REM sleep deprivation-induced noradrenaline stimulates neuronal and inhibits glial Na-K ATPase in rat brain: in vivo and in vitro studies

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- Impact factor = 3.228
- Issues per year = 16

Sleep
- Sleep is a complex, active phenomenon.
  - It is composed of physiologically distinct states, and it cycles at regular intervals.
- Sleep is not simply a reduction in activity of the reticular activating system; rather, it is generated by many different regions of the CNS working together.

Characteristics of sleep
- Episodic
- Promptly reversible
- Reduced awareness
- Reduced responsiveness
- Motor inhibition

Sleep control mechanism
- Homeostatic mechanism
  - Accumulation of adenosine
- Circadian mechanism
  - The suprachiasmatic nuclei are the centers responsible for the most important circadian rhythms
  - Zeitgebers = reset “biological clock”

Mechanisms of sleep-wake control
Sleep stage

- **NREM (non-rapid eye movement) sleep**
  - Stage I & II (light sleep)
  - Stage III & IV (deep sleep)

- **REM (rapid eye movement) sleep**
  - (dream sleep, paradoxical sleep)

**Neurophysiology of sleep**

- REM sleep
- NREM sleep stage II
- Deep sleep

**Neuroanatomy of sleep**

- **NREM sleep**
  - Ventrolateral preoptic area (VLPO)
    - GABAergic neurons

- **REM sleep**
  - Dorsolateral pons (LDT/PPT)
    - Cholinergic neurons (REM-on neurons)
  - Nucleus magnocellularis and nucleus paramedianus (medulla)
    - Inhibit motor neuron -> muscle atonia

**REM sleep**

- Paralysis or nearly absent muscle tone (except for control of breathing and erectile tissue)
- High levels of cortical activity (low voltage, mixed frequency) that are associated with dreaming
- Episodic bursts of phasic eye movements
- Irregular respiration and heart rate

**Neurotransmitter interactions:**

- Cholinergic and GABAergic neurons in REM sleep control

Roles of REM sleep

- Memory consolidation
  - Dreaming??
  - Forget a lot of unnecessary data
- Maintains brain excitability by maintaining the activity of Na-K ATPase in the rat brain
  - Gulyani and Mallick, 1993

REM sleep loss

- REM sleep loss has been associated with several signs and symptoms like increased anxiety, aggression, irritability, confusion, loss of concentration (mood and behavior changes)
  - Gulyani et al., 2000
- Recent studies have shown that longer duration of REM sleep deprivation (REMSD) results in morphological changes in neurons and neuronal death
  - Majumdar and Mallick, 2005
  - Biswas et al., 2006
  - Cordova et al., 2006

REM sleep loss

- REM sleep deprivation increased neuronal depolarization, which is the likely cause for increased brain excitability
- The Na-K ATPase activity also increased in the deprived sample
- Both the effects were mediated by NA acting on α1-adrenoceptors in the brain
  - Das and Mallick, 2008

Structure of Na-K ATPase

Function: exchange sodium & potassium and maintain transmembrane potential of neurons

Na-K ATPase in neuron and glia

- Developmental and tissue-specific expressions
- 4 isoforms of alpha subunit
- 3 isoforms of beta subunit
- α3 isoform is predominantly present in the neuron and is absent in the glia
Objectives

- Compares the effects of REM sleep deprivation and noradrenaline (NA) on neuronal and glial Na-K ATPase activities
- Investigated whether their mechanism of action is similar

In vivo study

- Male Wistar rats (250-280 gram), maintained on 12/12 light/dark cycle
- 4 groups of rat:
  - Free moving control (FMC) group
  - REM Sleep deprived (REMSD) group
    - Standard flowerpot method (diameter 6.5 cm) x 4 days
  - Recovery control (REC) group
  - Large platform control (LPC) group
    - Large platform = 13 cm in diameter

The Flowerpot Technique

- This technique is designed to allow NREM sleep but prevent REM sleep
- A rat is placed on top of an upside down flower pot which is placed in a bucket of water

Immunostaining

- Diluted in PBS (phosphate-buffered saline)
- Fixed with 4% paraformaldehyde in PBS for 15 min
- Incubate 45 min

- Primary antibodies:
  - Anti-Neu
  - Anti-GFAP

- Secondary antibodies:
  - Labeled with green fluorescein isothiocyanate + anti-Neu
  - Labeled with red phycoerythrin (PE) + anti-GFAP

- Minimized non-specific fluorescence by normal goat serum

Use “Flow cytometry” to confirm the purity of the separated neurons and glia

Neurons and glia were well separated with negligible contamination by the other type
**Preparation of synaptosomes**

- Whole brain / Neurons / Glia
- Homogenized in 10 vol of ice-cold buffer
- Centrifuged 6,000 rpm x 5 min
- Supernatant
- Pellet
- Centrifuged 12,000 rpm x 20 min
- Supernatant
- Pellet
- Ultra centrifuged 25,000 rpm x 2 hr
- Supernatant
- Pellet
- Washed with 3 vol of homogenizing buffer

