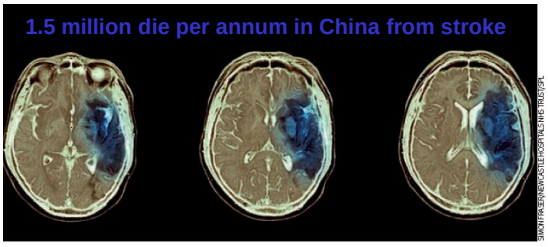
Stroke is the third most common cause of death in England and Wales and affects 150,000 people each year in the UK.



A major cause of disability

Mortality rate ~ 30% *Incidence of 250-400/100,000*

UK stroke outcomes in Europe

3 large European multicentre studies

Improving the outcome of stroke
Markus, H. 2007 BMJ **335,** 359-360.

Recommendations

- Rapid intervention in STROKE improves outcome
- Specialist Units (shortage in the UK) provide thrombolysis necessary within 3h improve outcome
- New neuroprotective drugs needed

Acute stroke

- Occlusion or haemorrhage of cerebral BVs:
  Transient cerebral ischaemia (TIA)
  Cerebral ischaemic stroke (CI)
  Primary intracranial cerebral haemorrhage (ICH)
  Sub-arachnoid haemorrhage (SAH)
- Current acute treatments for CI improve blood flow (tPA ,aspirin anti-platelet) - minority of pts
- Prophylaxis: cholesterol lowering statins (atherosclerosis), ACE inhibitors, anti-platelet, anti-hypertensives.

How can infarct size be reduced in the majority of patients???

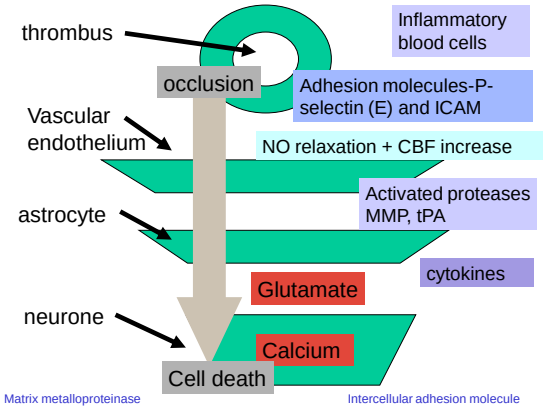
Cerebral ischaemia: a transient or permanent reduction in CBF

How can cell death be limited?

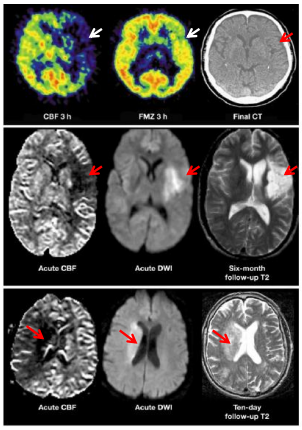
- **Stroke pathophysiology:** the role of glutamate and the ischaemic cascade
- **Balance between cell death and neuroprotection**.
- **Future directions for therapeutic intervention.**

Common mechanisms mediate acute CNS injury (stroke, trauma and seizure)

Stroke pathophysiology involves multiple tissue compartments



Stroke pathophysiology: spatial and temporal issues



Imaging the penumbra

PET (+ tPA)

69 year F, Final CT @ 3weeks
FMZ, flumazanil binds to the BZ site on the $GABA_A$ receptor

MRI (- tPA)

33 year M, hemiplegia and aphasia, Acute @ 4h
DWI ,Diffusion weighted image, images the water molecule and indicates "swelling"

MRI (+ tPA)

78 year M, Acute @ 4 - 5h

Lo et al 2003

Blood flow in cerebral ischaemia

> 50 ml/100g/min Normal

> 22 < 50 ml/100g/min olighaemia

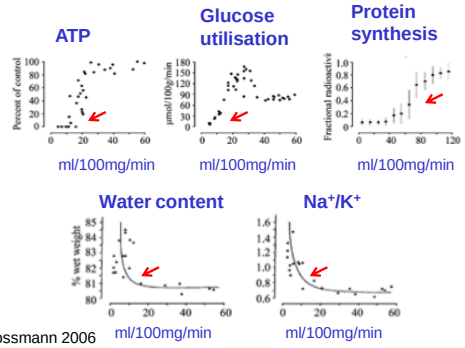
Hypoperfusion but likely to survive depending on factors such as collateral flow

