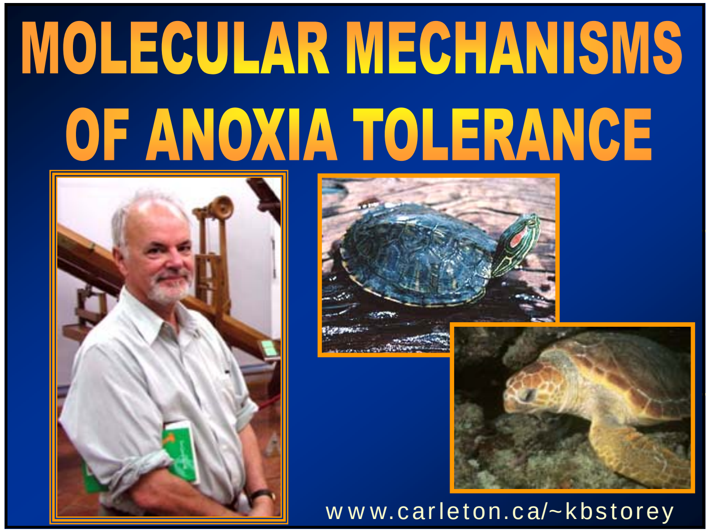
MOLECULAR MECHANISMS
OF ANOXIA TOLERANCE
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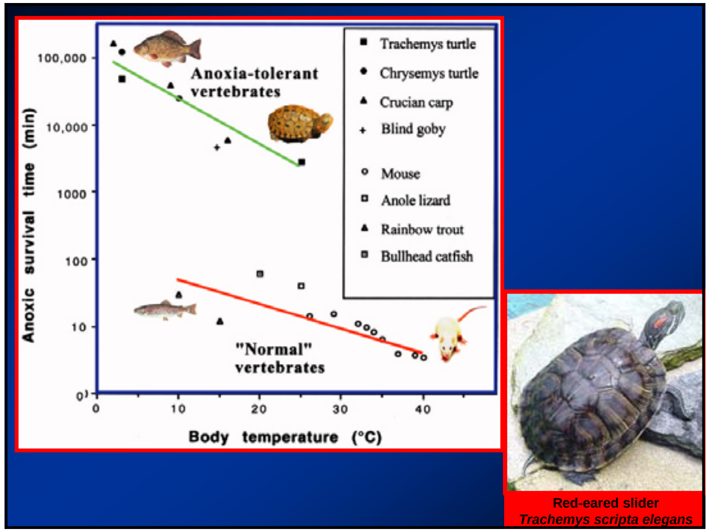
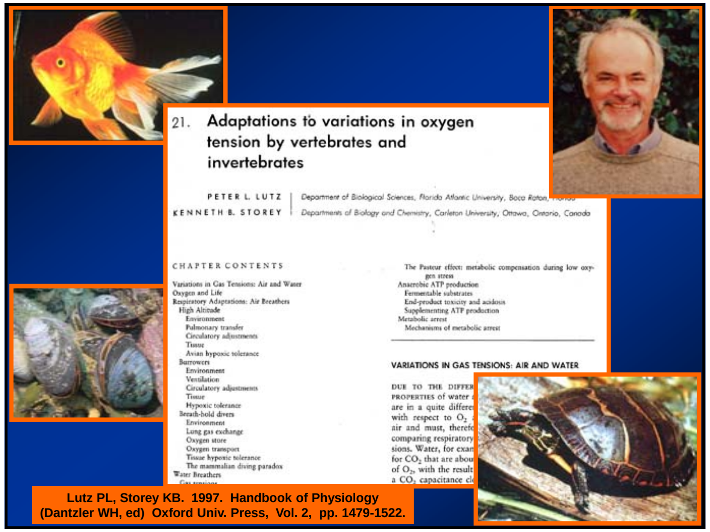
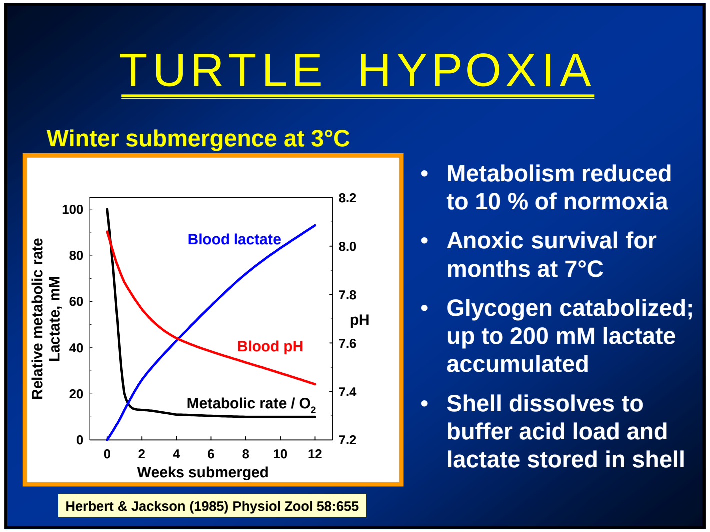
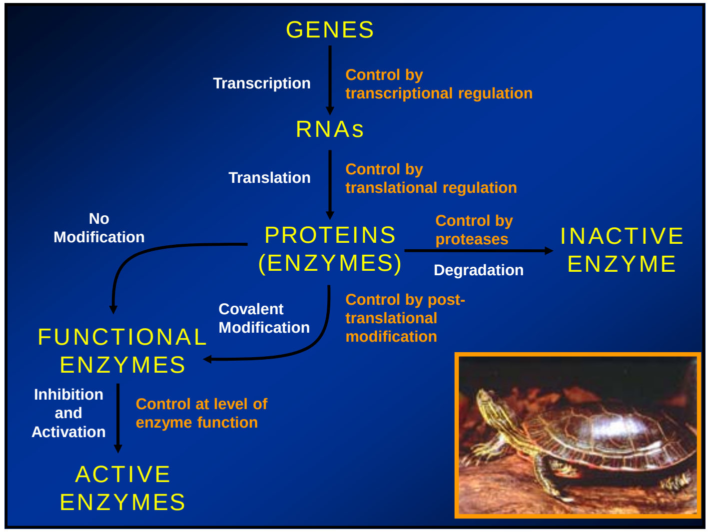
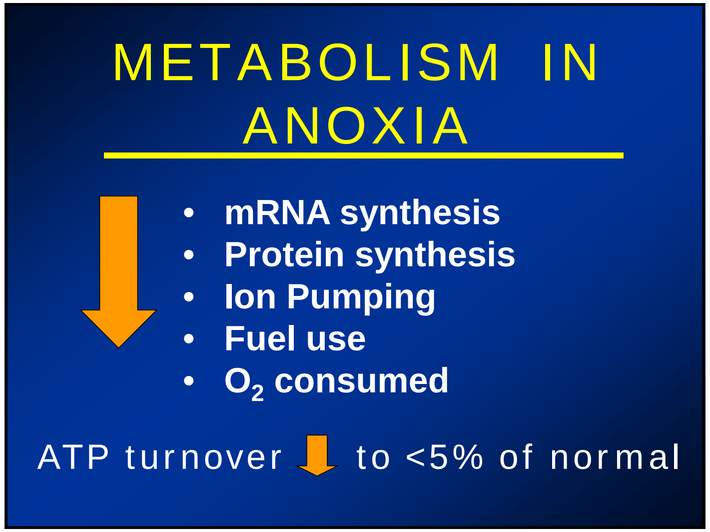
PETER
L.
LUTZ

