Alicante

eSI Meeting, Sept. 29-30

Pr Roméo Cecchelli
The ageing of the population gives to neurodegenerative diseases a frightening future because they relate to a population more and more important.
But

95% of new synthetised molecules do not reach the brain....

WHY?
The BBB was located in brain capillaries

The length in Human is around 650 km
Its surface area is around 12 m²

The mean distance between 2 capillaries is around 40 microns
• Brain capillaries are a complex structure
The intercellular cleft at a junction between two endothelial cells of a permeable capillary supplying a choroid plexus papilloma, is narrowed but not occluded. Unlike the tight junction shown in Fig. 1, this junction is patent (arrowheads) and is recognized as a junction by cytoplasmic densities situated symmetrically on either side of the cell membrane beneath the arrowheads. The vessel lumen is at top. $\times 250,000$. 
The intercellular cleft between two endothelial cells of a capillary within the brain of a mouse is occluded at two tight junctions (arrows) that have been sectioned transversely. In the transverse plane of this thin, plastic-embedded section, the junctions appear as short contacts (1). The vessel lumen is at top. × 380,000.
BBB: a physical barrier

Blood

Apical plasma membrane

Tight junction
Claudins
Occludin
JAMs
Adherens junction

Brain

ZO-1
ZO-2
ZO-3
Actin
Cingulin
AF6
7H6

α β/γ Cadherins
β/γ α Cadherins
Peripheral capillary
Peripheral capillary
BBB: a metabolic barrier

Blood

L-DOPA

Drug-metabolizing enzymes

MAO

Degradation

Brain

P-gp
CYP 450

MAO-B

Glutamyl aminopeptidase

Endothelial cells

BBB: A METABOLIC BARRIER

Pericyte

MAO-B

Glutamyl aminopeptidase

UGT-1A6

GST

CYP 1A1 (rat)

CYP 1B1 (human)

CYP 2B1 (rat)

CYP 2B6 (human)
Transport processes through a cerebral endothelium

Specific transport (receptor or transporter)
Different techniques used to study the BBB
Oldendorf (BUI)

(BRAIN RESEARCH, 1970)

Known fraction of $^3$H$_2$O in brain

Unknown fraction of $^{14}$C compound in brain

ext carotid and Pterygopalatine a.

10%

90%

unextracted tracers

Venous outflow

common carotid a.

bolus $^3$H$_2$O and $^{14}$C-compound

BUI % = ($^{14}$C / $^3$H in brain) / ($^{14}$C / $^3$H in perfusate)
IN SITU BRAIN PERFUSION TECHNIQUE

$^{3}\text{H}/^{14}\text{C}$-labelled drug
(to measure BBB permeability)

$^{14}\text{C}/^{3}\text{H}$ - sucrose/inulin
(to measure cerebrovascular volume)

RAT (Takasato et al., 1984; Smith, 1996)
MOUSE (Dagenais et al., J Cereb Blood Flow Metab, 2000)
<table>
<thead>
<tr>
<th>RESEARCH</th>
<th>DEVELOPMENT</th>
<th>BIRTH</th>
<th>LIFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 2 years</td>
<td>1 to 2 years</td>
<td>1 to 2 years</td>
<td>6 to 8 years</td>
</tr>
</tbody>
</table>

**SEARCH FOR CHEMICAL STARTING POINTS**
- Conception
- Synthesis

**SCREENING**
- Potency
- Selectivity
- Oral bio-availability
- Duration of action

**OPTIMISATION**
- In vitro & Animal Preclinical Evaluation
  - Potency
  - Selectivity
  - Oral bio-availability
  - Duration of action

**CLINICAL HUMAN EVALUATION**
- Phase I: Tolerance & Pharmacokinetics
  - Activity
  - Characterisation
  - Stability
  - Safety
  - Efficacy in whole organism
- Phase II: Biological Activity & research of a therapeutic effect
- Phase III: Confirmation of safety & therapeutic effect

**FILE SUBMISSION/REGISTRATION**
- Marketing Approval

**PHASE IV**
These techniques can not be used in screening to evaluate the brain penetration of a molecule.
In vitro methods
Cell lines

• All immortalized brain capillary endothelial cell lines (RBE4, MBEC line, TR-BBB) do not express most of the tight junction proteins and are very leaky.