< 22 ml/100g/min ischaemic penumbria

Misery perfusion likely to progress to infarction

< 10 ml/100g/min rapid cell death

Effect of focal ischaemia on brain metabolism

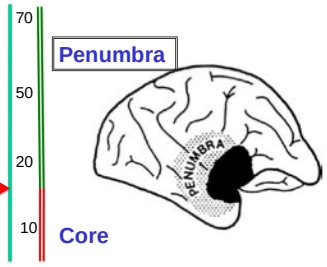


Hossmann 2006

Thresholds for metabolic disturbances

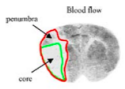
Protein synthesis decreased
HSP 70 up-regulation
Glucose utilisation decreased
Lactic acidosis

ATP depletion

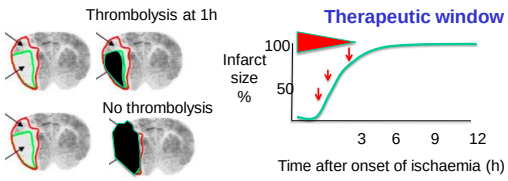


Cerebral blood flow (%)

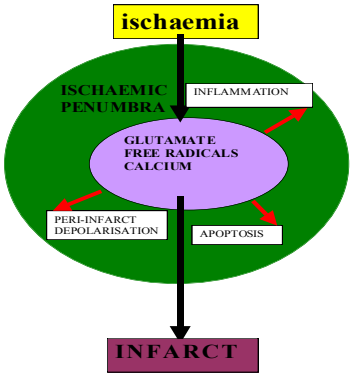
Hossmann 2006



The therapeutic window is limited to ~ 3hours



What causes cell death in the penumbra?
What are the sites for intervention and what is the effective timeframe



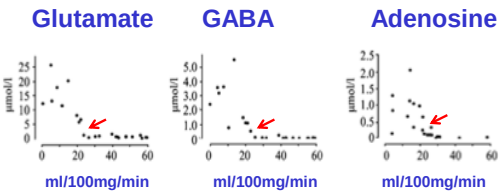
Stroke: time-dependent stages

- **ENERGY FAILURE** (minutes)
- **EXCITOTOXICITY** (minutes)
- **INDUCTION OF IMMEDIATE EARLY GENES** (hours)
- **INFLAMMATION** (hours/ days)
- **PROGRAMMED CELL DEATH / APOPTOSIS** (days)

I. ENERGY FAILURE

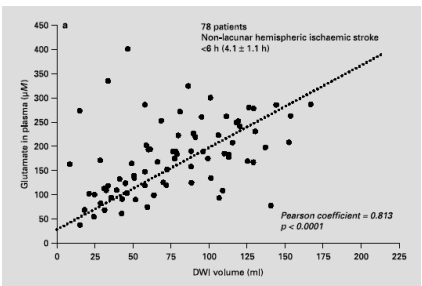
- Reduced blood flow
- ATP reduced (20% of total O_2 consumption used by the brain which is ~ 2% body weight)
- Ion gradients, Na^+ pump fails and hence membrane potential NOT maintained
- Extracellular glutamate (GLU) elevated
- Energy dependent GLU transporters inactivated
- Acidosis
- Na^+ and Cl^- entry accompanied by H_2O (passive) leads to oedema.