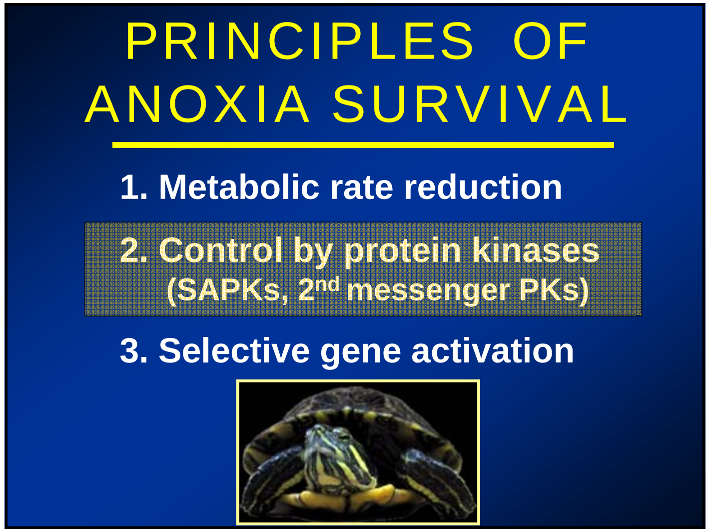

Anoxic survival time (min)
100,000
10,000
1000
100
10
0
Body temperature (°C)
0
10
20
30
40
Anoxia-tolerant vertebrates
"Normal" vertebrates
Trachemys turtle
Chrysemys turtle
Crucian carp
Blind goby
Mouse
Anole lizard
Rainbow trout
Bullhead catfish
Red-eared slider
Trachemys scripta elegans

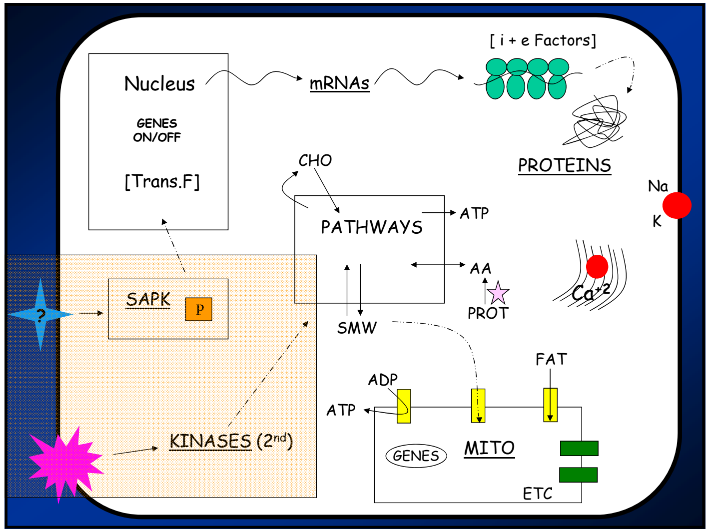

21. Adaptations to variations in oxygen tension by vertebrates and invertebrates
PETER L. LUTZ | Department of Biological Sciences, Florida Atlantic University, Boca Raton,
KENNETH B. STOREY | Departments of Biology and Chemistry, Carleton University, Ottawa, Ontario, Canada
CHAPTER CONTENTS
Variations in Gas Tensions: Air and Water
Oxygen and Life
Respiratory Adaptations: Air Breathers
High Altitude
Environment
Pulmonary transfer
Circulatory adjustments
Tissue
Avian hypoxic tolerance
Burrowers
Environment
Ventilation
Circulatory adjustments
Tissue
Hypoxic tolerance
Breath-hold divers
Environment
Lung gas exchange
Oxygen store
Oxygen transport
Tissue hypoxic tolerance
The mammalian diving paradox
Water Breathers
The Pasteur effect: metabolic compensation during low oxygen stress
Anaerobic ATP production
Fermentable substrates
End-product toxicity and acidosis
Supplementing ATP production
Metabolic arrest
Mechanisms of metabolic arrest
VARIATIONS IN GAS TENSIONS: AIR AND WATER
Lutz PL, Storey KB. 1997. Handbook of Physiology
(Dantzler WH, ed) Oxford Univ. Press, Vol. 2, pp. 1479-1522.


