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Hurdles with Using *In Vitro* Models to Predict Human Blood-brain Barrier Drug Permeability: A Special Focus on Transporters and Metabolizing Enzymes

Ramzi Shawahna[1,2], Xavier Declèves[1,2] and Jean-Michel Scherrmann[1,2,*]

[1]*Neuropsychopharmacologie des addictions (CNRS UMR 8206), Université Paris Descartes, Faculté de Pharmacie, Paris, France;* [2]*INSERM U705, Neuropsychopharmacologie des addictions, Paris, France*

Abstract: The penetration of drugs into the human brain through the blood-brain barrier (BBB) is a major obstacle limiting the development of successful neuropharmaceuticals. This restricted permeability is due to the delicate intercellular junctions, efflux transporters and metabolizing enzymes present at the BBB. The pharmaceutical industry and academic research relies heavily on permeability studies conducted in animals and *in vitro* models of the BBB. This text reviews the available animal and *in vitro* BBB models with special emphasis on the situation in freshly isolated human brain microvessels and the unique tightness between brain endothelial cells, drug transport pathways and metabolic capacity. We first outline the delicate structure of the intercellular junctions and the particular interaction between the brain endothelial cells and other components of the neurovascular unit. We then examine the differences in transporters and metabolizing enzymes between species and *in vitro* systems and those found in isolated brain microvessels. Finally, we review the possibilities of benchmarking *in vitro* models of the BBB in terms of gene and protein expression.

Keywords: ATP-binding cassette, blood-brain barrier, cytochromes P450, *in vitro* models, metabolizing enzymes, solute carrier.

1. INTRODUCTION

Drug permeability into the human brain is a huge obstacle challenging the development and success rate of neuropharmaceutical candidates [1]. In pharmaceutical industry, low permeability across the blood-brain barrier (BBB) is a classic motive to discontinue the vast majority of the candidates investigated to treat brain diseases [2]. Lead optimization involves determining the capacity of numerous candidate molecules to enter the brain, which is again not possible in humans. The pharmaceutical industry and academic research have developed in two directions to overcome this hurdle. The first involves studying the ideal physicochemical properties of successful neuropharmaceuticals in trials to optimize future candidates [1]. *In silico* computational approaches and quantitative structure-activity relationship (qSAR) models have became popular for this approach. The second involves characterizing the drug transport and metabolic processes at the BBB so as to identify exploitable biomarkers, such as drug uptake transporters, and to circumvent other efflux transporters and metabolizing enzymes.

Although *in vivo* data are always more relevant than any *in vitro* data in clinical research, it is extremely difficult to obtain data on the human BBB *in vivo* [3]. *In vivo* data are often obtained from *in situ* brain perfusion studies on other animal species, but these cannot be simply extrapolated to humans. The penetration of a drug into the human brain is more often approximated by measuring its concentration in the cerebrospinal fluid (CSF), which is not a direct measurement of its BBB permeability or of its concentration in the brain extracellular fluid.

The situation is further complicated by the huge differences observed in the brain uptake ratios between human and rodents. Even in higher primates which are considered very close to humans, recent studies showed considerable distribution differences of CNS drugs as compared to those observed in human [4]. These observations are linked to differences in the patterns and functions of drug transporters and enzymes at the BBBs of human and animal models, showing clearly the need to carry out studies in representative situations. Therefore, scientists began to develop *in vitro* models that reflect as closely as possible the *in vivo* situations at the BBB. These efforts have led to the appearance of several models using primary brain endothelial cells from different species [5, 6]. These *in vitro* models have evolved and now show some similarities to the BBB *in vivo*.

The recent advancements in brain microvessel isolation techniques, coatings, support materials, media and their supplements have revolutionized brain vasculature endothelial cell cultures. Endothelial cells are now co-cultured with other cells of the neurovascular unit (NVU) to obtain an improved BBB maturation and phenotype [7, 8].

In vitro models have often been claimed to closely reflect the human BBB in terms of phenotype, restricted paracellular permeability, and functional transporters and enzymes, regardless of their species origin [9]. The scientific and industrial communities presently rely greatly on the existing *in vitro* models as real representations of the human BBB, so that the influx, efflux and metabolic data generated in these models are often extrapolated to the human BBB during the various phases of drug development [10]. The great advances that have been made recently in molecular biology have led to the characterization and quantification of genes and proteins in human and animal brain microvessels [4, 11-14].

This review assesses the representative nature and the extent of closeness of different animal species and *in vitro* BBB models to those observed in freshly isolated human brain microvessels as the closest representation of the BBB *in vivo*, it focuses mainly on three features: a) *the unique tightness between brain endothelial cells which prevents paracellular transport across the BBB, and the complex network of structural proteins that lead to this remarkable resistance to drug permeability,* b) *the active transport pathways mediated by solute carrier (SLC) and ATP-binding cassette (ABC) transporters,* and c) *the metabolic capacity resulting from the spectrum of phase I and phase II metabolizing enzymes*.

2. THE HUMAN BBB AND BRAIN MICROVESSEL ENDOTHELIAL CELLS (BMVECS) IN THE NVU

It is now universally accepted that the BBB is formed by the endothelial cells lining the brain blood microvessels [15, 16], also known as brain capillary endothelial cells [17] and cerebral capillary endothelial cells [18]. These cells are referred to as brain microvessel endothelial cells (BMVECs) in this review. Only microvessels possess the properties of a mature BBB, since leakiness

*Address correspondence to this author at the INSERM U705 CNRS UMR 8206, Faculté de Pharmacie, 4 avenue de l'Observatoire, 75006 Paris, France; Tel/Fax: (+33)-1-53 73 97 19;
E-mail: jean-michel.scherrmann@inserm.fr

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