Effect of focal ischaemia on:
extracellular neurotransmitters

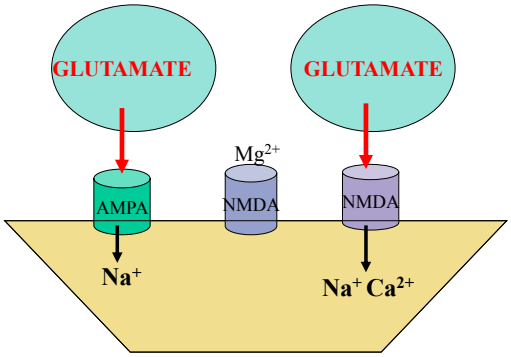


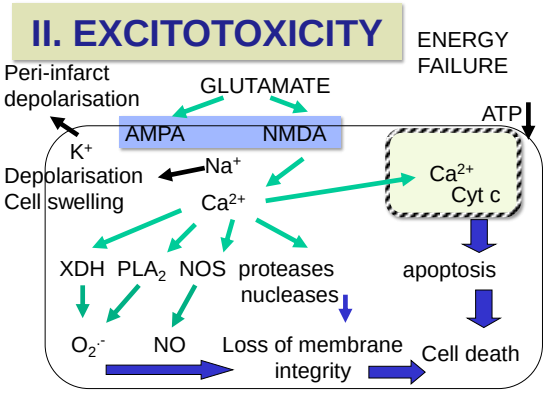
Hossmann 2006

Early changes in GLUTAMATE can be detected in plasma (< 6h)



Castillo and Rodriguez 2004 Cerebrovasc Dis. 17(suppl 1):7–18





Calcium overload

- Caused by **NMDA** receptor activated **calcium** entry and depolarisation
- Leads to activation of :
- Proteolytic enzymes (actin degradation)
- Phospholipase A2 and Cyclooxygenase (**free radical generation**).
- Nitric acid synthase (NO generation)
- Calcium causes mitochondrial swelling, reduced oxidative phosphorylation (loss of mitochondrial transmembrane potential-proton motive force), cytochrome c loss (mitochondrial transition pore) leading to APOPTOSIS

Nitric oxide synthase

nNOS	retrograde messenger	Toxic levels of NO free radicals -neuronal lesion
eNOS	vasodilator (relaxes sm. muscle)	Improves cerebral blood flow
iNOS	immune mediator	Toxic effects enhanced in ischaemia

Endogenous antioxidants and free radical scavengers

Important in the ischaemic period and also in the subsequent reperfusion when tissue is exposed to high levels of oxygen ("oxidative stress)

NO and $O_2^{\cdot -}$

Superoxide dismutase (SOD) Catalase
Glutathione peroxidase
Alpha-tocopherol Ascorbic acid

Exogenous SOD, or iNOS and nNOS KOs protect

NMDA receptor mediated neurotoxicity

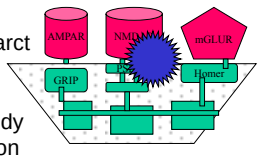
SEVERE INSULT
Ca^{2+} entry
Ca^{2+} uptake into mitochondria
Free radical generation
Severe ATP depletion
Mitochondrial swelling

NECROSIS

MILD INSULT
Transient depolarisation
ATP levels reduced
Ca^{2+} loaded mitochondria
P38 MAPkinase and c-jun N-terminal transferase
Cytochrome c release from mitochondria
APOPTOSIS

Glutamate receptors mediate tissue damage

NMDA receptors

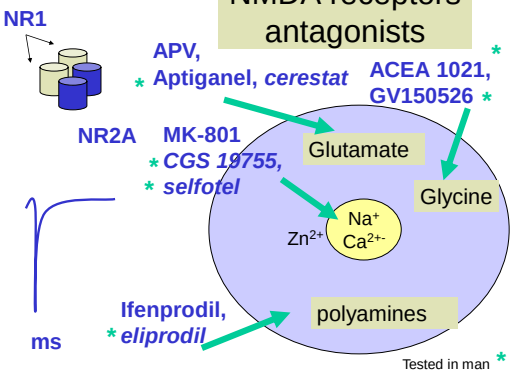


- NR2A KO decreases infarct size (focal ischaemia)
- Interruption of signalling using a 2B subunit antibody affecting PSD95 interaction reduces ischaemic damage

AMPA receptors

GluR2 antisense knockdown increases injury (global)- AMPA receptor more Ca $^{2+}$ permeable.