TURTLE HYPOXIA
Winter submergence at 3°C
Relative metabolic rate
Lactate, mM
100
80
60
40
20
0
8.2
8.0
7.8
7.6
7.4
7.2
pH
Blood lactate
Blood pH
Metabolic rate / O2
0 2 4 6 8 10 12
Weeks submerged
Herbert & Jackson (1985) Physiol Zool 58:655
• Metabolism reduced to 10 % of normoxia
• Anoxic survival for months at 7°C
• Glycogen catabolized; up to 200 mM lactate accumulated
• Shell dissolves to buffer acid load and lactate stored in shell


GENES
Transcription
Control by transcriptional regulation
RNAs
Translation
Control by translational regulation
No Modification
PROTEINS (ENZYMES)
Control by proteases
Degradation
INACTIVE ENZYME
Covalent Modification
Control by post-translational modification
FUNCTIONAL ENZYMES
Inhibition and Activation
Control at level of enzyme function
ACTIVE ENZYMES


METABOLISM IN ANOXIA
• mRNA synthesis
• Protein synthesis
• Ion Pumping
• Fuel use
• O2 consumed
ATP turnover to <5% of normal


PRINCIPLES OF ANOXIA SURVIVAL
1. Metabolic rate reduction
2. Control by protein kinases (SAPKs, 2nd messenger PKs)
3. Selective gene activation

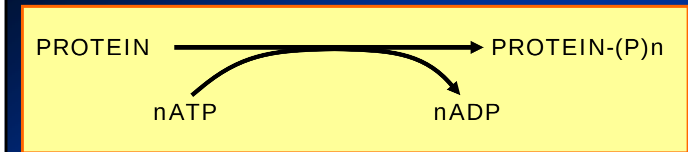


AONXIA INDUCED CHANGES

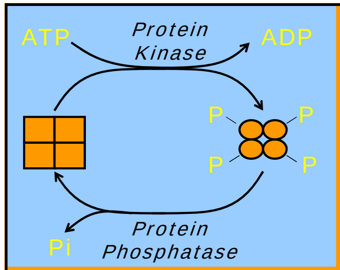
- Protein Synthesis slows to 1%
- Pumps & Channels closed
- Energy Production slows to 5%
- Energy Utilization slows to 2%
- Few 'SAP' kinases activated

- Gene 'inactivation' (↓ mRNA)
- Few Genes activated (1-2%)

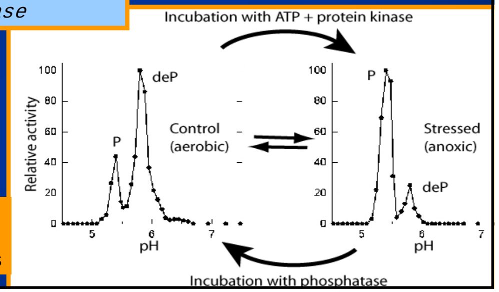
PROTEIN KINASES



- Covalent modification by phosphorylation
- Families of protein kinases: PKA (cAMP), PKG (cGMP), CaM (Ca^{2+}), PKC (Ca^{2+}, PL,DG)
- SAPKs : daisy chain phosphorylations
- Regulation is via interconversion of active vs subactive forms of protein substrates



Reversible phosphorylation control of enzymes



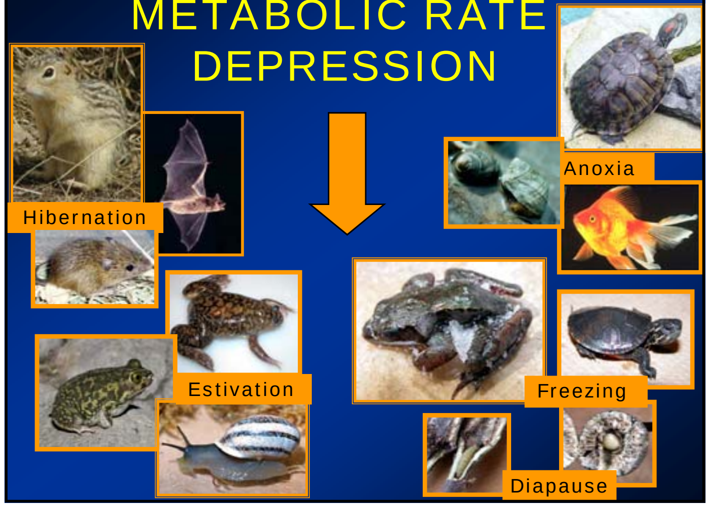
P & deP enzymes separate on ion exchange columns

PROTEIN PHOSPHORYLATION & GLYCOLYSIS

- **Protein kinase A, PKG**
- **Protein kinase C (Brain)**
- **Protein phosphatase 1, 2A, 2C**

Storey, K.B. 1996. Metabolic adaptations supporting anoxia tolerance in reptiles: recent advances. Comp. Biochem. Physiol. B 113, 23-35.

METABOLIC RATE DEPRESSION



ANOXIA INDUCED CHANGES

- **Protein Synthesis slows to 1%**
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- **Energy Production slows to 5%**
- **Energy Utilization slows to 2%**
- **Few 'SAP' kinases activated**

- **Gene 'inactivation' (↓ mRNA)**
- **Few Genes activated (1-2%)**

ROLE OF TRANSCRIPTION

- **Global rate of mRNA synthesis depressed. Method: nuclear run-on**
- **Are selected genes up-regulated ?**
- TO ASSESS GENE UPREGULATION:

What new mRNAs are created?
- **cDNA library**
- **Gene Chip**

cDNA Arrays
- Methods
- Materials
- Sources
- Publications

GENE CHANGES IN TURTLE ANOXIA

- cDNA Library & Chip
  - (~2% putative up-regulated)
  - -Transcription Factors
  - - Mitochondrial Genes
  - - Protease inhibitors
  - - Shock proteins (Hsps)
  - - Antioxidant enzymes
  - - Ferritin H & L

ANTIOXIDANT DEFENSE

- **Iron storage:**
  - **- Ferritin (H & L chains)**
  - **- Transferrin receptor 2**
- **Antioxidant enzymes**
  - **- SOD (1)**
  - **- GST (M5, A2)**
  - **- GPX (1, 4)**
  - **- Peroxiredoxin 1**

