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Purines: Forgotten Mediators in Traumatic Brain Injury

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Abstract

Recently, the topic of traumatic brain injury has gained attention in both the scientific community and lay press. Similarly, there have been exciting developments on multiple fronts in the area of neurochemistry specifically related to purine biology that are relevant to both neuroprotection and neurodegeneration. At the 2105 meeting of the National Neurotrauma Society, a session sponsored by the International Society for Neurochemistry featured three experts in the field of purine biology who discussed new developments that are germane to both the pathomechanisms of secondary injury and development of therapies for traumatic brain injury. This included presentations by Drs. Edwin Jackson on the novel 2′,3′ cAMP pathway in neuroprotection, Detlev Boison on adenosine in posttraumatic seizures and epilepsy, and Michael Schwarzschild on the potential of urate to treat central nervous system injury. This mini review summarizes the important findings in these three areas and outlines future directions for the development of new purine-related therapies for traumatic brain injury and other forms of central nervous system injury.

Keywords

adenosine; cyclic-AMP; seizure; neuroprotection; urate; uric acid

In the last 5–10 years, the topic of traumatic brain injury (TBI) has garnered incredible attention in both the scientific community and lay press. This has resulted from the emerging recognition of the importance and consequences of both repetitive mild traumatic brain injury in the civilian population (repeated sports concussion) and blast-induced TBI in combat casualty care and terrorist attacks. The potential linkage of TBI to a variety of

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neurodegenerative diseases such as chronic traumatic encephalopathy, among others has further fueled this interest. There have been exciting developments in the field of purine biology that may have relevance to TBI. At the 2015 meeting of the National Neurotrauma Society, a session sponsored by the International Society for Neurochemistry featured three experts in the field of purine biology who discussed new developments germane to the pathomechanisms and development of therapies in TBI. This included presentations by Drs. Edwin Jackson on the novel 2′,3′ cAMP pathway in neuroprotection, Detlev Boison on adenosine in posttraumatic seizures and epilepsy, and Michael Schwarzschild on the potential of urate to treat CNS injury.

The 2′,3′ cAMP pathway in neuroprotection

Discovery of Nucleoside 2′,3′-Cyclic Monophosphates

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a powerful tool that couples the resolving capability of ultra-performance liquid chromatography with the sensitivity and specificity of tandem mass spectrometry. Jackson and coworkers measured, using LC-MS/MS, purines in the renal venous outflow from isolated, perfused rat kidneys (Ren et al., 2009). They observed a large chromatographic peak that was due to a 330 m/z precursor ion and 136 m/z product ion consistent with what would be expected for 3′,5′–cAMP. Surprisingly, however, the retention time of the compound was shorter than that for authentic 3′,5′-cAMP, thus eliminating the possibility that the signal was due to 3′5′-cAMP. They identified the unknown compound as adenosine 2′,3′–cyclic monophosphate (2′,3′–cAMP), and these studies were arguably the first unequivocal identification of 2′,3′–cAMP in any biological system. The discovery of 2′,3′-cAMP in the rat kidney was rapidly followed by other publications providing evidence for the existence in biological systems of not only 2′,3′-cAMP, but other nucleoside 2′,3′-cyclic monophosphates (2′,3′-cNMPs) (Pabst et al., 2010; Van Damme et al., 2012; Burhenne et al., 2013; Bähre and Kaever, 2014; Bordeleau et al., 2014; Jia et al., 2014; Van Damme et al., 2014). It is now clear that there exist a family of non-canonical cNMPs with 2′,3′-cyclic, rather than 3′,5′-cyclic, phosphodiester bonds (Figure 1).

