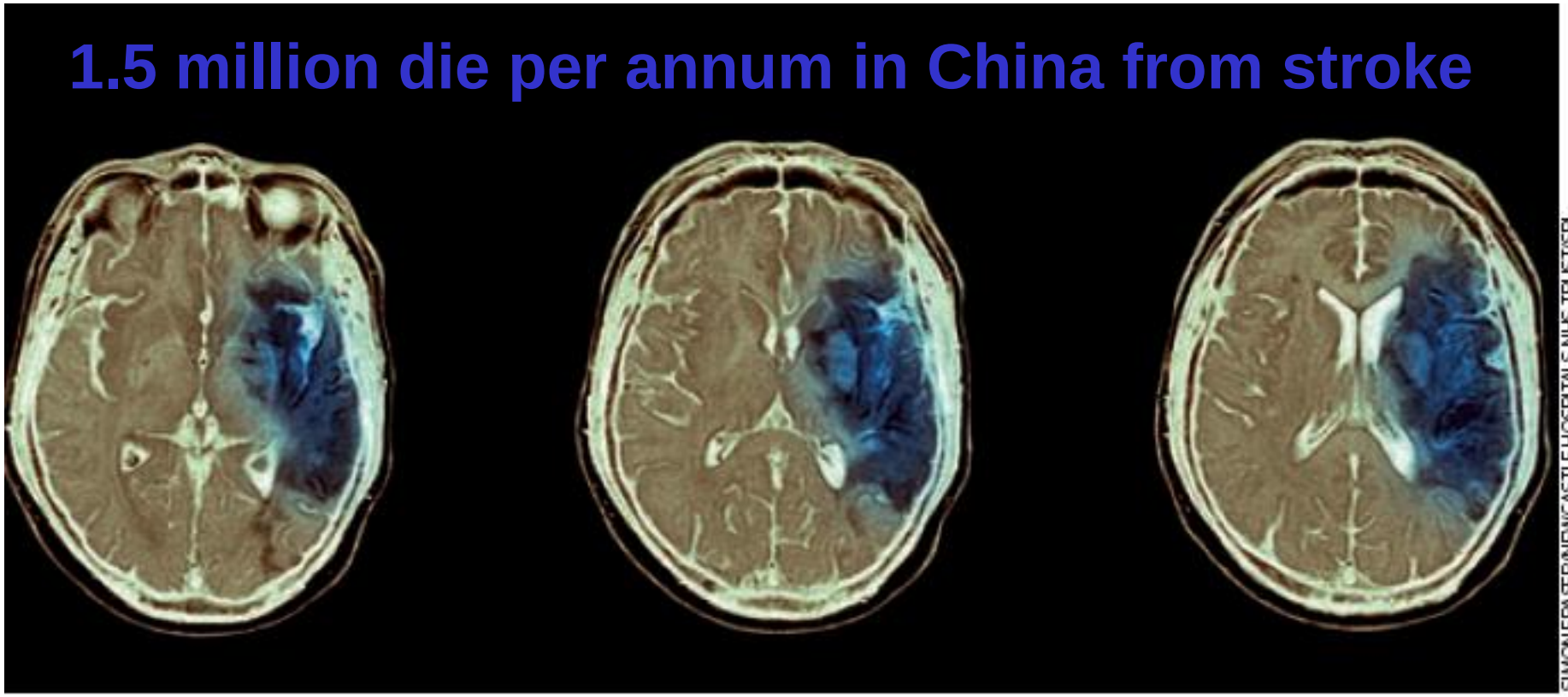
Stroke is the third most common cause of death in England and Wales and affects 150,000 people each year in the UK.



A major cause of disability

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

Improving the outcome of stroke

Markus, H. 2007 BMJ **335,** 359-360.

- Rapid intervention in STROKE improves outcome
- Specialist Units (shortage in the UK) provide thrombolysis necessary within 3h improve outcome (20-30% of eligible patients in US, Australia and Europe but <1% in UK)
- 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 (anti-platelet, cholesterol lowering statins (athersclerosis)), ACE inhibitors and anti-hypertensives

How can infarct size be reduced???

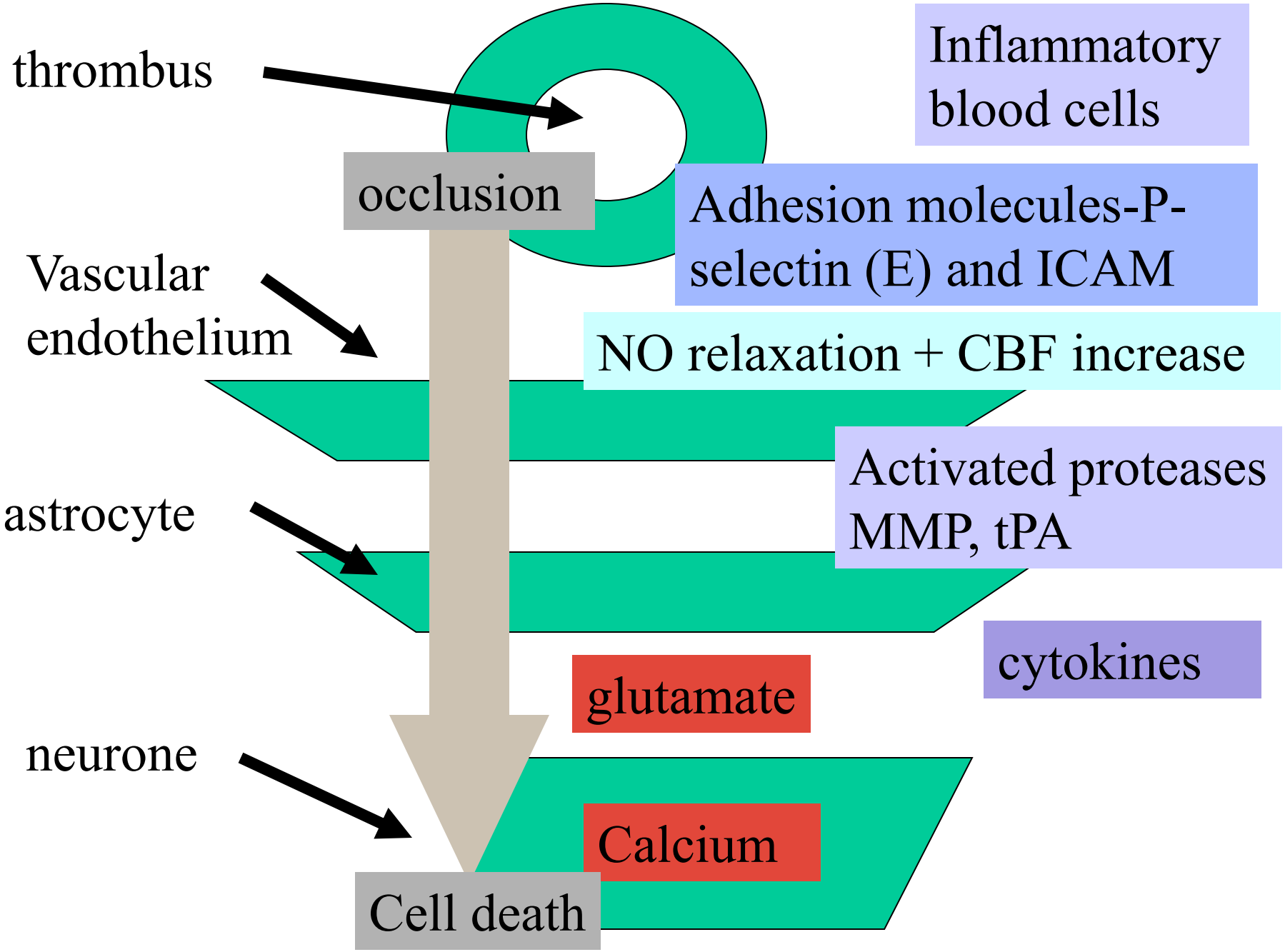
Cerebral ischaemia: a transient or permanent reduction in CBF