• They cannot be used to study transcellular transport.

⇒ I just want to focus my presentation on primary and long term culture of brain capillary endothelial cells.
Classical Method

Gray matter

Enzymatic digestion + Centrifugation

Microvessel endothelial cells and pericytes

Capillaries  Arterioles  Venules  Pericytes

Primary culture of microvessel endothelial cells
Gray matter

Mechanical homogenization

Filtration

Isolated microvessels

Arterioles  
**Capillaries**  
Veinules

Extracellular Matrix

Pure capillary endothelial cells
What about the Blood Brain Barrier marker?
Freeze fracture examination of endothelial cells

Transendothelial electrical resistance = 400 Ohms.cm²
Histochemical detection of g-GT in brain capillaries
Gamma-GT expression

<table>
<thead>
<tr>
<th>Isolated capillaries</th>
<th>BBCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1</td>
</tr>
<tr>
<td>P</td>
<td>2</td>
</tr>
<tr>
<td>P3</td>
<td>5</td>
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<td>P4</td>
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<td>P</td>
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</tr>
<tr>
<td>P6</td>
<td></td>
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<tr>
<td>P</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>% positive cells</th>
<th>Tight junctions</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>MAO</td>
</tr>
<tr>
<td>+</td>
<td>ACE</td>
</tr>
<tr>
<td>+</td>
<td>gamma-GT</td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>+</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Units / (mg prot.min)</th>
<th>F VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Gamma-GT expression
Consequently, endothelial cells in culture alone cannot be considered as a relevant blood-brain barrier model.
The BBB localisation

Astrocytes

Endothelial cell
The *in vitro* BBB model

- Blood
- Brain
- Endothelial cells
- Glial cells
Glial cell repartition

Astrocytes  Oligodendrocytes  Microglia
What about the g-GT activity?
Histochemical detection of g-GT in brain capillaries endothelial cells
Statistical study of 2D-PAGE area from BCECs

- Co-culture
  - Important variation: overexpressed in co-culture
  - No statistical significant variation

- Solo-culture
The transendothelial electrical resistance raises from 400 Ohms.cm² in soloculture to 800 Ohms.cm² in coculture.
P-gp detection by Western Blot analysis

- Isolated capillaries
- BBCE in coculture
- BBCE in solo culture

k Da

205

140
Analysis of OCTN2 and P-gp immunofluorescent staining in apical and basolateral membrane of endothelial cells

- OCTN2
- P-gp
- Nuclei

Fluorescence intensity vs. Slides (1 = 0.16μm)

Apical membrane
Basolateral membrane
Inhibition study (Uptake)

Control

S9788 (1μM)

VINCRISTINE

% / control
Conclusion

In coculture with astrocytes, brain capillary endothelial cells present most of the characteristics that are known to have important functions in vivo.
How can we use this model to predict the drug brain penetration?
General working process

- **Glial Cells**
  - Preparation of glial cells culture medium
  - Defrosting glial cells vial

- **BCEC**
  - Preparation of BCEC culture medium
  - 60mm dishes coating
  - Defrosting BCEC vial
  - Filters coating
  - Trypsinisation
  - Seeding

- **Co-culture**
  - Transport experiment

- **Experiments**
  - Analytics
  - Raw data processing

- **Refresh**
  - Refreshing BCEC medium (every 2 days)
  - Refreshing glial cells medium (twice a week)