The 3′,5′-cAMP-Adenosine Pathway

Extracellular adenosine biosynthesis occurs via several pathways activated by diverse stimuli to produce adenosine for different purposes. The classical pathway is catalyzed by the ecto-enzyme CD39 working in tandem with the ecto-enzyme CD73; a pathway that produces adenosine in the interstitium as follows: ATP → ADP → 5′-AMP → adenosine. Hypoxia and inflammation activate the “CD39/CD73 pathway” which produces large amounts of extracellular adenosine that restore tissue perfusion and down-regulate inflammation (Eltzschig, 2009; Eltzschig and Carmeliet, 2011; Eltzschig et al., 2012; Eltzschig, 2013). The “extracellular 3′,5′-cAMP-adenosine pathway” is another mechanism for producing adenosine in the interstitium. It involves: 1) the intracellular conversion of ATP to 3′,5′-cAMP by adenylyl cyclases; 2) rapid export of 3′,5′-cAMP from cells by cyclic nucleotide transporters such as MRP4 (Cheng et al., 2010); 3) conversion of extracellular 3′,5′-cAMP to 5′-AMP by ecto-3′,5′-cyclic nucleotide 3′-phosphodiesterases; and 4) metabolism of extracellular 5′-AMP to adenosine by CD73 and tissue non-specific alkaline phosphatase

(TNAP; an ecto-enzyme structurally similar to CD73). The extracellular adenosine produced by the extracellular 3′,5′-cAMP-adenosine pathway engages adenosine receptors to expand/modulate the initial effects of adenylyl cyclase activation. The first explicit formulation of the extracellular 3′,5′-cAMP-adenosine pathway was postulated in 1991 (Jackson, 1991); evidence for this mechanism and the role of 3′,5′-cAMP as a "3rd messenger" is extensive (Mi et al., 1994; Mi and Jackson, 1995; Dubey et al., 1996; Jackson et al., 1997; Dubey et al., 1998; Mi and Jackson, 1998; Hong et al., 1999; Dubey et al., 2000a; Dubey et al., 2000b; Jackson and Mi, 2000; Dubey et al., 2001; Jackson et al., 2003; Jackson et al., 2006; Do et al., 2007; Jackson et al., 2007; Chiavegatti et al., 2008; Giron et al., 2008; Jackson and Mi, 2008; Müller et al., 2008; Dubey et al., 2010; Kuzhikandathil et al., 2011; Duarte et al., 2012; Sciaraffia et al., 2014).

The 2′,3′-cAMP-Adenosine Pathway

Since biological systems can express an extracellular 3′,5′-cAMP-adenosine pathway, Jackson and coworkers (Jackson et al., 2009) considered whether an analogous "extracellular 2′,3′-cAMP-adenosine pathway" may also exist: intracellular synthesis of 2′,3′-cAMP → egress of 2′,3′-cAMP → extracellular conversion of 2′,3′-cAMP to 2′-AMP plus 3′-AMP → extracellular catabolism of 2′-AMP and 3′-AMP to adenosine. The rationale for this hypothesis was based on four observations. First, intracellular ribonucleases (RNases) degrade RNA by facilitating the hydrolysis of the P-O5′ bond of RNA via transphosphorylation of RNA to yield 2′,3′-cNMPs (Wilusz et al., 2001). NMR spectroscopy (Thompson et al., 1994) demonstrated that 2′,3′-cNMPs formed by transphosphorylation of RNA are released form RNases as intact 2′,3′-cNMPs. Consequently, most 2′,3′-cNMPs, such as 2′,3′-cAMP, likely are formed from nucleotide bases in RNA via RNase catalyzed transphosphorylation (Thompson et al., 1994). Recent studies (Gu et al., 2013; Sokurenko et al., 2015) using a variety of approaches reveal a detailed molecular mechanism for 2′,3′-cNMP biosynthesis from RNA. Inasmuch as mRNA has a large number of adenosine monophosphates in the poly-A tail (Alberts et al., 1989), mRNA degradation can generate large amounts of 2′,3′-cAMP.