How can cell death be limited?

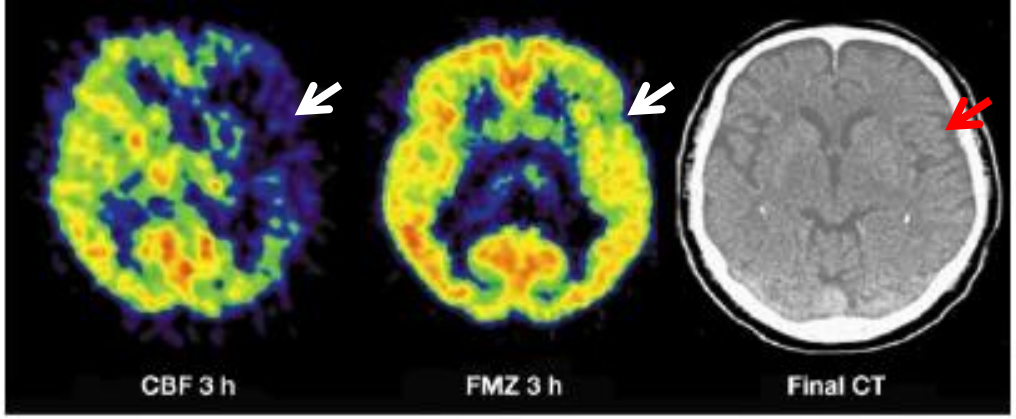
- **Stroke pathophysiology:** the role of glutamate and the ischaemic cascade
- **Balance between cell death and neuroprotection**.
- **Future directions for therapeutic intervention.**

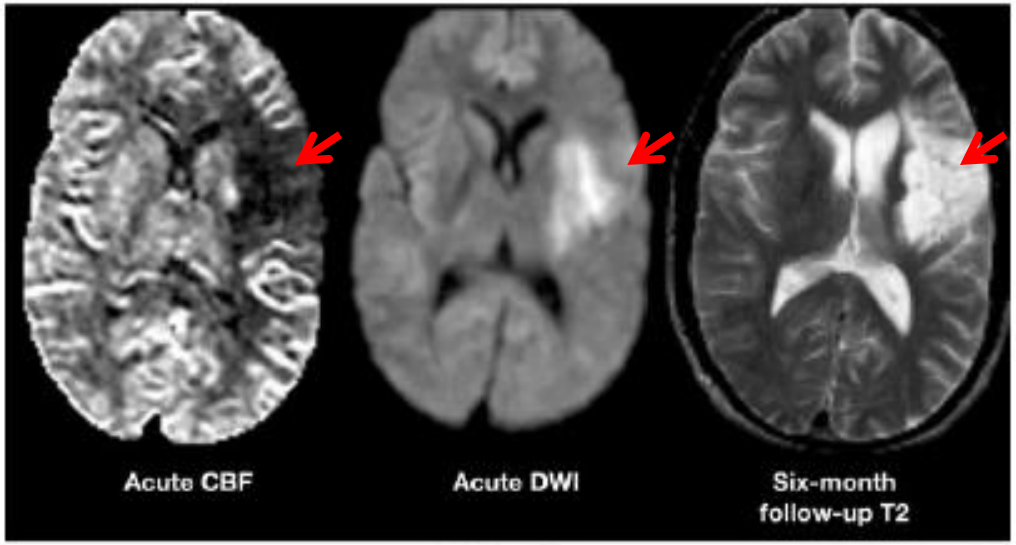
Common mechanisms mediate acute CNS injury (stroke, trauma and seizure)

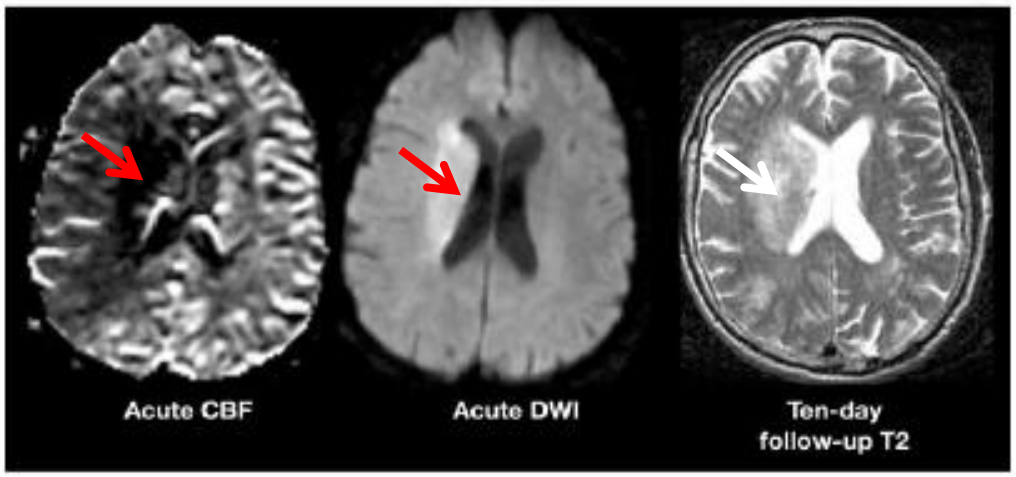
Stroke pathophysiology involves multiple tissue compartments


thrombus
Inflammatory blood cells
occlusion
Adhesion molecules-P-selectin (E) and ICAM
Vascular endothelium
NO relaxation + CBF increase
Activated proteases MMP, tPA
astrocyte
cytokines
glutamate
neurone
Calcium
Cell death

