treat CNS pathologies in the fetus. Selective serotonin reuptake

263 inhibitors (SSRIs) have been identified as a class of drug that inhibit P-gp transporters at the

264 BBB [63]. However, sertraline (a member of the SSRI family) has the opposite effect on

265 function of P-gp at the placenta. Mice treated with sertraline had increased efflux function of

266 the P-gp transporter at the placenta, and decreased P-gp transporter function at both the fetal

267 and maternal BBB [63]. These tissue specific effects emphasise the need to understand

268 transport functions at both the placenta and BBB in the development of novel therapies.

269

11

270   Illicit drug use during pregnancy in the United States has been estimated to be approximately

271   16% in teenagers, and 7% in women 18-25 years of age [64]. There is a population of women

272   who seek assistance from physicians for opioid maintenance with methadone or

273   buprenorphine [65]. Exploiting the developing BBB in the fetus has been proposed as a novel

274   therapy to prevent neonatal abstinence syndrome without significant effects on maternal

275   maintenance. A selective opioid antagonist has been identified that crosses the placenta and

276   fetal BBB without substantial effects on the maternal BBB opioid receptors [66]. Oberdick

277   and colleagues suggest that is it the immaturity of the BBB in the neonatal mouse (equivalent

278   to a fetal human) that allows the opioid antagonist 6β-naltrexol to cross into the brain and

279   exert its effect. While such results are encouraging, species differences in BBB development

280   is an important consideration. Significant alterations in the timing of BBB development have

281   been noted in humans when compared with other mammals. It is also worth noting that there

282   are several other routes into the brain such as the blood CSF barrier which may affect studies

283   that manipulate developmental differences [67].

284

285   Other avenues to exploit the BBB include development of therapies to increase biochemical

286   barrier function at the BBB and placenta and therefore decrease fetal exposure and toxicity.

287   Nanoparticles are a promising area of research where a drug can be encapsulated for targeted

288   delivery to specific tissues. In the case of maternal CNS disorders, direct targeting would

289   increase the proportion of drug reaching the maternal brain, reducing the dose available to

290   cross the placenta and reach the fetus [68]. Liposomal encapsulation may significantly reduce

291   placental transfer of drugs such as the anti-epileptic drug valproic acid [69]. It was postulated

292   that this technique may be useful in the treatment of maternal CNS disorders such as epilepsy

293   without exposing the fetus to significant amounts of drug. However, it is unclear how this

294   modification would affect transport through the maternal BBB and therefore disease control.

295 This technique reduces the risk in manipulation of placental transporters and potential off

296 target effects such as unintended alteration of BBB transport function. However,

297 advancement in encapsulation technology needs to occur before it can be applied clinically

298 [68].

299

300 **Conclusion**

301 Despite the presence of placental efflux transporters, the placenta is an imperfect drug barrier.

302 Given sufficient time and dosage most drugs can breach the placenta and enter the fetal

303 circulation, posing a teratogenic risk to the fetal brain. Although the placenta and BBB have

304 several efflux transporters in common, the BBB is a far more structurally complex and

305 restrictive system. To cross the BBB and enter the CNS, drugs need to be small and lipophilic

306 or have dedicated transport systems. Drugs with these properties would easily cross the

307 placental barrier even with functional efflux transporters, as quick diffusion surpasses the

308 ability of efflux transporters to pump substances from the fetal circulation. Understanding

309 how the BBB functions during development of the fetus is essential to ensuring optimal brain

310 growth and protection from drugs and toxins once they cross the placenta. Given the

311 differences in BBB development across species, refinement of animal models is critical

312 before application to the human.

313

314 Minimising drug exposure in the fetus is vital to reducing teratogenic effects, and long-term

315 neurological disease. Recently it has been shown that valproate, and other anti-epileptic drugs

316 such as phenytoin and topiramate during pregnancy can result in long-term reductions in IQ

317 and behavioural consequences [70]. Stolp and Dziegielewska (2009) have previously

318 reviewed the role of disruption to the BBB during development in the onset of serious

319 neurological diseases such as Alzheimer's disease and multiple sclerosis [71]. It is unclear at

320 this stage the long-term functional consequences of changes to transporters at the BBB during

321 development.

322

323 It is important to note that there are a number of relevant additional factors to consider that

324 have not been addressed in this review. Drug metabolising enzymes at the BBB and placenta

325 may significantly affect drug transfer through these barriers, and biotransformation by these

326 enzymes may affect movement of substances by barrier efflux transporters [72, 73]. Drug

327 metabolising enzymes with actions at the BBB include phase I and phase II enzymes such as

328 cytochrome P450 and glutathione *S*-transferases [74]. In addition, there are other routes into

329 the brain other than the BBB, these include the blood-CSF barrier between the systemic

330 blood supply and the choroid plexus, the meningeal barrier, and the fetal-specific CSF-brain

331 barrier [11]. These factors are an additional source of complexity when considering how best

332 to protect the developing brain.

333

334 A significant clinical challenge is the protection of the vulnerable fetal brain from drugs in

335 the maternal environment. The changes in placental or BBB function due to drug exposure

336 from the maternal environment have significant clinical relevance as the functional outcomes

337 vary and are often unknown. Greater consideration needs to be paid to the relationship

338 between placental adaptation to the maternal environment and changes to other barrier

339 systems in the fetus such as the BBB to fully understand the functional consequences of these

340 changes. In addition, transporter systems of the placenta and BBB and the effect of substrates

341 and inhibitors needs to be further elucidated. A more comprehensive understanding of these

342 systems may allow exploitation of transport in order to better treat and protect the fetal brain.

343

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Blood - Luminal/apical membrane
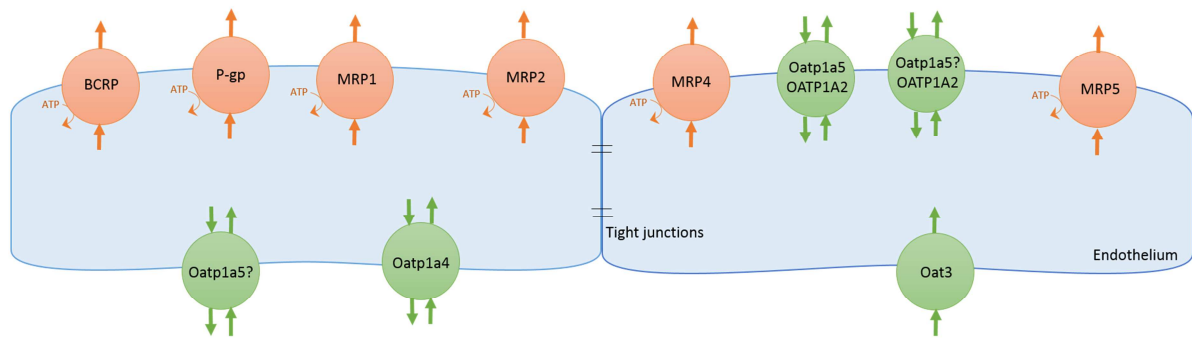
Brain - Subluminal/basolateral membrane

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