**Timeline**
- 3 weeks: Glial Cells
- 12 days: BCEC
- Co-culture
- Experiments
BBB Permeability studies

Dosage
SUCROSE

$Pe = 0.24 \times 10^{-3} \text{ cm/min}$

$PSf = 14.38 \mu L/min$

$R^2 = 0.99$

$PSt = 0.95 \mu L/min$

$R^2 = 0.99$
CAFFEIN

\[ Pe = 59.60 \times 10^{-3} \text{ cm/min} \]

\[ PSf = 16.16 \mu\text{L/min} \]
\[ R^2 = 0.99 \]

\[ PSt = 15.18 \mu\text{L/min} \]
\[ R^2 = 0.99 \]
Correlation between permeability values obtained *in vivo* with the *in vivo* techniques (Brain perfusion and Oldendorf) and *in vitro* with the BBB model.
157 SERVIER COMPOUNDS
(High diversity of compounds)

R² = 0.2415
In Silico / In vitro correlation

Correlation between BBB cell culture permeability and in silico prediction

$R^2 = 0.52 \Rightarrow R = 0.72$

$Q^2 = 0.50$

Compounds measured by Stefan Lundquist and Mila Renftel Research DMPK, Södertälje. In silico model developed by Markus Haeberlein, Chemistry Dept, Södertälje
Reproducibility

- 6 last months
- 3 different clones (B1, PK, Lau1) between P4 et P7
- 4 different technicians

\[
\text{Pe (sucrose)} = 0.28 \pm 0.12 \ (n=81)
\]

Astrazeneca (Sweden)

\[
\text{Pe (sucrose)} = 0.32 \pm 0.18 \ (n=52)
\]
## Ranking

<table>
<thead>
<tr>
<th>Example of product</th>
<th>Pe (x 10^{-3} cm/min)</th>
<th>Brain penetration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>59.90</td>
<td>VERY GOOD</td>
</tr>
<tr>
<td>Nicotine</td>
<td>58.76</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>19.86</td>
<td>GOOD</td>
</tr>
<tr>
<td>Antipyrine</td>
<td>18.74</td>
<td></td>
</tr>
<tr>
<td>DiPhenylHydantoine</td>
<td>7.37</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>2.22</td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>Verapamil</td>
<td>1.74</td>
<td></td>
</tr>
<tr>
<td>Warfarin</td>
<td>1.72</td>
<td></td>
</tr>
<tr>
<td>L. Dopa</td>
<td>1.29</td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
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<td></td>
</tr>
<tr>
<td>Sucrose</td>
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<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

30 \times 10^{-3} \text{ cm/min}  
2.1 \times 10^{-3} \text{ cm/min}  
1.1 \times 10^{-3} \text{ cm/min}
This *in vitro* model constitutes a RELEVANT model to the BBB
SEARCH FOR CHEMICAL STARTING POINTS

CONCEPTION

SYNTHESIS

OPTIMISATION

IN VITRO & ANIMAL PRECLINICAL EVALUATION

PHASE I

Potency

Activity

Tolerance & pharmacokinetics

PHASE II

Selectivity

Characterisation

Biological Activity & research of a therapeutic effect

PHASE III

Oral bioavailability

Stability

Safety

Confirmation of safety & therapeutic effect

PHASE IV

Duration of action

Efficacy in whole organism

CLINICAL HUMAN EVALUATION

FILE SUBMISSION/REGISTRATION

MARKETING APPROVAL

RESEARCH DEVELOPMENT BIRTHLIFE

1 to 2 years

1 to 2 years

1 to 2 years

6 to 8 years

1 to 2 years
**General working process**

**Preparation of glial cells culture medium**
- Defrosting glial cells vial

**Preparation of BCEC culture medium**
- 60mm dishes coating
- Defrosting BCEC vial
- Filters coating
- Trypsinisation
- Seeding