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 oligohaemia

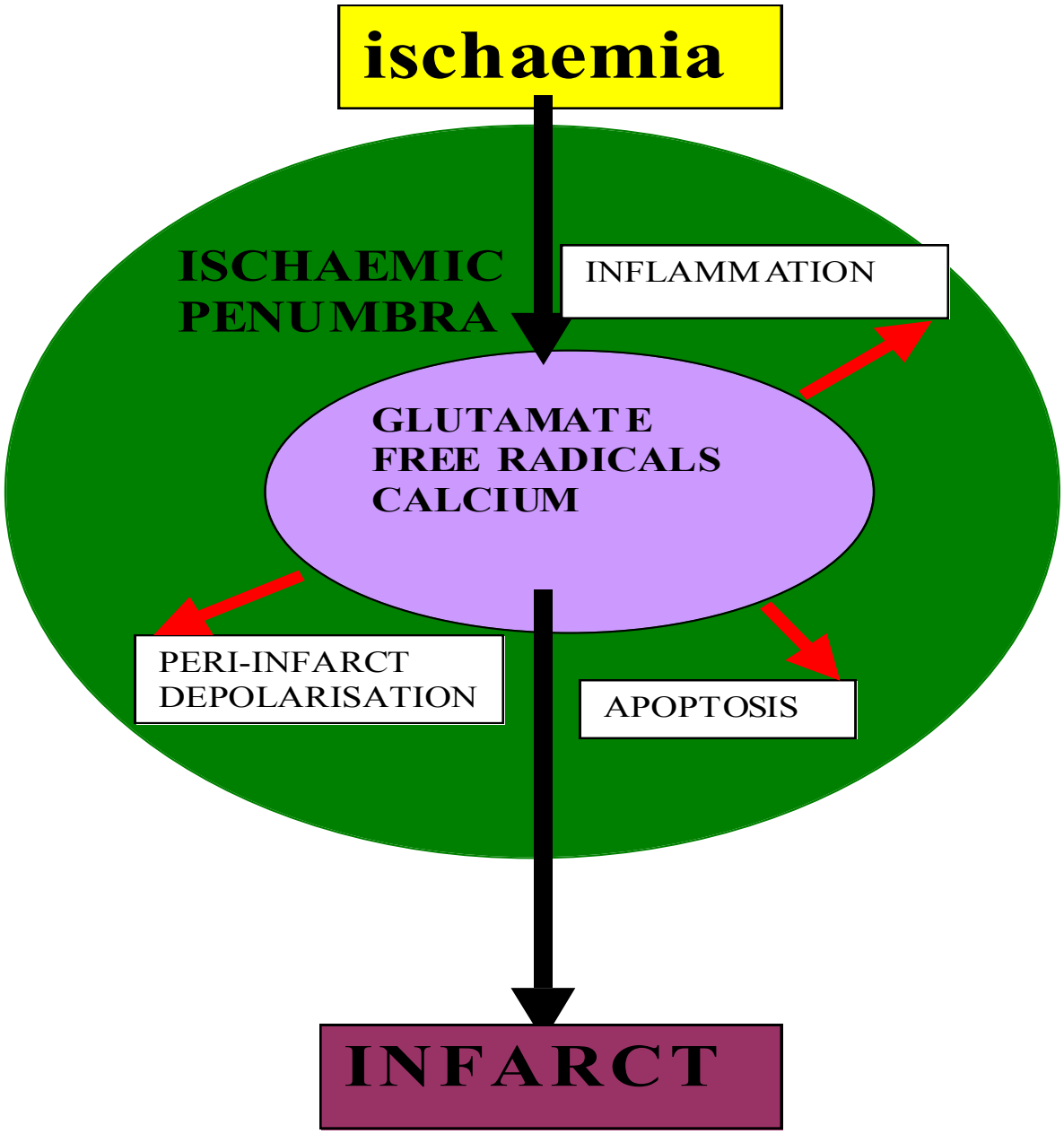
Hypoperfusion but likely to survive depending on factors such as collateral flow