C. picta **hatchlings liver & heart**

Storey KB. 2005. Gene hunting in hypoxia and exercise.
In: R.C. Roach *et al.*, eds. Hypoxia and Exercise, Springer, NY

The Good And The Bad Of Oxygen

The Good

1) Fuels normal aerobic metabolism
2) More than 200 enzymes use O_2
3) Eliminates toxins (xenobiotics) via cytochrome P450
4) Produce O_2 via photosynthesis

The Bad

1) Reactive oxygen species (ROS) damage macromolecules, deplete GSH, vitamins
2) ROS produced by normal aerobic metabolism & must be destroyed
3) Heavy metals catalyze formation of particularly dangerous ROS
4) Associated with disease & ageing

Reactive Oxygen Species: The Bad Guys

O_2 Oxygen —(e^-)→ $O_2^{\bullet -}$ Superoxide —(e^-, $2H^+$)→ H_2O_2 Hydrogen Peroxide —(e^-, metals)→ •OH Hydroxyl Radical

Superoxide - forms when O_2 acquires a single electron
- relatively short-lived

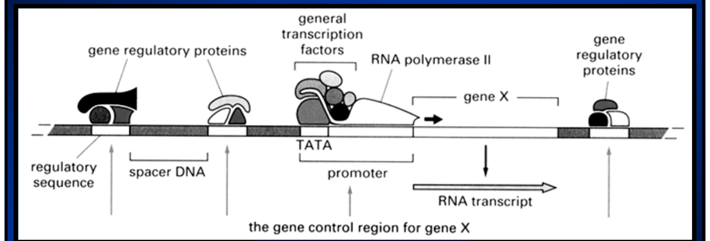
Hydrogen Peroxide - formed from superoxide
- not a radical, is long-lived
- passes readily through membranes

Hydroxyl Radical - formed from H_2O_2 (with Fe^{2+} or Cu^+)
- HIGHLY REACTIVE - very short-lived

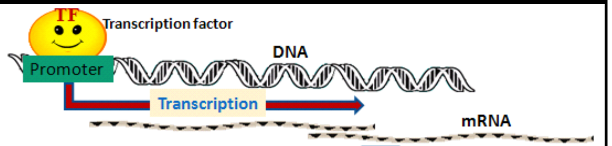
GENE CHANGES IN TURTLE ANOXIA

- cDNA Library & Chip
  (~2% putative up-regulated)
  - -Transcription Factors
  - - Mitochondrial Genes
  - - Protease inhibitors
  - - Shock proteins (Hsps)
  - - Antioxidant enzymes
  - - Ferritin H & L

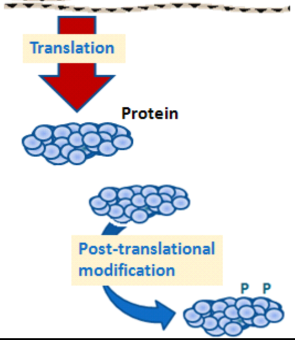
CONTROL REGION OF A TYPICAL EUKARYOTIC GENE



Changes in Gene Expression and Regulation

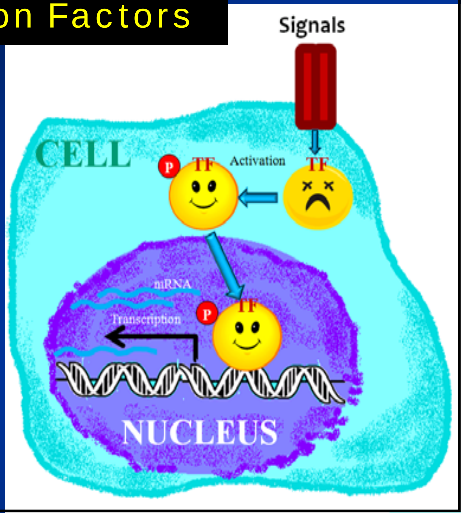


We Study:
- Transcriptional regulation
  - Changes in mRNA levels
- Translational regulation
  - Changes in protein levels
- Post-translational regulation
  - Changes in post-translational modifications
  - Changes in subcellular distribution



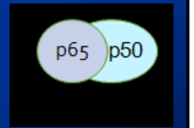
Transcription Factors

- Master regulators of gene expression
- Respond to intra- or extracellular signals
- Bind to promoter regions of specific genes
- Mediate DNA transcription to mRNA



Nuclear Factor kappa B (NF-kB)

- Dimeric transcription factor, composed of subunits including p65, p50, p52, c-Rel and Rel B



- Activated by: Stress ,Cytokines Free radicals, UV
- Functions :
  - Immune response, Development
  - Cell growth, Apoptosis, Stress response

NF-kB

CONTROL:

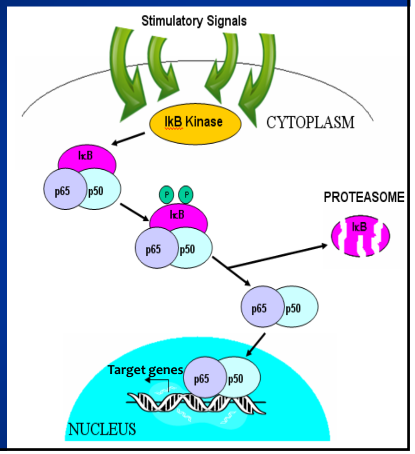
NF-kB dimer is sub-active in cytoplasm, bound to IkB

STRESS:

IkB phosphorylated & degraded

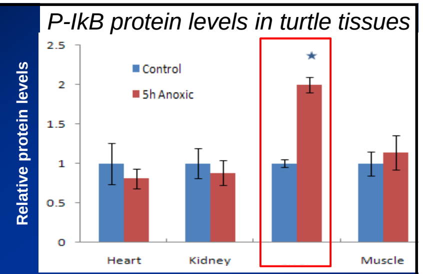
Free NF-kB dimer:

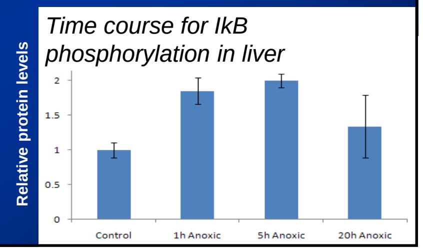
- moves to nucleus
- binds to DNA
- transcription of downstream genes



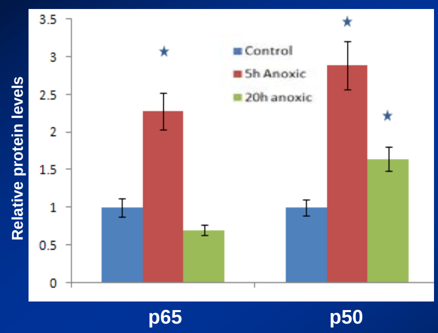
IkB Phosphorylation

- IkB is (P) in liver & brain at 5h of anoxia
- Elevated (P) of IkB frees NF-kB dimer to move into the nucleus





Turtles: NF-kB Protein Levels

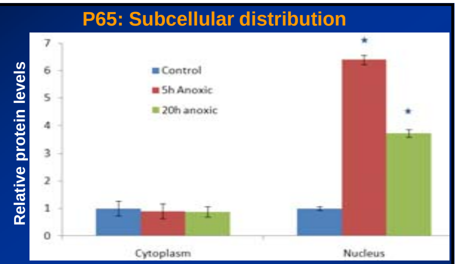


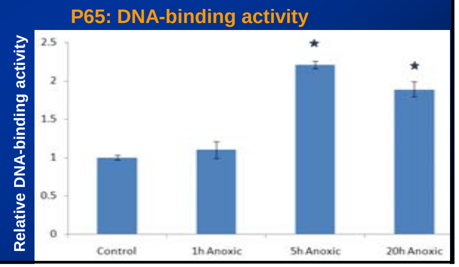
- NF-kB p65 and p50 are upregulated during 5 h of anoxia

NF-kB

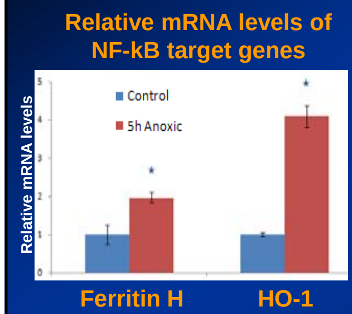
- P65 moves into nucleus in anoxia
- DNA-binding activity of p65 elevated after 5 & 20 h of anoxia

NF-kB pathway is activated in turtle liver and brain in anoxia !





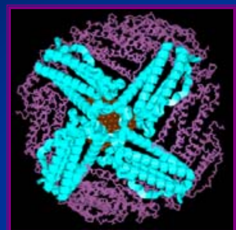
NF-kB: Target Gene Levels



Ferritin heavy chain and Heme oxygenase-1 (HO-1) are upregulated after 5 h of anoxia

Ferritin heavy chain

- Sequesters iron
- Can hold up to 4,500 atoms of iron
- 24 subunits: light (19 kDa) and heavy (21 kDa)
- Limits iron-catalyzed ROS production via the Fenton reaction



$O_2 + e^- \rightarrow O_2^{\cdot-}$ superoxide radical

$O_2^{\cdot-} + H_2O \rightarrow HOO^{\cdot} + OH^-$ hydroperoxyl radical

$HOO^{\cdot} + e^- + H^+ \rightarrow H_2O_2$ hydrogen peroxide

$H_2O_2 + e^- \rightarrow {}^{\cdot}OH + OH^-$ hydroxyl radical

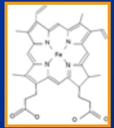
70's radicals

$Fe(III) + O_2^{\cdot-} \rightarrow Fe(II) + O_2$

$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\cdot}OH + OH^-$ Fenton reaction

$O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + {}^{\cdot}OH + OH^-$ Haber-Weiss reaction

Ferritin and Heme Oxygenase -1



Help minimize free iron levels in cells

- Ferritin: Binds iron; Heavy & Light chains
- Heme oxygenase -1:
  - Degrades heme, a source of redox active iron
  - Free iron then stored into ferritin

Iron can be a source of oxidative stress:
• Catalyzes production of Hydroxyl radicals via Fenton reaction:

$H_2O_2 \xrightarrow{Fe^{2+}} \cdot OH + OH^-$

Hydroxyl radical is very reactive and responsible for most oxidative stress-mediated damage