NMDA receptors antagonists



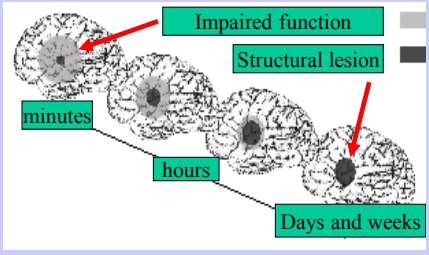
Glutamate antagonists and ion channel blockers in stroke models

- **NMDA, AMPA** antagonists - HIGHLY effective up to ~2h after insult BUT have psychotomimetic (NMDA) and respiratory depressive properties
- **Window of therapeutic opportunity difficult to translate to application in man**
- **Metabotropic** receptors: Group 1 receptors antagonists (postsynaptic and associated with NMDAR action) and Group II / III mGluR agonists (presynaptic, inhibit glutamate release) effective
- Ca^{2+} channel (L, P/Q and N), Ca^{2+}-dependent K^+, channel and proton activated Ca^{2+} permeable channel (ASIC1a) blockers reduce brain injury.

Ischaemic cascade

- A cascade of reactions is set up which are self perpetuating and no longer subject to physiological regulation ('vicious cycle') and lead to cell death initially necrotic and later apoptotic.
- In parallel, neuroprotective mechanisms are activated and the balance between the two mechanisms determines the fate of the cell.

Progression of lesion in the penumbra

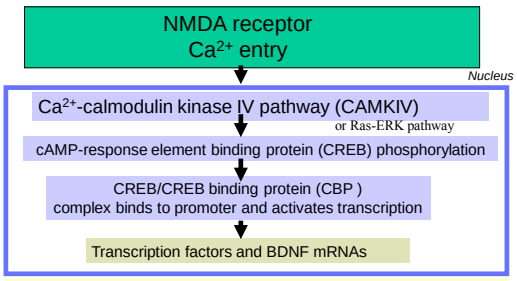


III. Glutamate activates an extensive transcriptional cascade

A co-ordinated activation of multi-potential early response genes occurs during normal neuronal activity which contributes to physiological responses and is also recruited in response to noxious insults

- **inducible transcription factors (IEGs)** which activate/repress other genes
- **enzymes such as COX-2** which underlie developmental and behavioural responses
- **neuroprotective proteins** e.g. HSPs which counter damaging effects

GLUTAMATE ACTIVATES TRANSCRIPTION

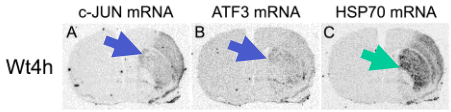


Mice lacking CREB during development show enhanced apoptosis postnatal gene deletion leads to neurodegeneration in hippocampus and cortex

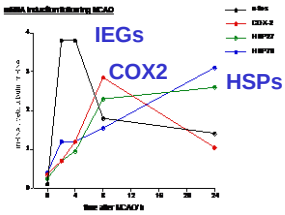
In the adult brain this pathway is involved in the injury response which can contribute to **cell survival** or **cell death** depending on the balance between transcription factors

PENUMBRA / PERI-INFARCT effects

- Elevated extracellular K^+ and glutamate depolarisation in penumbra
- IEG, COX2 and HSP induction
- Extends area of infarct
- Sensitive to glutamate antagonists



Cyclooxygenase 2 (COX-2)
Prostaglandin endoperoxidase synthase 2

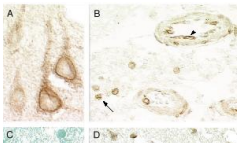


- First enzyme in the PG biosynthetic pathway
- Induced in parallel with IEGs
- COX-2 mRNA and protein are induced by physiological synaptic activity
- Localised in dendrites especially dendritic spines

Immunocytochemistry in MCA ischaemic stroke
COX2 in neurones (A) and endothelial cells (B)