< 22 ml/100g/min ischaemic penumbria

Misery perfusion likely to progress to infarction

< 10 ml/100g/min rapid cell death

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


ischaemia
ISCHAEMIC PENUMBRA
INFLAMMATION
GLUTAMATE
FREE RADICALS
CALCIUM
PERI-INFARCT DEPOLARISATION
APOPTOSIS
INFARCT

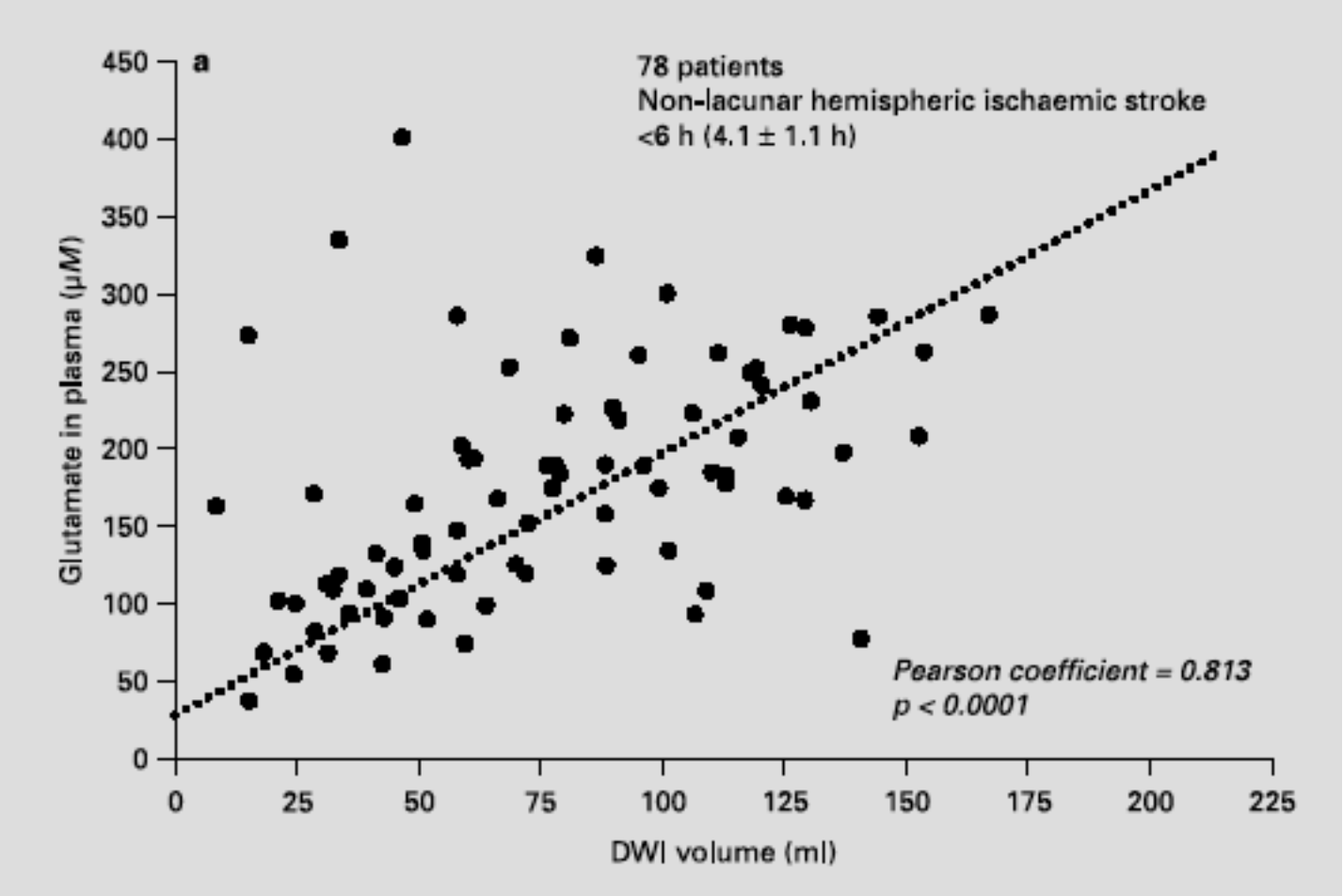
Stroke: time-dependent stages

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

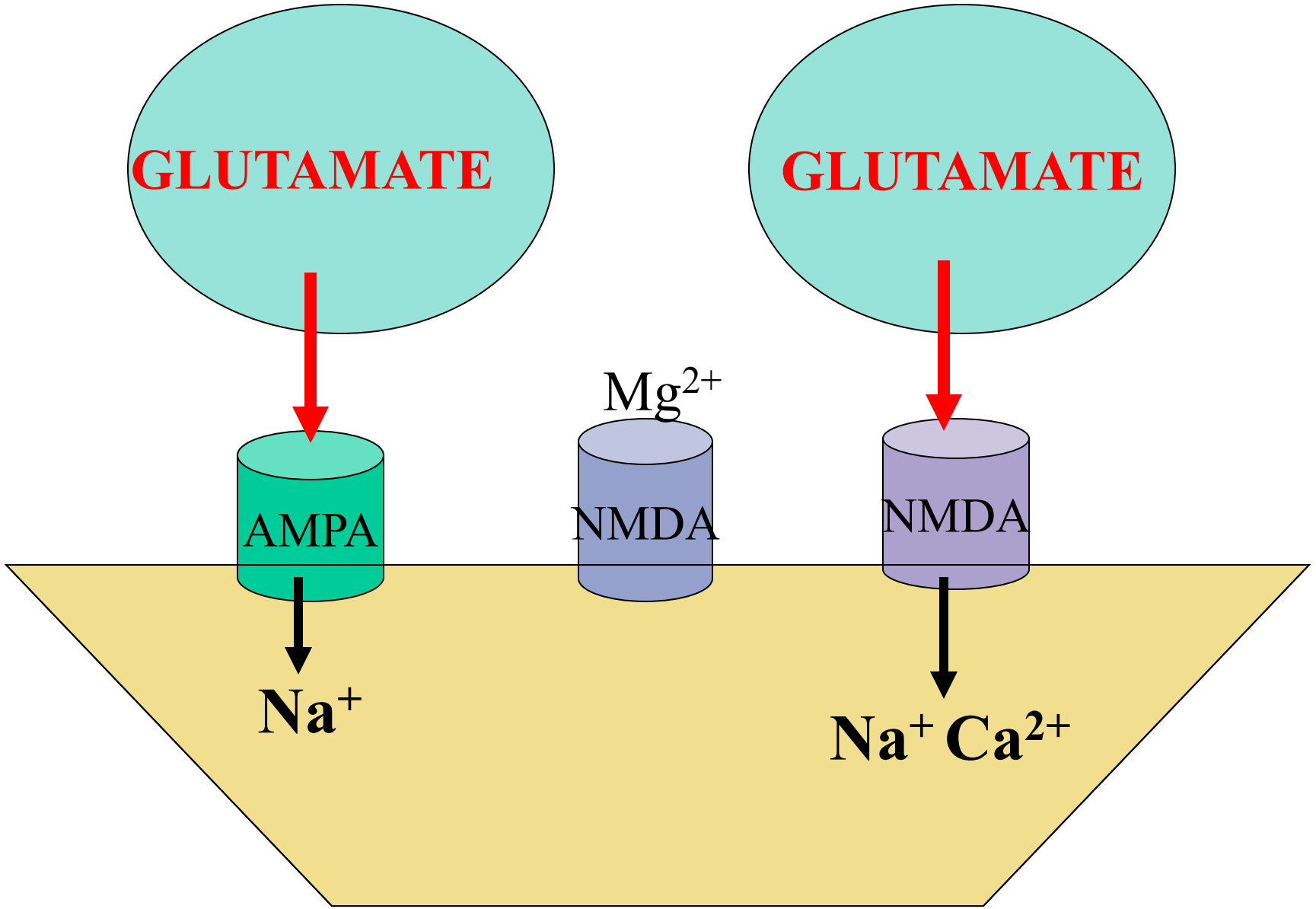
I. ENERGY FAILURE

- 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 elevated
- Energy dependent transporters inactivated
- Acidosis
- Na^+ and Cl^- entry accompanied by H_2O (passive) leads to oedema.