Second, nucleotide transporters, for example MRP4 and MRP5, both rapidly and actively export a diverse number of nucleotides (linear and cyclic), into the extracellular space (Kruh et al., 2001; van Aubel et al., 2002; Deeley et al., 2006; Borst et al., 2007). Likely then nucleotide transporters would also export 2′,3′-cAMP; release of endogenous 2′,3′-cAMP into the renal circulation (Jackson et al., 2009; Ren et al., 2009; Jackson et al., 2011a) unquestionably indicates that 2′,3′-cAMP reaches the extracellular compartment.

Third, there exist enzymes that could catalyze the pathway. For example, 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) can metabolize 2′,3′-cAMP to 2′-AMP *in vitro* (Vogel and Thompson, 1988; Sprinkle, 1989; Thompson, 1992). Some secreted RNases can hydrolyze 2′,3′-cAMP to 3′-AMP (Sorrentino and Libonati, 1997; Sorrentino, 1998). Rao et al. (Rao et al., 2010) report that six different phosphodiesterases containing three different families of hydrolytic domains can generate 3′-AMP from 2′,3′-cAMP. Consistent with the existence of ecto-2′,3′-cyclic nucleotide 2′-phosphodiesterases and ecto-2′,3′-cyclic nucleotide 3′-phosphodiesterases are the findings that: 1) 3′-AMP is present in rat spleen,

kidney, liver, heart, and brain (Bushfield et al., 1990; Fujimori and Pan-Hou, 1998; Fujimori et al., 1998; Miyamoto et al., 2008); and 2) 2′-AMP and 3′-AMP are present in human cerebrospinal fluid (CSF) and their concentrations correlate with the concentrations of 2′,3′-cAMP (Verrier et al., 2012).

Fourth, there are ecto-nucleotidases that process extracellular 2′-AMP and 3′-AMP to adenosine. Ohkubo et al. (Ohkubo et al., 2000) showed that in NG108-15 cells TNAP can release orthophosphate from 2′-AMP or 3′-AMP. Moreover, in kidneys TNAP metabolizes 2′-AMP and 3′-AMP to adenosine (Jackson, E.K.; unpublished observations). Also, adenosine levels in human CSF correlate with those of 2′,3′-cAMP, 2′-AMP, and 3′-AMP (Verrier et al., 2012)—consistent with the metabolism of these compounds to adenosine in the extracellular compartment.

Jackson and coworkers confirmed the existence of the extracellular 2′,3′-cAMP-adenosine pathway. In kidneys, arterial infusions of 2′,3′-cAMP dramatically increased renal venous 3′-AMP, 2′-AMP, and adenosine (Jackson et al., 2009; Jackson et al., 2011a); and infusions of 2′-AMP and 3′-AMP augmented secretion of adenosine similar to that achieved by 5′-AMP (prototypical adenosine precursor). Energy depletion can activate the degradation of RNA (Akahane et al., 2001a; Akahane et al., 2001b; Almeida et al., 2004) and should engage the extracellular 2′,3′-cAMP-adenosine pathways. As predicted, treatment of kidneys with metabolic inhibitors increased renal venous 2′,3′-cAMP, 2′-AMP, 3′-AMP, and adenosine (Jackson et al., 2009; Jackson et al., 2011a). The extracellular 2′,3′-cAMP-adenosine pathway may exist in many cells, tissues, and organs. For example, preglomerular vascular smooth muscle cells (Jackson et al., 2010), preglomeular vascular endothelial cells (Jackson and Gillespie, 2012), glomerular mesangial cells (Jackson et al., 2010), renal epithelial cells (Jackson and Gillespie, 2012; Jackson and Gillespie, 2013), aortic vascular smooth muscle cells (Jackson et al., 2011b), coronary artery vascular smooth muscle cells (Jackson et al., 2011b), microglia (Verrier et al., 2011), astrocytes (Verrier et al., 2011), oligodendrocytes (Verrier et al., 2013), neurons (Verrier et al., 2013), Schwann cells (Verrier et al., 2015), and intact brain in vivo (Verrier et al., 2012) metabolize exogenous 2′,3′-cAMP, 2′-AMP, and 3′-AMP to downstream purines.