COX2 in neurones

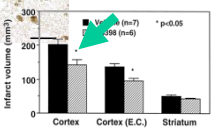
COX2 in endothelial cells



iNOS in bvs

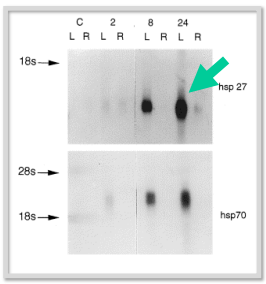
iNOS in neutrophils

COX2 selective inhibitor-NS398 reduces infarct volume (Nogawa et al 1997)



HEAT SHOCK PROTEINS

•Act as protein chaperones facilitating the transfer of proteins between subcellular compartments and
•Following a noxious stimulus (heat, ischaemia) HSPs are induced which target abnormal proteins for degradation
•HSPs are also anti-apoptotic and antioxidant (HSP27).

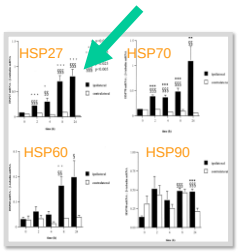


Unilateral MCAO (L) induces HSP protein

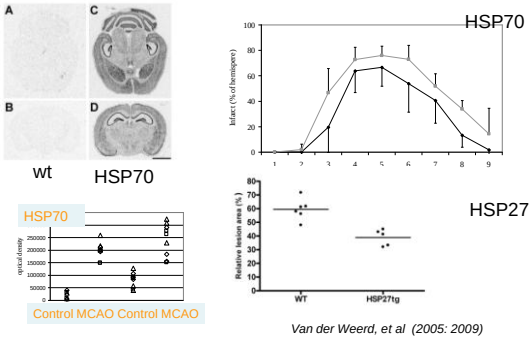
Wagstaff, Collaço-Moraes, Aspey, Coffin, Harrison, Latchman and de Belleroche (1996)

Induction of HSPs in MCAO

Unilateral MCAO (L) induces HSP mRNA



Infarct size in MCAO is reduced in HSP70 and HSP27 transgenic mice.

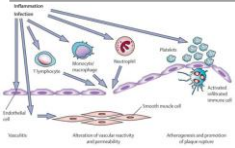


Van der Weerd, et al (2005: 2009)

Heat shock proteins play a role in ischaemic preconditioning (IPC)

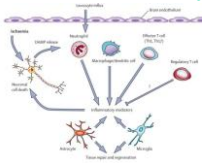
- IPC is a process in which brief exposure to ischaemia provides robust protection/tolerance to subsequent prolonged ischaemia (~TIA)
- HSP involvement in IPC has been demonstrated in cardiac and cerebral ischaemia (Sun et al 2010)
- NF-kB is necessary for late phase IPC (Tranter et al 2010), the major NF-kB-dependent genes being heat shock response genes, including the genes encoding Hsp70.1/70.3.
- Hsp70.3 is protective after IPC.

IV. INFLAMMATION



Macrez et al 2011

Neutrophils enter the brain parenchyma (30 min) accompanied by monocytes and later, macrophages (5-7 days) (iNOS elevated). Enabled by the disruption in the Blood brain barrier



- Production of mediators of inflammation: tumour necrosis factor alpha, platelet activating factor (PAF), interleukin1beta and adhesion molecules on endothelial cell surface (ICAM-1, p and E-selectins).

Cellular inflammatory response

- Neutrophils accumulate within 30 minutes on vascular endothelial cells
- Cell adhesion molecules (Selectins, Integrins, Immunoglobulins) promote adherence leading to infiltration of cells into the brain parenchyma.
- Neutrophils cause tissue damage by releasing O_2 free radicals & proteolytic enzymes
- Other cells entering the tissue e.g. lymphocytes promote tissue damage (24h)

Cytokines and chemokines

- Produced by a range of activated cell types (endothelial cells, microglia, neurones, astrocytes, platelets, leukocytes, fibroblast) within the first few hours after ischaemia.
- IL-1 and TNFα upregulate adhesion molecules promoting neutrophil migration
- CSF levels of IL-1, IL-6 and TNFα at 24h correlate with infarct size
- Chemokines (e.g. CINC and MCP-1) detected in the brain between 6 and 24h attract neutrophils & infiltration.