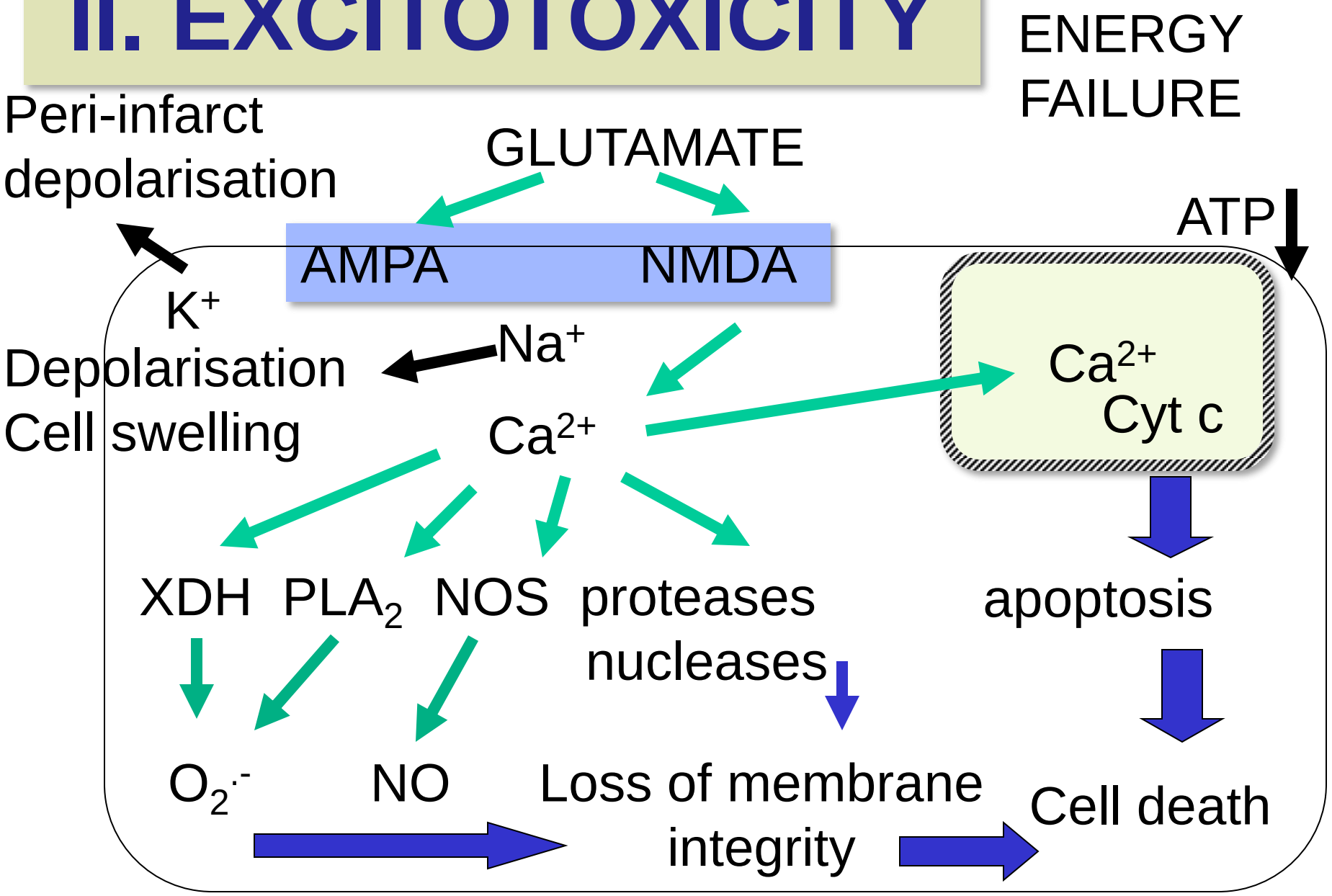
Early changes in GLUTAMATE can be detected in plasma (< 6h)



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


GLUTAMATE
GLUTAMATE
Mg2+
AMPA
NMDA
NMDA
Na+
Na+ Ca2+

II. EXCITOTOXICITY



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

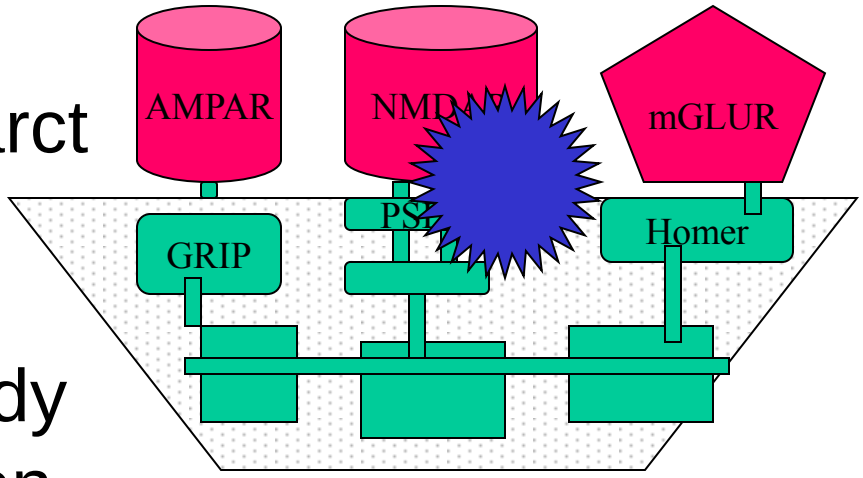
SEVERE INSULT
Ca^{2+} entry
Ca^{2+} uptake into mitochondria
Free radical generation
Severe ATP depletion
Mitochondrial swelling

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

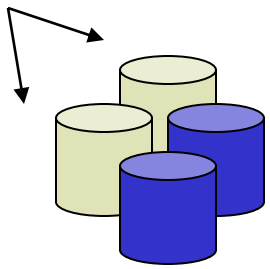


- NR2A KO decreases infarct size (focal ischaemia)
- Interruption of signalling using a 2B subunit antibody affecting PSD95 interaction reduces ischaemic damage