CNPase and the Role of the 2′,3′-cAMP-Adenosine Pathway in Neurotrauma

CNPase is the most abundant protein in non-compact CNS myelin, and is the 3rd most abundant protein overall in CNS myelin (Raasakka and Kursula, 2014). Yet, the role for CNPase remained an enigma from the time of its discovery (Whitfeld et al., 1955) until the discovery of 2′,3′-cAMP in rat kidneys in 2009 (Ren et al., 2009). Before 2009, 2′,3′-cAMP was not known to exist in biological systems; therefore the ability of CNPase to convert 2′,3′-cAMP to 2′-AMP *in vitro* was viewed as an epiphenomenon (Vogel and Thompson, 1988; Thompson, 1992; Schmidt, 1999). With the discovery of 2′,3′-cAMP pathway, the role of the enzymatic activity of CNPase is being reconsidered (Raasakka and Kursula, 2014).

Jackson and coworkers (Verrier et al., 2012) were the first to reveal an important role for the enzymatic activity of CNPase. Employing microdialysis to infuse 2′,3′-cAMP and 2′-AMP into the extracellular compartment of the mouse brain they demonstrated that the brain converts exogenous 2′,3′-cAMP to 2′-AMP and adenosine and metabolizes exogenous 2′-

AMP to adenosine. Notably the increase in interstitial levels of 2′-AMP and adenosine following delivery of 2′,3′-cAMP to the brain interstitial compartment caused significant increases in 2′-AMP and adenosine within 30 minutes. Similarly the increase in interstitial levels of adenosine following delivery of 2′-AMP also occurred within 30 minutes. However, the conversion of exogenous 2′,3′-cAMP to 2′-AMP was attenuated in brains from CNPase knockout (KO) mice. In wild-type mice, TBI increased brain interstitial levels of 2′,3′-cAMP, 2′-AMP, 3′-AMP, adenosine, and inosine (adenosine metabolite) within a time frame of 30 minutes. In CNPase KO mice, TBI induced higher levels of interstitial 2′,3′-cAMP, yet lower levels of 2′-AMP, adenosine, and inosine. Thus deficiency of CNPase impairs the 2′, 3′-cAMP-adenosine pathway (Verrier et al., 2012) and activity of CNPase importantly participates in this system. Furthermore, histology suggested greater hippocampal neuronal injury in CNPase KO vs. wild-type and functional outcomes were worse in CNPase KO. This is consistent with observations by others that CNPase KO mice have enhanced astrogliosis, microgliosis, axon degeneration, and defects in working memory following brain injury (Wieser et al., 2013); and an aging associated psychiatric disease (catatonia-depression syndrome) (Hagemeyer et al., 2012).

Neuroprotective Mechanism of CNPase and the 2′,3′-cAMP-Adenosine Pathway

Adenosine is neuroprotective (Kochanek et al., 2013) and 2′,3′-cAMP rapidly (within minutes) opens mitochondrial permeability transition pores (mPTPs) (Azarashvili et al., 2009; Azarashvili et al., 2010) possibly triggering apoptosis, necrosis, and autophagy (mitophagy). Therefore, metabolism of endogenous 2′,3′-cAMP to 2′-AMP by CNPase would rid the brain cells of an intracellular neurotoxin (2′,3′-cAMP) while metabolism of 2′-AMP and 3′-AMP to adenosine would provide a neuroprotectant/anti-inflammatory agent (adenosine). With regard to inflammation, 2′,3′-cAMP, 3′-AMP, and 2′-AMP inhibit the release of proinflammatory TNF-α and CXCL10 in murine microglia via production of adenosine leading to activation of A_{2A} receptors (Newell et al., 2015). It is important to note that the effects of brain injury on components of the 2′,3′-cAMP-adenosine pathway occurs within 30 minutes. Thus, the pathway is activated in a time frame rapid enough to affect early events following brain injury. Indeed, in TBI patients, CSF levels of 2′,3′-cAMP are increased for 12 hours after injury and correlate with CSF 2′-AMP, 3′-AMP, adenosine, and inosine (Verrier et al., 2012). These clinical findings suggest that the 2′,3′-cAMP-adenosine pathway is engaged post TBI in humans in a time frame consistent with affecting early events after injury. Although activation of the 2′,3′-cAMP-adenosine pathway requires RNA metabolism, this does not mean that the pathway is activated only in dying cells. In addition, it would be expected that even cells destined to die could produce extracellular adenosine via the 2′3′-cAMP-adenosine pathway, and this adenosine could increase the survival rate of surrounding cells via paracrine effects.