**In vitro study**

**Cell culture condition**

- 2 types of cell lines
  - Rat brain glioblastoma cell line, C6
  - Mouse neuroblastoma cell line, Neuro2a
- Temperature 37°C
- Humidified atmosphere containing 5% CO₂ in an incubator
- 100 mm culture petri dishes containing Dulbecco Modified Eagles Medium
- 10% FBS
- 1% Antibiotic mix

**Membrane preparation**

- Scraped cells
- Homogenized in 1 ml of homogenizing buffer
- Centrifuged 12,000 rpm x 10 min, 4°C
- Supernatant
- Pellet
- Centrifuged 25,000 rpm x 75 min, 4°C
- Supernatant
- Pellet
- Protease inhibitor + (Aprotinin)

**Enzyme assay**

- Measure of Na-K ATPase activity (ATP -> ADP + Pi)
- Reaction mixture (NaCl, KCl, MgCl₂, ATP, Tris pH 7.4)
- Incubate at 37°C x 15 min
- Stop reaction with 10% ice cold TCA
- Centrifuged 2,000 rpm
- Supernatant
- Pellet
- Measure Pi level by Shimadzu UV160 spectrophotometer

**Basal Na-K ATPase activities**

<table>
<thead>
<tr>
<th>Basal Na-K ATPase activities</th>
<th>μmol of Pi released/(mg protein h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons from rat brain</td>
<td>8.16±1.09</td>
</tr>
<tr>
<td>Neuro2a cell line</td>
<td>7.18±1.18</td>
</tr>
<tr>
<td>Glia from rat brain</td>
<td>6.13±0.68</td>
</tr>
<tr>
<td>C6 cell line</td>
<td>9.65±1.58</td>
</tr>
</tbody>
</table>
Na-K ATPase activity: Whole brain

Discussion

- REM sleep maintains "brain excitability" by maintaining the activity of Na-K ATPase
- REMSD affects neuronal and glial Na-K ATPase activities in an opposite manner
  - Na-K ATPase activity in neurons is increased while it is decreased in the glial membrane

Discussion

- Both the neurons and the glia in rat brain possess Na-K ATPase
- Frequently, the neurons and the glia respond in an opposite manner, particularly for neuronal excitability and K⁺ exchange
- NA stimulates Na-K ATPase activity in neurons, while it inhibits the enzyme activity in glia
Na-K ATPase activity
Neuro2a cell line + NA +
Prazosin

Na-K ATPase activity
C6 cell line + NA + Prazosin

Prazosin effects

- **Experiments:** 50 µM of prazosin was added into culture medium
- **Results**
  - Prazosin did not significantly affect the enzyme activities
  - Prazosin blocked the NA-induced changes in Na-K ATPase activity in neurons and glia
- **Conclusion:** NA action was mediated through α1-adrenoreceptor in both neurons and glia

Discussion

- REMSD induced increased Na-K ATPase activity is mediated by increased levels of NA acting on α1 adrenoceptors
- NA exerts a reciprocal influence on the neuronal and glial Na-K ATPase activities, which thus help maintaining ionic balance (esp. K+) across neuronal membrane
  - = maintenance of transmembrane potential

Na-K ATPase activity

Neuron +WB 4101 (10 µM)
Glia +WB 4101 (10 µM)

Blocked by WB4101 (α1,α-adrenoceptor blocker)
**WB4101 effects**

- **Experiment:** separated neurons and glia from rat brain were treated with WB4101 before treating with NA.
- **Results**
  - WB4101 did not affect the basal activity of enzyme
  - WB4101 blocked the NA-induced changes in Na-K ATPase activity
- **Conclusion:** NA-induced effect on the neuronal and glial Na-K ATPase activities are mediated by $\alpha_1$-adrenoreceptor

**Discussion**

- Opposite effect of NA on neuronal and glial Na-K ATPase
  - Difference in the functional units of Na-K ATPases?
  - Tissue-specific isoform
  - Role of Ca$^{2+}$ (inhibit Na-K ATPase)
  - Other adrenoceptors?
  - Direct action of NA on the membrane lipid peroxidation?

**Discussion**

- The glial Na-K ATPase activity is higher in the presence of EDTA
- Calcium has a role in maintaining normal glial Na-K ATPase activity

**Na-K ATPase activity C6 cell line: EDTA effect**

[Graph showing Na-K ATPase activity with and without EDTA]

**Final conclusion**

- Increased NA after REMSD has opposite effects on neural and glial Na-K ATPase activities
- An opposite effect of NA is likely to maintain normal brain function at an optimum level
Normal condition
REM sleep deprivation

Neuron
Glia
Na-K ATPase
Intracellular mechanism Ca??
Stimulation
Inhibit

Further studies
- Glial function and neural & brain excitability
  - Epilepsy
  - Psychiatric disorders
- Underlying mechanism of Na-K ATPase action in neurons and glia
  - Role of calcium
  - Combination of noradrenaline and calcium effects

Thank you for your attention