AMPA receptors

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

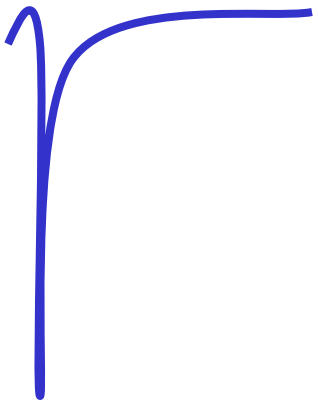
NMDA receptors antagonists



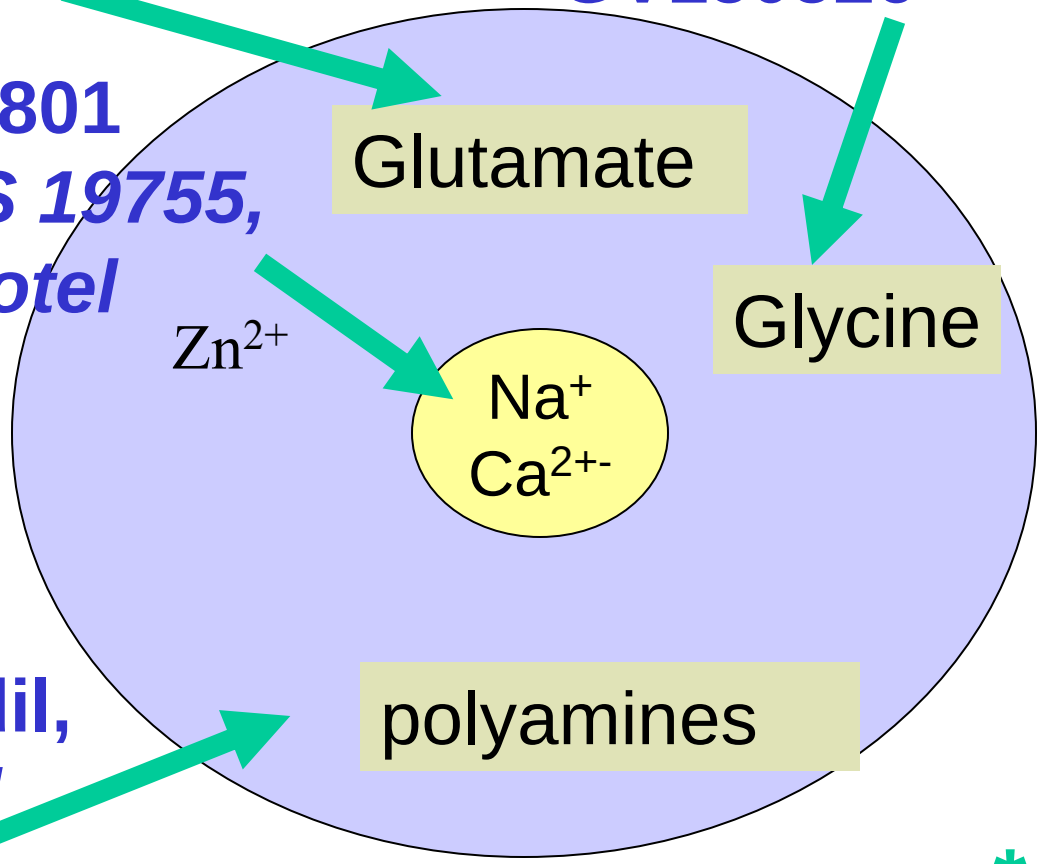
APV,
* Aptiganel, *cerestat*

ACEA 1021,
GV150526 *

NR2A MK-801
* *CGS 19755,*
* *selfotel*



Ifenprodil,
* *eliprodil*



Tested in man *

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) have efficacy.
- 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.

III. Glutamate activates an extensive transcriptional cascade

A co-ordinated activation of multipotential 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

NMDA receptor
Ca^{2+} entry

Nucleus

Ca^{2+}-calmodulin kinase pathway (CAMKIV)

or Ras-ERK pathway

cAMP-response element binding protein CREB)
phosphorylation at serine133

CREB/CREB binding protein (CBP)
complex binds to promo

Transcription activation

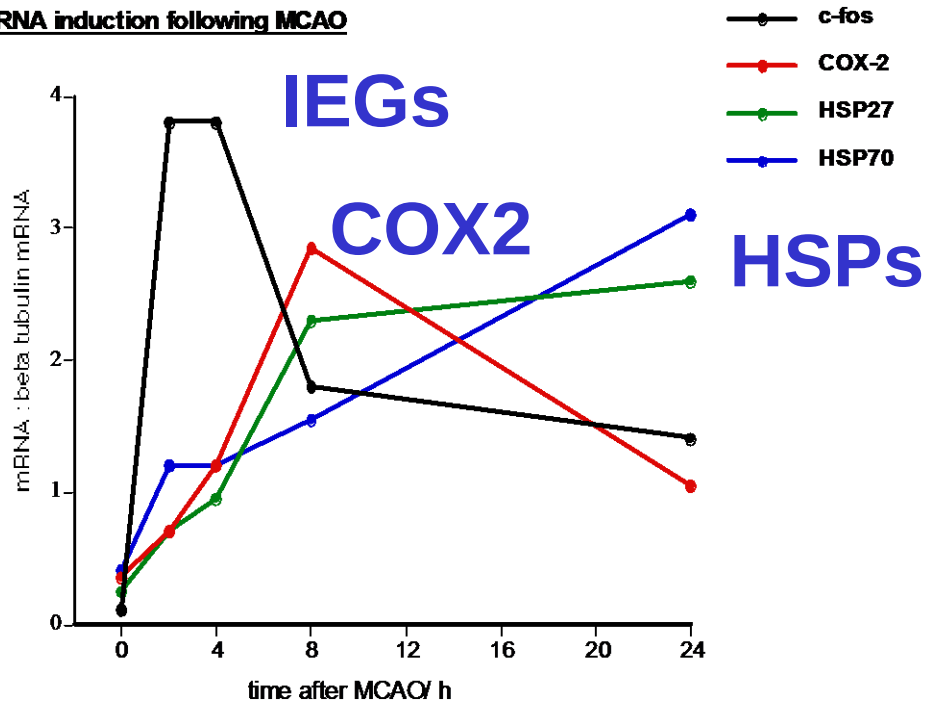
Transcription factors and BDNF m

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

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

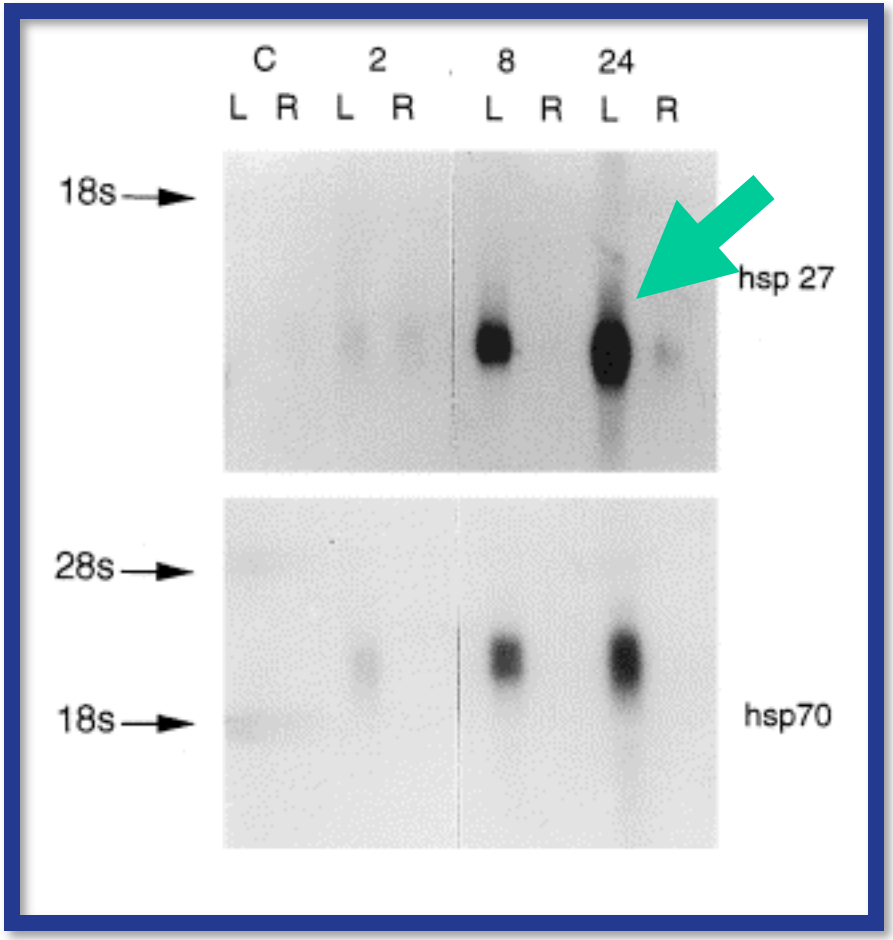


- First enzyme in the PG biosynthetic pathway
- Induced in parallel with IEGs
- COX-2 mRNA and protein are induced by physiological synaptic activity
- Localised in dendrites especially dendritic spines

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).

