Thrombin and Protease Activated Receptor-1 (PAR-1) in Minimal Traumatic Brain Injury (mTBI) in Mice

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Why mTBI?

- Affects millions every year
- Associated with cognitive dysfunction and epilepsy
- Definite biochemical markers are still sparse
- Pathophysiology is yet to be determined conclusively
- Simple and well established model
Why PAR-1 (and Thrombin) ?

- Present in the brain – neurons and glia, activated by serum and CNS proteases
Why PAR-1 (and Thrombin)?

- Involved in neural damage, neuro-inflammation and neuro-protection
Starting Hypothesis A

Proteases in the brain

- Traumatic BBB disruption can result in serum proteases such as thrombin leakage to brain tissue.

- Therefore traumatized brain is expected to exhibit elevation in thrombin or thrombin-like activity.
Starting hypothesis - B

PAR-1

- Proteolytic activation is known to downregulate PAR-1 by internalization and/or degradation

- Thus PAR-1 levels are expected to decline following proteases elevation in mTBI
Starting hypothesis - C

Protease inhibitors

- Elevation of proteolytic or inflammatory activity is expected to draw a contra-regulatory response.

- Such a response in the CNS has been linked to PN−1,KPI−APP and increased thrombin activity inhibition in CNS inflammation (Beilin et al., 2005)

  - Thus levels of these proteins are expected to increase following mTBI and subsequent peak of proteolytic activity
Experimental model
Trauma induction in mice (male)

- Right Temporal side
- 30 gr.
Experimental model

Assays

In Vitro

- Fluorometric enzymatic activity of brain slices (= cleavage of fluorogenic thrombin substrate)

\[
\text{N-p-Tos-Gly-L-Pro L-Arg 7-amido-4 methylcoumarin}
\]

1. prolylendopeptidase

2. aminopeptidase
Results

Thrombin like activity in mTBI brains rises **acutely and chronically**

And is reduced to baseline by thrombin inhibitor
Results
Immunoblot
PN-1 levels rise

Intact  5 min  1 hour  24 hours  72 hours

42 kDa

PN-1 immunoreactivity (mean density)

sham (n=6)  5 min post mTBI (n=6)  1 hour post mTBI (n=6)  24 hours post mTBI (n=8)  72 hours post mTBI (n=10)
Results
Immunoblot
KPI-APP levels rise

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5 min post mTBI</th>
<th>1 hour post mTBI</th>
<th>24 hours post mTBI</th>
<th>72 hours post mTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td></td>
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</table>

Bar graph showing APP immunoreactivity (mean density) across different time points:
- Intact (n=9)
- 5 min post mTBI (n=6)
- 1 hour post mTBI (n=9)
- 24 hours post mTBI (n=8)
- 72 hours post mTBI (n=10)

APP immunoreactivity:
- Intact: 100
- 5 min post mTBI: 116%
- 1 hour post mTBI: 119%
- 24 hours post mTBI: 100.7%
- 72 hours post mTBI: 129%

Significance:
- **: p < 0.01
Results
Immunoblot
PAR-1 levels rise

<table>
<thead>
<tr>
<th>Intact</th>
<th>5 min</th>
<th>1 hour</th>
<th>24 hours</th>
<th>72 hours</th>
</tr>
</thead>
</table>

PAR-1 immunoreactivity (mean density)

- Sham (n=8)
- 5 min post mTBI (n=9)
- 1 hour post mTBI (n=6)
- 24 hours post mTBI (n=7)
- 72 hours post mTBI (n=14)
Results

Immunofluorescence of the brain

Immunofluorescence images showing the localization of PAR-1 in the brain tissue. The images compare Sham and mTBI conditions in the CA3 region. Arrows indicate the areas of fluorescence. The images are labeled with different magnifications (X10, X63).
Results
Immunofluorescence
PAR-1 migrates to the nuclei in CA3

- PAR-1 nuclear localization ratio comparison between sham (n=16) and 72 hours post mTBI (n=16).

* denotes statistical significance.
<table>
<thead>
<tr>
<th>Brain thrombin or like activity</th>
<th>Expected: immediate</th>
<th>Observed: late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain protease inhibition</td>
<td>Expected:</td>
<td>Observed:</td>
</tr>
<tr>
<td>Brain PAR-1 levels</td>
<td>Expected:</td>
<td>Observed: late</td>
</tr>
<tr>
<td>Brain PAR-1 localization</td>
<td>?</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>
Emerging hypothesis B
Behavioral effect of PAR-1 elevation

- Thus, mice with elevated PAR-1 (=mTBI) are likely to exhibit more epileptic like activity following thrombin exposure.

Experimental model

Assays

In vivo

- Intracerebroventricular thrombin injections
- Intact vs. 72 hours post mTBI mice
- Quantification of freezing (in a mouse = epileptic like seizure) periods during the first 20 minutes post recovery
- EEG validation of the seizure response

Sundaresan et al., J.Virol. 2000
## Results

**Behavior**

Proportion of thrombin induced seizure episodes **rises**

<table>
<thead>
<tr>
<th></th>
<th>mTBI Injection</th>
<th>intact</th>
<th>72 hours post mTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>thrombin (n=8)</td>
<td>thrombin (n=9)</td>
<td></td>
</tr>
</tbody>
</table>
Results

Behavior

And PAR-1 antagonism abolishes epileptic like response

![Graph showing % of seizure like episodes](image-url)
Results

EEG

Epileptiform EEG activity of thrombin injected mice

Saline injection (n=3)

Thrombin injection (n=2)
<table>
<thead>
<tr>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin responsiveness in mTBI animals</td>
<td>epileptiform</td>
</tr>
<tr>
<td>Electrophysiological pattern of the behavioral response</td>
<td>epileptiform</td>
</tr>
</tbody>
</table>
Emerging hypothesis C

Behavioral effect of elevated thrombin activity

- Thus, elevation of thrombin in brain tissue (via mTBI or direct injection) may result in memory formation impairment.