THE BRAINS OF THE OPERATION

GENE ⟷ PROTEIN

INTEGRATION

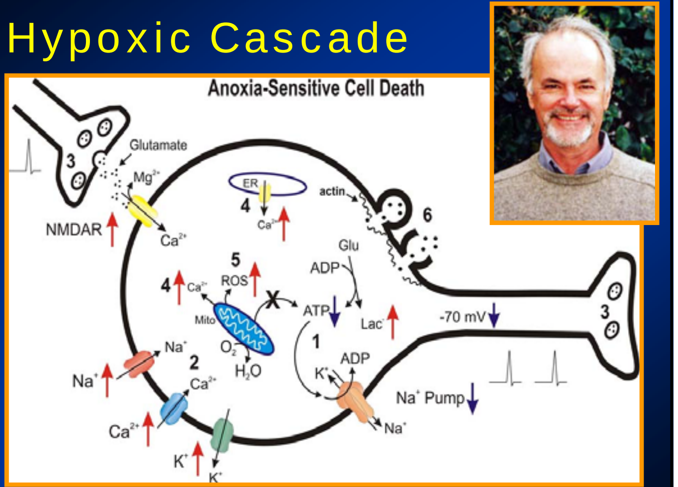
PHYSIOLOGY ⟷ BIOCHEMISTRY

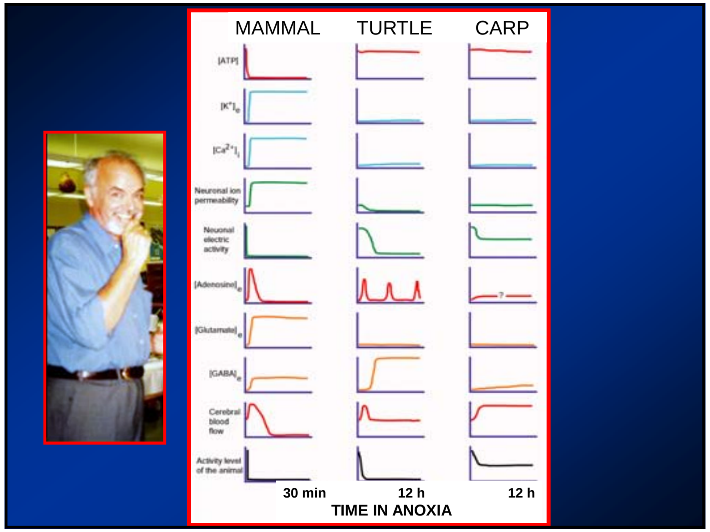
PETER LUTZ  ⟷  TERRRTLE

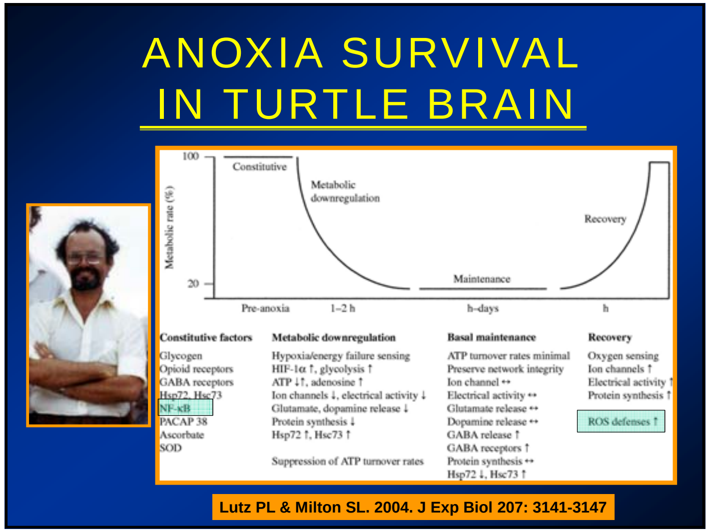
Hypoxia / Ischemia

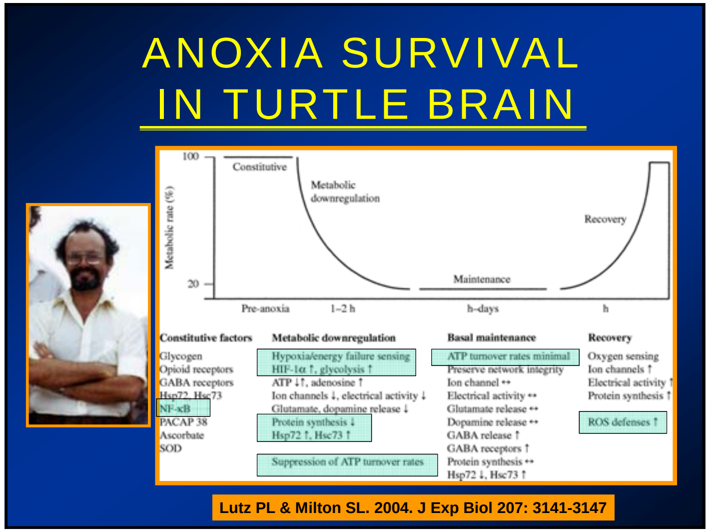
- Sensitive Animals (most mammals)
  - Energy deficit (high ATP demand)
  - Disruption of ions and depolarization
  - Release of excitotoxic GLU,
  - Excess intracellular Ca^{2+}
  - Oxidative Stress (+ reperfusion)
  - Cell Death
- Tolerant Animals (e.g. turtles, carp)
  - Decrease ATP demand (Metabolic Arrest)
  - Adenosine as a Retaliatory Molecule

Hypoxic Cascade




MAMMAL
TURTLE
CARP
[ATP]
Neuronal ion permeability
Neuronal electric activity
Cerebral blood flow
Activity level of the animal
30 min
12 h
12 h
TIME IN ANOXIA


ANOXIA SURVIVAL
IN TURTLE BRAIN
100
20
Metabolic rate (%)
Constitutive
Metabolic downregulation
Maintenance
Recovery
Pre-anoxia
1–2 h
h-days
h
Constitutive factors
Glycogen
Opioid receptors
GABA receptors
Hsp72, Hsc73
NF-κB
PACAP 38
Ascorbate
SOD
Metabolic downregulation
Hypoxia/energy failure sensing
HIF-1α ↑, glycolysis ↑
ATP ↓↑, adenosine ↑
Ion channels ↓, electrical activity ↓
Glutamate, dopamine release ↓
Protein synthesis ↓
Hsp72 ↑, Hsc73 ↑
Suppression of ATP turnover rates
Basal maintenance
ATP turnover rates minimal
Preserve network integrity
Ion channel ↔
Electrical activity ↔
Glutamate release ↔
Dopamine release ↔
GABA release ↑
GABA receptors ↑
Protein synthesis ↔
Hsp72 ↓, Hsc73 ↑
Recovery
Oxygen sensing
Ion channels ↑
Electrical activity ↑
Protein synthesis ↑
ROS defenses ↑
Lutz PL & Milton SL. 2004. J Exp Biol 207: 3141-3147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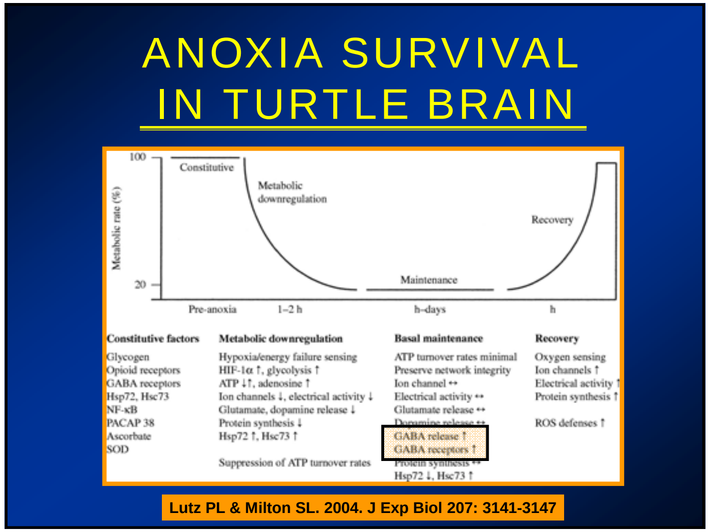
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100
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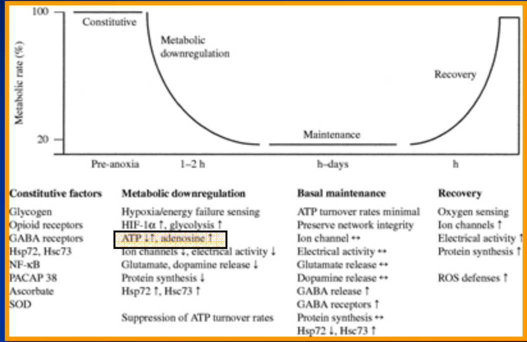
Lutz PL & Milton SL. 2004. J Exp Biol 207: 3141-3147