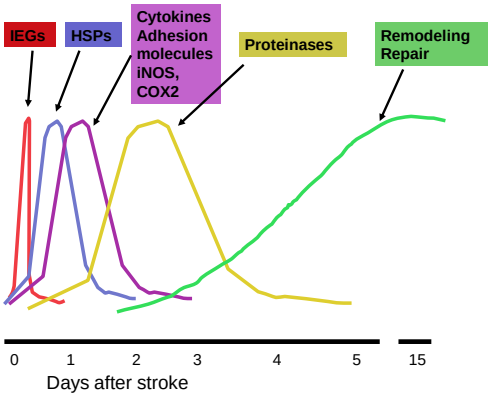
Cytokine-induced neutrophil chemoattractant 1, Monocyte chemotactic protein-1,

Anti-inflammatory agents are neuroprotective

- IL-1β receptor antagonists are protective
- TNFα neutralising antibodies and antisense nucleotides are protective

Some cytokines are neuroprotective

- TGF β and IL-10 produced by lymphocytes limit leukocyte invasion and reduce immune responses
- Complex protective/harmful effects are seen due to multiple sites of action.



Cell death

SEVERE ISCHAEMIA	MILD ISCHAEMIA
Necrotic following proteolysis loss of membrane integrity **Core**	Apoptosis Caspase dependent/ caspase-independent ATP required Delayed cell death **penumbria**

V. APOPTOSIS

- Triggered by free radicals, death receptor, DNA damage, protease action, ion imbalance.
- Release of cytochrome c from mitochobndria activates the formation of an apoptosome complex (APAF1 + procaspase 9) and caspase 3 activation (detected at ~8h) leading to DNA fragmentation
- Caspase 3 selective inhibitors (zDEVD.FMK) are effective up to 9h after reversible ischaemia.
- Broad specificity caspase inhibitors (zVAD)/ caspase 1 deletion protects against ischaemia.
- Delayed cell death occurs in man (MRI)
- Most relevant to damage in the penumbra (e.g. delayed cell death)

Bcl-2 family of proteins: PROMOTE (Bax, Bak, Bad, Bim, Bid) or PREVENT (Bcl-2, Bcl-XL) mitochondrial pore formation involved in cytochrome c release

- Basal Bcl-2 is high in ischaemia resistant pyramidal cells of CA3 and brainstem cells controlling autonomic function but low in ischaemia-sensitive cortical and hippocampal CA1 cells.
- Viral mediated gene transfer of Bcl-2 and Bcl-XL are neuroprotective

VI. LATE STAGE REPAIR

- Growth factors are secreted by neurones, astrocytes, microglia, macrophages,vascular and peripheral cells e.g. IGF1, erythropoietin
- Glutamate-mediated synaptic activity increases BDNF transcription and secretion
- Neuronal sprouting occurs in an attempt to form contacts

Neuroprotective drugs in stroke

What is the verdict on current drug trials and their design?

Where should the focus lie in future trials and what is in the pipeline?

Bench to bedside: nothing since tPA in 1996
(<5% patients treated, <4.5h, safety concerns)

Prevention: lifestyle/ diet

↓

Ischaemic effects

↓

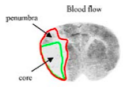
Functional recovery: specialised intensive care units

Neuroprotective drugs in stroke: what is the progress?

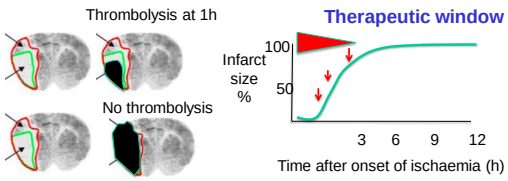
Rescue of the penumbra
50% of maximum infarct size

What are the ESSENTIAL criteria for Drug trial design?