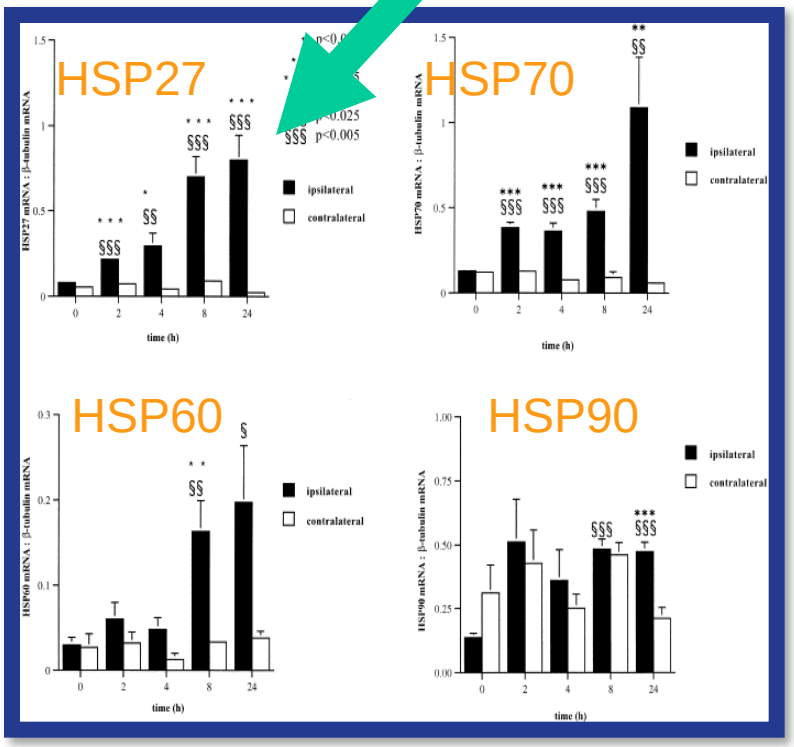
Induction of HSPs in MCAO



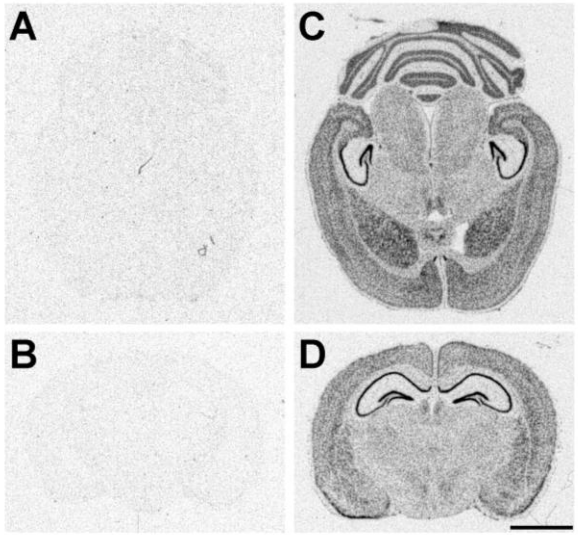
Unilateral MCAO (L) induces HSP protein

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

Unilateral MCAO (L) induces HSP mRNA

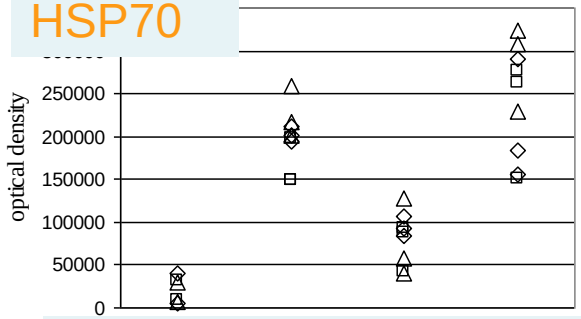


Infarct size in MCAO is reduced in HSP70 and HSP27 transgenic mice.



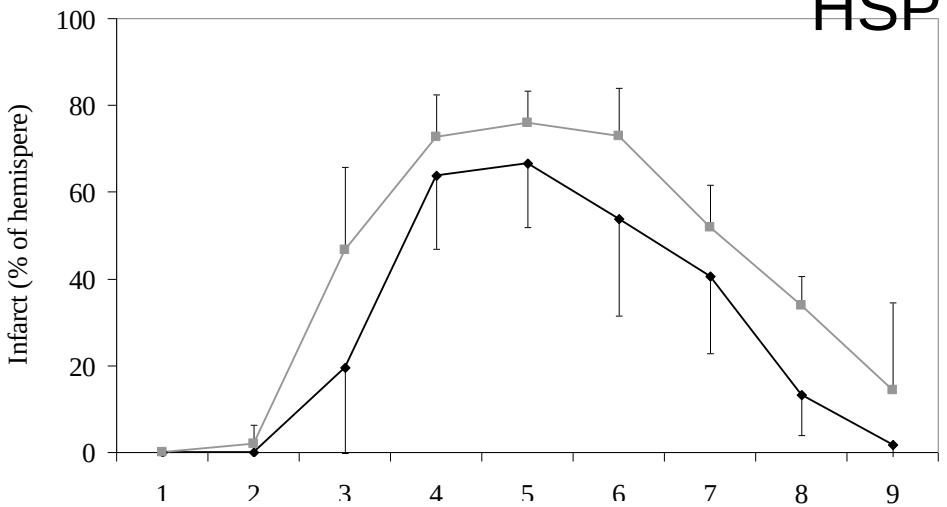
wt HSP70

HSP70

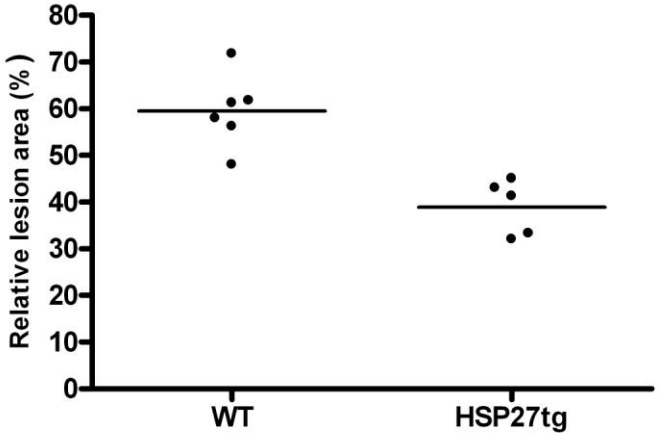


Control MCAO Control MCAO

HSP70



HSP27



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

- Neutrophils enter the brain parenchyma (30 min) accompanied by monocytes and later, macrophage (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 and proteolytic enzymes
- Other cells entering the tissue e.g. lymphocytes promote tissue damage (24h)