Experimental model

- Novel Object Recognition Test Sequence.
- Intracerebroventricular thrombin injections; mTBI induction. Mid Sequence intervention.
- Intact vs. mTBI mice, saline vs. thrombin injected mice.
- Evaluation of Preference Index 24 hours post intervention.
- Electrophysiological assessment of LTP formation ability 24 hours post intervention.

Preference Index = \( \frac{(\text{time new} - \text{time familiar})}{(\text{time new} + \text{time familiar})} \)
Results

Recognition memory of mice is impaired following mTBI or thrombin exposure. And is restored following PAR-1 antagonist administration.
Results

Long term LTP formation in hippocampal slices is impaired following mTBI or thrombin ICV injection and is restored following PAR-1 antagonist administration.
<table>
<thead>
<tr>
<th>Expected</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mTBI and thrombin effect on cognitive performance</strong></td>
<td>negative</td>
</tr>
<tr>
<td><strong>mTBI and thrombin effect on LTP formation</strong></td>
<td>negative</td>
</tr>
</tbody>
</table>
Conclusions

- mTBI results in increased thrombin like activity in the brain – acute and prolonged
- mTBI results in upregulation of CNS protease inhibitors
- mTBI results in upregulation of PAR-1
- mTBI results in nuclear relocalization (possibly by trafficking) of PAR-1 in the hippocampus
- These changes lead to neurophysiological and cognitive deficits – which are reversed by PAR-1 blockade
Proposed model

Resting phase  Early phase  Late phase  Long term changes
0  minutes  hours  days-weeks

- Elevation in PAR-1 activation by thrombin
- PAR-1 down regulation
- PAR-1 and thrombin rebound upregulation

- Nuclear translocation
- Memory Impairment
- Epileptiform activity
Thank you !!!

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(BGU)
Conclusions

- Thrombin exposure leads to PAR-1 upregulation in glial cells, a prominent player in tri-partide synapse.

- These changes lead to increased sensitivity to thrombin-induced epileptiform seizures.
<table>
<thead>
<tr>
<th>Protease-activated receptors: subtypes, agonists, and antagonists</th>
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<tbody>
<tr>
<td><strong>Cleavage sites</strong></td>
</tr>
<tr>
<td>$S^{41}$S$^{42}$-LLRN (h)</td>
</tr>
<tr>
<td>$R^{41}$S$^{42}$-FFLRN (r, m)</td>
</tr>
<tr>
<td><strong>Protease agonists</strong></td>
</tr>
<tr>
<td>Thrombin</td>
</tr>
<tr>
<td>Trypsin</td>
</tr>
<tr>
<td>Mesotrypsin/Trypsin IV (?)</td>
</tr>
<tr>
<td>FVIIa</td>
</tr>
<tr>
<td>FXa</td>
</tr>
<tr>
<td>APC</td>
</tr>
<tr>
<td>Granzyme A</td>
</tr>
<tr>
<td>Arginine-specific gingipains-R</td>
</tr>
<tr>
<td><strong>Peptide agonists</strong></td>
</tr>
<tr>
<td>SFLLR-NH$_2$</td>
</tr>
<tr>
<td>TFLLR-NH$_2$</td>
</tr>
<tr>
<td>TRag$^b$</td>
</tr>
<tr>
<td>TFRIFD</td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
</tr>
<tr>
<td>3-mercapto-propionyl-F-Cha-Cha-RKPNDK-amide</td>
</tr>
<tr>
<td>Trans-cinnamoyl-parafuororo-F-paraguanidino-LLRR-amide</td>
</tr>
<tr>
<td>N-palmitoyl-RCLSSAVANRS-amide</td>
</tr>
<tr>
<td>RPPGF-OH</td>
</tr>
<tr>
<td>RWJ56110</td>
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<td>RWJ58259</td>
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<td>SCH79797</td>
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<tr>
<td>FR171113</td>
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<tr>
<td>Merck isoxazole 1</td>
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<tr>
<td><strong>PAR-1</strong></td>
</tr>
<tr>
<td>$R^{36}$S$^{37}$LIGKV (h)</td>
</tr>
<tr>
<td>$R^{36}$S$^{37}$LIGRL (r)</td>
</tr>
<tr>
<td>$R^{36}$S$^{35}$LIGRL (m)</td>
</tr>
<tr>
<td><strong>PAR-2</strong></td>
</tr>
<tr>
<td>$K^{38}$T$^{39}$FRGAP (h)</td>
</tr>
<tr>
<td>$K^{38}$S$^{39}$FNGGP (m)</td>
</tr>
<tr>
<td><strong>PAR-3</strong></td>
</tr>
<tr>
<td>$R^{37}$G$^{38}$YPGQV (h)</td>
</tr>
<tr>
<td>$R^{38}$G$^{39}$FPK (r)</td>
</tr>
<tr>
<td>$R^{35}$G$^{36}$YPKF (m)</td>
</tr>
<tr>
<td><strong>PAR-4</strong></td>
</tr>
<tr>
<td>$K^{37}$S$^{38}$FNGGP (m)</td>
</tr>
<tr>
<td><strong>Note.</strong></td>
</tr>
<tr>
<td>a: standard PAR activating peptide. c: most potent and selective PAR-2 peptide agonist. b: putative and selective PAR-1 peptide agonist. h, human; r, rat; m, mouse; ı, cleavage site. ?: It is still open which PARs are activated by mesotrypsin/trypsin IV in the brain.</td>
</tr>
</tbody>
</table>