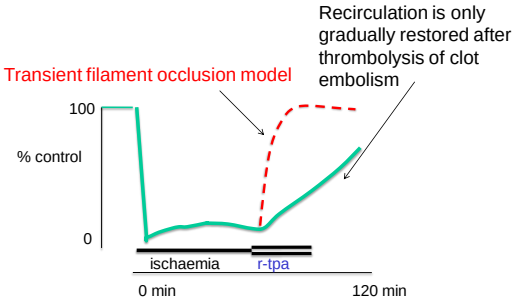
- Critical time window
- Using the right model
- Replicate effects of post ischaemic reperfusion on blood pressure
- Effect of age and co-morbidity
- Stratify for severity



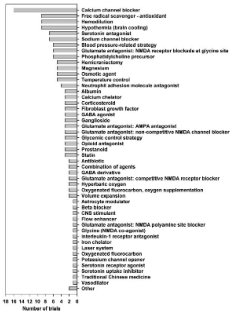
The therapeutic window is limited to ~ 3hours



Blood flow in transient vascular occlusion with reperfusion



Clinical trials on Neuroprotectants (Ginsberg 2008)



A large number of clinical trials have been carried out but most do **not** satisfy the basic requirements:

- A robust rationale
- Initiated within 6h
- Adequate dose levels
- Statistical power

Neuroprotection trials in stroke: lack of efficacy to date. What are the problems?

- Brief therapeutic window when ischaemic penumbral neurones remain viable **NOT USED**
- Studies are **underpowered** to detect small effects 40/160 trials with >200 subjects
- Stratification by severity: No allowance made for different effectiveness mild to severe strokes.
- Disability scores difficult to quantify and non-linear
- Combination treatments with multiple targets**?**
- **Primate models needed** (BBB)

"Rigorously conducted experimental studies in animal models of cerebral ischaemia provide incontrovertible proof of principal that high grade protection is achievable" Ginsberg 2008

Few trials have used the 4-6h therapeutic window within which efficacious neuroprotection is considered achievable, which may account for disappointing results

Focussing on timing

Combination treatments: thrombolytics ± free radical scavengers ± anti-inflammatory ± anti-apoptotic drugs – 3h limit applies

- **FIELD ADMINISTRATION:** Magnesium sulphate (FAST-MAG in progress) NMDA blockade, calcium channel antagonism and maintenance of CBF. No significant effect in 12h trials but earlier use in trial
- **HYPOTHERMIA** (reduced O_2 demand & inflamm^n)
- **ISCHAEMIC TOLERANCE/PRECONDITIONING-** also involved in cardiac protection, hypoxia, seizures

Mild (34-36ºC) **to Moderate Hypothermia** (32-34ºC)

- Therapeutic therapy for cardiac arrest (comatose patients) and in neonates with acute perinatal asphyxia: trials indicate better neurological outcome and survival.
- Acute ischaemic stroke??
  - Intensive care units
  - Pneumonia risk
- Thrombolysis (3-6h) plus endovascular hypothermia 33ºC reached after 60 min. Effects at 3 months were NS but demonstrates feasibility (Hemmen et al 2010).

external cooling with cooling blankets, cold infusions. Cooling via ice-packs and rapid intravenous administration of cold crystaloids.

Preconditioning: limits damage

Early effects: occur within minutes, last for a few hours but do not require protein synthesis

GPCRs (e.g. Adenosine): εPLC activation elevates IP_3 and Ca^{2+} mobilisation, PKC activation, enhanced ATP currents and inhibits opening of mPTP and apoptosis

Late effects: occur after 12-72 h, last days /weeks, involve transcriptional activation (e.g. HIF, CREB, HSP) and epigenetics (e.g. Sirtuin1 histone deacetylases)

Applicable at the time of the ischaemic insult (peri-conditioning) or after (post-conditioning) Remote preconditioning by limb ischaemia (cardiac ischaemia)

mPTP (mitochondrial permeability transition pore)

References

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- Stroke and the immune system: from pathophysiology to new therapeutic strategies. Macrez et al (2011) The Lancet Neurology 10, 2011 471 - 480

Learning Objectives

- Describe the main events that occur in cerebral ischaemia including energy failure, excitotoxicity, inflammation and cell death and relevant localisation and timescale of these events.
- Understand the transcriptional changes that contribute to endogenous neuroprotective processes and delayed cell death
- Identify the main molecular targets that could improve outcome but also appreciate the problems encountered in developing treatments.