Studies by Lappe-Siefke et al. (Lappe-Siefke et al., 2003) suggested that myelin and axonal morphology are normal in CNPase −/− mice up to about 3.5 months of age. Then axonal pathology begins to emerge and gradually increases with age. In aged CNPase −/− mice, the main axonal pathology is axonal swellings, whereas the myelin sheath remains relatively normal (only minor changes at the paranodal regions). However, subsequent studies by Edgar et al. (Edgar et al., 2009) indicated early changes (i.e. swelling) in the inner tongue of

the myelin sheath in paranodal regions of small (but not large) axons associated with some degeneration of small axons. However, these changes were not associated with a significant reduction in axon number until about 6 months of age. In preliminary studies, we examined with transmission electron microscopy white matter tracts of CNPase KO mice and CNPase wild-type mice that were about 3 months old. We did not detect any changes in axonal morphology, autophagosome number and area or mitochondrial number and area. Because our TBI experiments were performed in CNPase KO mice that were about 3 months of age, it is unlikely that background white matter pathology accounted for the differential response to TBI in CNPase KO versus CNPase wild-type mice. Although, we cannot completely rule out this possibility, it is important to consider that subtle changes in background axonal health may indeed be mediated by chronic deficiency of the 2′,3′-cAMP-adenosine pathway. That is to say, not only may acute changes in the pathway determine the response to an acute injury, it is conceivable that chronic deficiency causes underlying pathology that determines the response to acute TBI as well as the risk of neurodegenerative processes such as chronic traumatic encephalopathy.

Brain Cells that Mediate the 2′,3′-cAMP-Adenosine Pathway

The aforementioned findings suggest that: 1) the 2′,3′-cAMP-adenosine pathway exists in vivo in the CNS of mice and humans; 2) brain CNPase converts endogenously generated 2′,3′-cAMP to 2′-AMP; and 3) the 2′,3′-cAMP-adenosine pathway and CNPase are neuroprotective. What CNS cell types mediate the 2′,3′-cAMP-adenosine pathway? Although astrocytes, microglia, oligodendrocytes, and neurons all can metabolize 2′,3′-cAMP to 2′-AMP, oligodendrocytes are preeminent in this regard (Verrier et al., 2013); likely because oligodendrocytes are enriched in CNPase. In oligodendrocytes from CNPase KO mice, the metabolism of 2′,3′-cAMP to 2′-AMP is impaired (Verrier et al., 2013). In contrast, microglia are the most efficient at converting 2′-AMP to adenosine (Verrier et al., 2011). Although brain injury increases extracellular 2′,3′-cAMP levels, the major sources of 2′,3′-cAMP have yet to be identified. Likely a collaboration among CNS cell types is required to constitute a complete brain 2′,3′-cAMP-adenosine pathway.