**Transport experiment**
- Analytics
- Raw data processing

**Experiments**
- 12 days

**General working process**
- Refreshing BCEC medium (every 2 days)
- Refreshing glial cells medium (twice a week)

**Glial Cells**
- 3 weeks

**BCEC**
- 12 days
General working process

4D@Screen Pack

1 day: CELLIAL BBB-inducing medium

Preparation of BCEC culture medium
60mm dishes coating
Defrosting BCEC vial
Filters coating
Trypsinisation
Seeding

Transport experiment
Analytics
Raw data processing

Preparation of in vitro BBB model

Experiments

4 days
General working process

CT Bovial@Screen Pack

Preparation of glial cells culture medium
Defrosting glial cells vial

Preparation of BCEC culture medium
60mm dishes coating
Defrosting BCEC vial
Filters coating
Trypsinisation
Seeding

Transport experiment
Analytics
Raw data processing

Glial Cells
BCEC
Co-culture
Experiments

1 day: CELLIAL BBB-inducing medium

4D@Screen Pack

CT Bovial@Screen Pack

Preparation of in vitro BBB model
Experiments

12 days
3 weeks
12 days
4 days

Refreshing glial cells medium (twice a week)
Refreshing BCEC medium (every 2 days)
Culture Time

3 weeks (glial cell culture)

Co-culture

60mm-culture dish

4 day-culture

CT Bovial@Screen Pack / 4D@Screen Pack

+ 7 days (confluence) + 5 days : BBB ready

4 days : BBB ready
BBB characteristics

Cell monolayer morphology:

Vimentin

Actin

Bar = 25 μm
BBB characteristics

Tight junctions:

- **Occludin**
- **ZO-1**
- **Claudin-1**
- **Claudin-5**

Bar = 25 μm
Control of BBB integrity

Sucrose permeability

Integrity threshold

12 day-Co-culture
4 day-culture
P-glycoprotein

Presence of the protein (Western Blot)  
Functionality of P-gp (inhibition assay)

MW (kDa)

220 -
94 -
66 -
### Transporters (mRNA)

RT-PCR from co-cultures, capillaries and 4 day-endothelial cells

<table>
<thead>
<tr>
<th>Transporter</th>
<th>coc</th>
<th>C</th>
<th>4 day-e</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
</tr>
<tr>
<td>MRP1</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
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<tr>
<td>MRP4</td>
<td><img src="#" alt="Image" /></td>
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<tr>
<td>MRP5</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
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</tr>
<tr>
<td>MRP6</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
</tr>
<tr>
<td>β-actin</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
</tr>
</tbody>
</table>
1 vial of endothelial cells

3 x 60mm-culture dishes

CT Bovial@Screen Pack / 4D@Screen Pack

90 filters

12 day-system
6 well-filters

226 filters

4 day-system
24 well-filters
Correlation between \textit{in vitro} BBB (24w-4d permeability) and \textit{in vivo} BU (brain perfusion)

\[ R^2 = 0.8538 \]

All parameters were normalized for molecular weight
Correlation between in vivo BU (brain perfusion) and in vitro MDCK (Papp)

All parameters were normalized for molecular weight

\[ R^2 = 0.3956 \]
How to regulate, dose, improve the cerebral transport?

- By testing hits or leads on the *in vitro* model to have an experimental result of reference for permeability.
- By testing optimized compounds (affinity, selectivity) on the *in vitro* model to check the increase in permeability and select the best candidates.
- By testing selected compounds on *in vivo* model to check the *in vivo* cerebral penetration.

The *in vitro* model is an complementary tool which provide data to help for decisions.

It allows mechanistic studies on a cellular and a molecular level (Pgp- Specific transporters, pathological conditions i.e. stroke, inflammation).
Use of the transcytotic pathway.

Blood

Low density lipoproteins (LDL)

Brain
Conclusion

Lamp 1
Légèrement acide
Nanoparticules : biovectors
Transport of albumin-loaded biovectors

Pe (x10^-3 cm/min)

Albumin

Albumin-loaded biovectors

X 27