BRAIN GENES
Up-regulated in turtle anoxia (DNA array)

- GABA transporter
- GABA receptor

Adult *T. s. elegans*

ANOXIA SURVIVAL IN TURTLE BRAIN



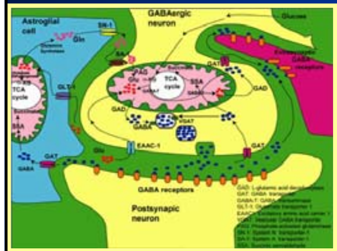
Lutz PL & Milton SL. 2004. J Exp Biol 207: 3141-3147

BRAIN GENES
Up-regulated in turtle anoxia (DNA array)

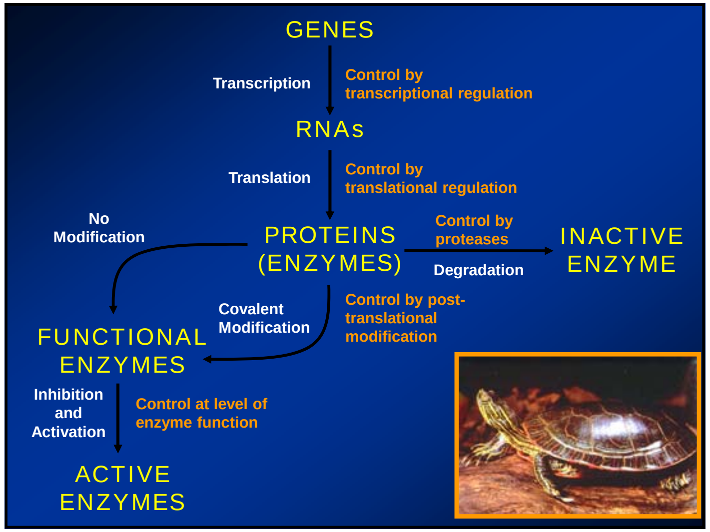
- Adenosine receptor
- 5'Nucleotidase

Adult *T. s. elegans*

BRAIN GENES



- GABA transporter
- GABA receptor
- Adenosine receptor
- 5'Nucleotidase
- Serotonin receptor



ANOXIA

- J. STOREY
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- Q. CAI
- W. WILLMORE
- H. MEHRANI
- D. DOUGLAS
- J. DUNCAN
- S. GREENWAY
- A. KRIVORUCHKO
- M. HERMES-LIMA
- T. ENGLISH
- K. LARADE
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- B. MICHAELIDIS
- J. ZHOU

