

## **Bax-dependent, but not cyclophilin D dependent, mitochondrial permeabilization contributes to neonatal hypoxic ischemic brain injury**

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**Objective:** Mitochondria play a critical role in mediating cell death in both the adult and immature brain. It has previously been shown that hypoxia-ischemia (HI) in the immature brain induces mitochondrial permeabilization, release of pro-apoptotic proteins from the mitochondria and leading to the initiation of caspase- and apoptosis-inducing factor (AIF)-dependent cell death. Cyclophilin D (CyD), a regulator of mitochondria membrane permeability transition pore (PTP) is important in necrotic cell death triggered by ischemia/reperfusion injury in adult brain, whereas the role of CyD in hypoxic-ischemic (HI) injury in the immature brain is unknown. The aim of this study was to evaluate the mechanism of mitochondrial permeabilization in the neonatal brain after HI.

**Study design:** At postnatal day (PND) 9 or 60, littermates of CyD KO, WT and Het were subjected to left carotid artery ligation and 10% O<sub>2</sub> for 40 minutes or 30 minutes, respectively. Mice were sacrificed either at 6h and 24h after HI for activity measurement and western blot, or at 7 days after HI for evaluation of the tissue loss. Control brains were collected for analysis at PND 3, 9, 21 and 60 of both WT and KO mice. In a separate group of mice, 5 $\mu$ l of Bax inhibiting peptide (BIP) (5mg/ml) or vehicle (0.9%NaCl) was injected intracerebroventricularly (icv) immediately before HI.

**Results:** 1) At PND9, tissue loss was more pronounced in CyD deficient mice (20.6  $\pm$  3.5 mm<sup>3</sup>; n=19) than in WT (10.5  $\pm$  1.7 mm<sup>3</sup>) mice (p=0.017). 2) Tissue loss at PND60 was 2.3  $\pm$  1.9 mm<sup>3</sup> in CyD KO mice (n=13) vs 14.3  $\pm$  4.8 mm<sup>3</sup> in WT mice (n=18) (p=0.035). 3). Electronmicroscopy did not reveal any differences in mitochondrial morphology between normal control WT and CyD KO mice at PND9 or PND60. 4) There is slight increase of CyD expression and a marked decrease of hexokinase and Bax level in mitochondria during development from PND3, 9, 21 to PND 60. 5) Caspase-2, -9 and -3 activation as well as release of cytochrome C, AIF and Smac from the mitochondria were more pronounced in KO than in WT mice at PND9. 6) There is an increased level of hexokinase at 1.5h and Bax at 3h in mitochondria after HI in KO compared with Wt mice and BIP reduced brain injury and caspase-3 activation (by 47%) in CyD KO mice at PND9.

**Conclusions:** CyD-dependent PTP appears to play a different role in neonatal and adult HI brain injury. This vulnerability conferred by CyD deficiency in the immature brain may partly be due to more pronounced mitochondrial permeabilization as judged by the enhanced release of CytC, AIF, and increased activation of downstream caspases. We suggest that HI triggers mitochondria permeabilization and CytC release in a Bax- but not CyD/mPTP-dependent manner in neonatal brain, which could be important in selecting novel targets for brain protection in newborns.