Cytokines and chemokines

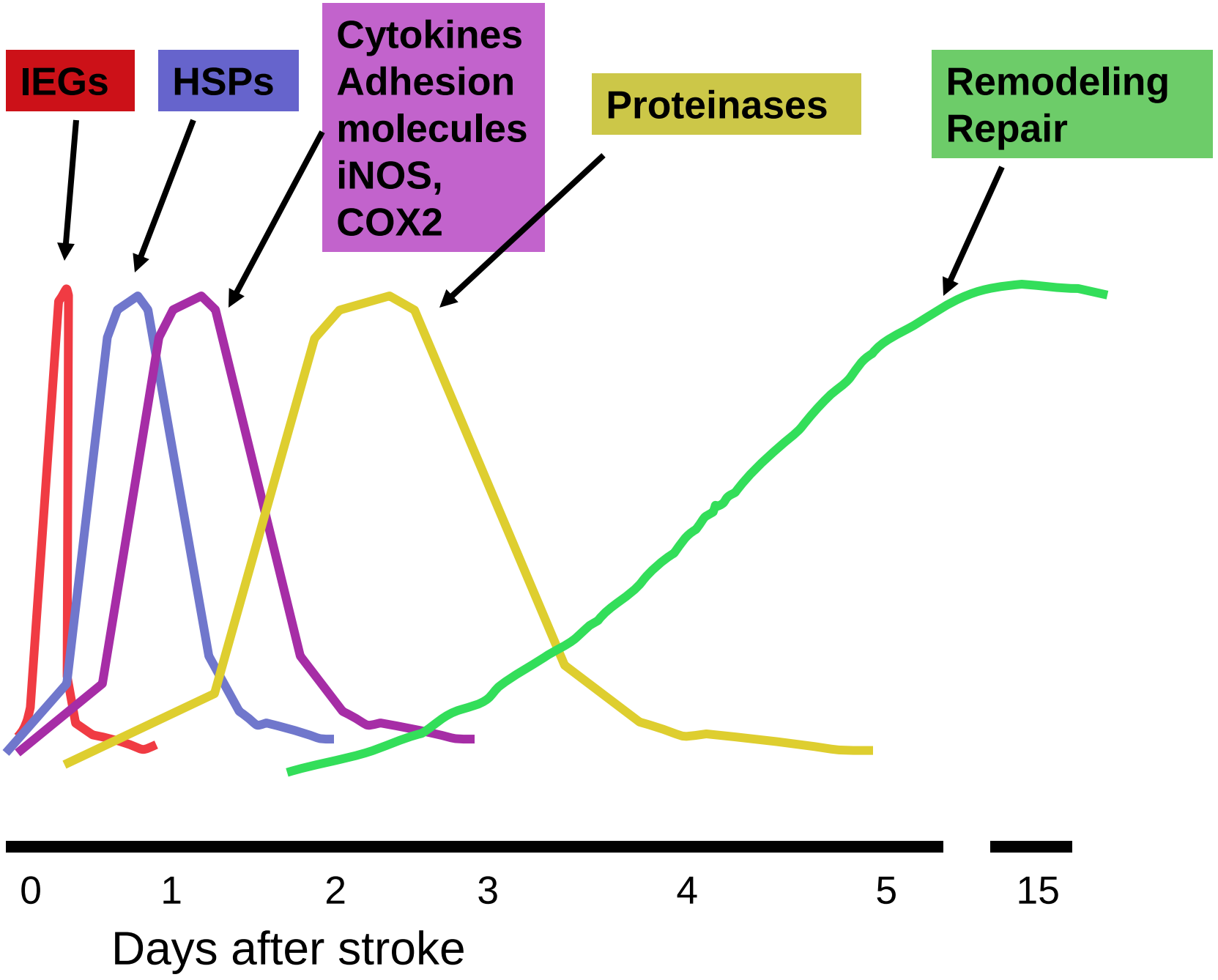
- Produced by a range of activated cell types (endothial cells, microglia, neurones, astrocytes, platelets, leukocytes, fibroblast) within the first few hours after ischaemia.
- IL-1 and TNFα upregulate adhesion molecules promoting neutrophil migration
- CSF levels of IL-1, IL-6 and TNFα at 24h correlate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

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.


IEGs
HSPs
Cytokines
Adhesion
molecules
iNOS,
COX2
Proteinases
Remodeling
Repair
0
1
2
3
4
5
15
Days after stroke

Cell death

SEVERE

Necrotic following proteolysis loss of membrane integrity

MILD ISCHAEMIA
Apoptosis
Caspase dependent and caspase-independent
ATP required
Delayed cell death
penumbria

What controls the balance between physiological and pathological signalling?

Synaptic NMDA receptors
CREB phosphorylation (Ser 133)
CREB-dependent transcription
Neuronal survival

Extrasynaptic NMDARs

Transient CREB phosphorylation but not gene expression

MAPK signalling & transcription activated

Cell death

Hardingham et al 2002 Nature Neuroscience 5, 405.

Signalling pathway is defined by the nature of the modulatory subunit NR2A or NR2B

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

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 verdict?

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
- Inadequate dose levels
- Statistical power

“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

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

- Brief therapeutic window when ischaemic penumbral neurones remain viable
- Complex process: combination treatments with multiple targets
- 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
- Primate models needed (BBB)

New approaches

- Combination treatments: thrombolytics + free radical scavengers + anti-inflammatory + anti-apoptotic drugs
- Magnesium sulphate (field administration)

Local and remote self-protective mechanisms

- Hypothermia (reduced O_2 demand and inflamm^n)
- ISCHAEMIC TOLERANCE/PRECONDITIONING- also involved in cardiac protection, hypoxia, seizures
- Remote preconditioning by limb ischaemia (cardiac ischaemia)

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. At 3 months effects were NS but demonstrates feasibility (Hemmen et al 2010).

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 leads to IP_3 generation, Ca^{2+} mobilisation, PKC activation, enhanced mK^+ ATP currents and inhibit opening of mPTP (mitochondrial permeability transition pore) 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)

References

- Mechanisms, Challenges and opportunities in stroke. Lo et al (2003) Nature Reviews Neuroscience 4, 399-415
- Stroke research at the crossroads: asking the brain for directions. Iadecola and Anrather (2011) Nature Neuroscience 14, 1363-1368

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.