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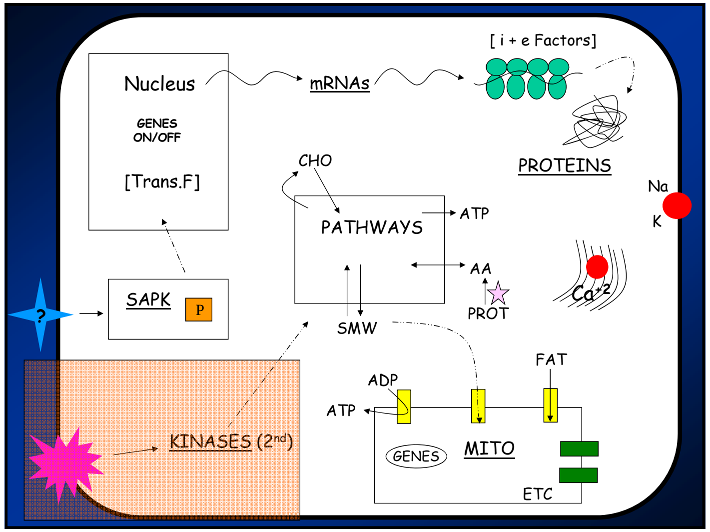
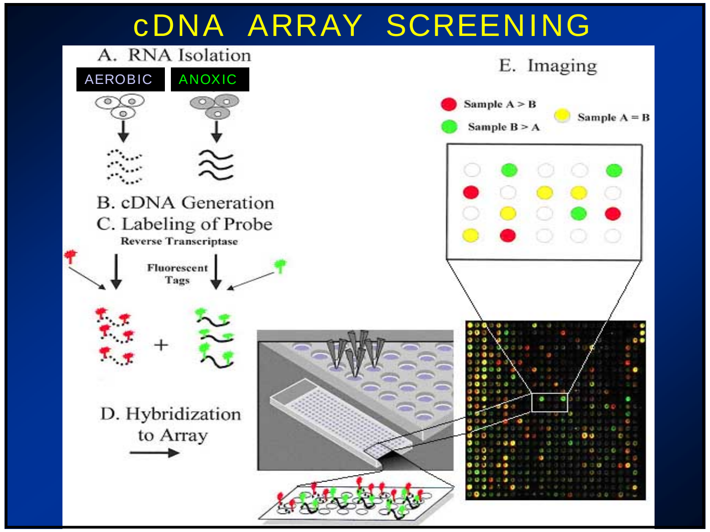
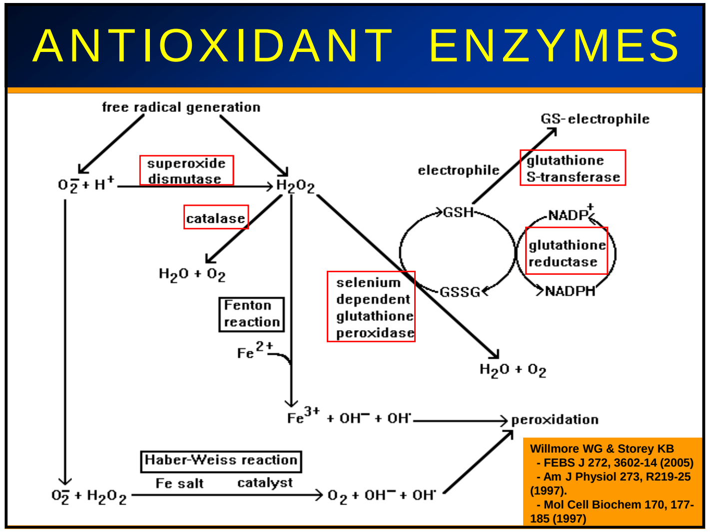
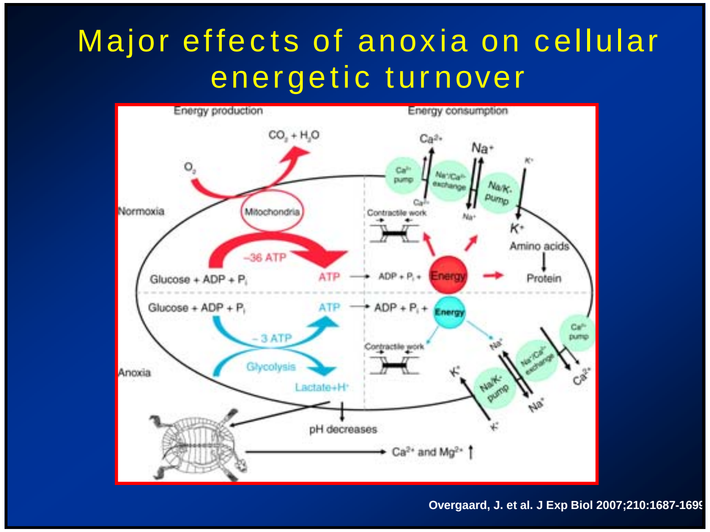
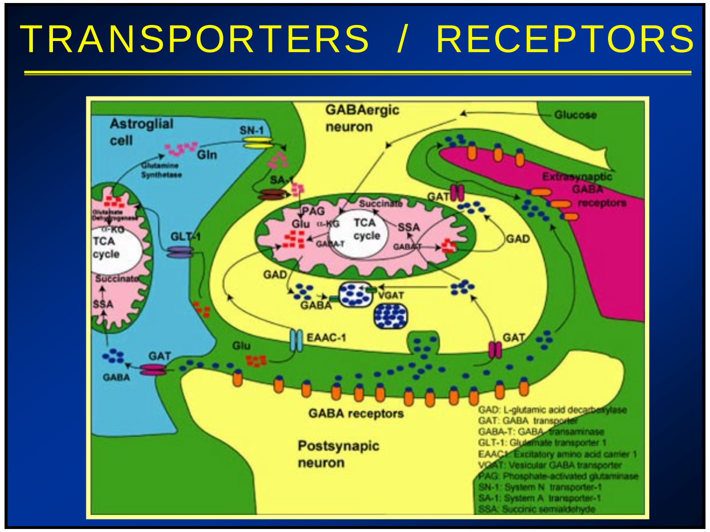



cDNA ARRAY SCREENING
A. RNA Isolation
AEROBIC
ANOXIC
B. cDNA Generation
C. Labeling of Probe
Reverse Transcriptase
Fluorescent Tags
D. Hybridization to Array
E. Imaging
Sample A > B
Sample B > A
Sample A = B


ANTIOXIDANT ENZYMES
free radical generation
superoxide dismutase
catalase
Fenton reaction
selenium dependent glutathione peroxidase
glutathione S-transferase
glutathione reductase
GS-electrophile
electrophile
GSH
GSSG
NADP+
NADPH
peroxidation
Haber-Weiss reaction
Fe salt catalyst
Willmore WG & Storey KB
- FEBS J 272, 3602-14 (2005)
- Am J Physiol 273, R219-25 (1997).
- Mol Cell Biochem 170, 177-185 (1997)


Major effects of anoxia on cellular energetic turnover
Energy production
Energy consumption
Normoxia
Anoxia
Mitochondria
~36 ATP
~3 ATP
Glycolysis
Lactate+H+
Contractile work
Amino acids
Protein
Energy
pH decreases
Overgaard, J. et al. J Exp Biol 2007;210:1687-1699


TRANSPORTERS / RECEPTORS
Astroglial cell
GABAergic neuron
Glucose
Extrasynaptic GABA receptors
TCA cycle
GABA receptors
Postsynapic neuron
GAD: L-glutamic acid decarboxylase
GAT: GABA transporter
GABA-T: GABA transaminase
GLT-1: Glutamate transporter 1
EAAC1: Excitatory amino acid carrier 1
VGAT: Vesicular GABA transporter
PAG: Phosphate-activated glutaminase
SN-1: System N transporter-1
SA-1: System A transporter-1
SSA: Succinic semialdehyde