Summary

Evidence is mounting that 2′,3′-cAMP is an important molecule that is metabolized to adenosine. TBI activates the 2′,3′-cAMP-adenosine pathway, and this mechanism is a determinant of outcome. The challenge going forward is to discover ways to manipulate this pathway to benefit patients after TBI. In this regard, there are a number of feasible strategies for using the knowledge generated by studying the 2′,3′-cAMP-adenosine pathway in the brain to treat TBI. For example, using the structure of 2′,3′-cAMP as a starting point, it is feasible to develop antagonists that block the effects of 2′,3′-cAMP on mPTPs thus preventing 2′,3′-cAMP-induced apoptosis, necrosis, and autophagy (mitophagy). Also, inhibitors of RNases that manufacture 2′,3′-cAMP could be developed to temporarily reduced 2′,3′-cAMP production. Other approaches would be to induce the expression (with pharmacological agents) of transporters that mediate cellular egress of 2′,3′-cAMP, CNPase, or TNAP so as to increase the rate at which 2′,3′-cAMP is exported and converted to adenosine. In addition to treating TBI, polymorphisms in CNPase, the relevant transport proteins, and TNAP may serve to identify individuals susceptible to TBI so that they can be

advised not to participate in contact sports or other activities that increase the risk of TBI or chronic traumatic encephalopathy.

Role of adenosine in posttraumatic seizures and epilepsy

Posttraumatic epilepsy accounts for ~10–20% of all symptomatic epilepsies in the general population (Englander *et al.* 2003). Predicting persons who might develop epilepsy and preventing its development are consequently of utmost importance. Adenosine is a well-known endogenous anticonvulsant and seizure terminator. Since adenosine deficiency, caused by enhanced metabolic clearance through reactive astrocytes and overexpression of the adenosine removing enzyme adenosine kinase (ADK), is a hallmark of epilepsy, therapeutic adenosine augmentation is a rational approach to suppress seizures in the epileptic brain (Boison 2012). Seizure suppression by adenosine is mediated by increased activation of adenosine A_1 receptors, whereas a lack of A_1 receptors is associated with lethal seizures after exposure of the brain to trauma or an excitotoxin (Kochanek *et al.* 2006, Fedele *et al.* 2006). Whereas the receptor-dependent effects of adenosine are well characterized and have been the subject of drug development efforts (Chen *et al.* 2013) new findings demonstrate that adenosine has additional, adenosine receptor independent, unprecedented properties to prevent the development of epilepsy through an epigenetic mechanism.

Epileptogenic brain areas in chronic epilepsy, in the clinic and in rodent models, are characterized by overexpression of ADK in reactive astrocytes (Aronica et al. 2011, Boison 2012) and a hypermethylated state of DNA (Miller-Delaney *et al.* 2015, Williams-Karnesky *et al.* 2013, Kobow *et al.* 2013). As stated in the “methylation hypothesis of epileptogenesis” originally proposed by Kobow and Blumcke in 2011 (Kobow & Blumcke 2011) seizures by themselves may induce epigenetic chromatin modifications, thereby aggravating the epileptogenic condition. Consequently, hypermethylation of DNA was considered a driving force for the progression of epilepsy (Kobow & Blumcke 2012). DNA methylation is an epigenetic modification whereby S-adenosylmethionine (SAM) contributes a methyl group to the formation of 5-methylcytosine bases in the DNA. This leads to the formation of S-adenosylhomocysteine (SAH), which is cleaved by S-adenosylhomocysteine hydrolase (SAHH) into adenosine and homocysteine. Since the thermodynamic equilibrium of the SAHH reaction is on the side of SAH formation and since SAH is a product inhibitor of DNA methyltransferases (DNMTs), DNA methylation can only occur if adenosine is effectively removed by ADK. Consequently, increased expression of ADK, as occurs in epilepsy, drives increased DNA methylation, whereas therapeutic adenosine augmentation blocks DNA methylation and induces a hypomethylated status of DNA (Williams-Karnesky et al. 2013) (Figure 2).

If increased DNA methylation is functionally implicated in epilepsy progression, then therapeutic adenosine augmentation, by reducing the methylation status of DNAshould block epileptogenesis. To test this hypothesis we used a rat model of status epilepticus-induced progressive temporal lobe epilepsy and silk-based brain implants engineered to release a defined dose of adenosine (250 ng adenosine/day/per ventricle) only transiently for 10 days (Williams-Karnesky et al. 2013). Transient drug use followed by a drug-free

'washout' period is a standard strategy to distinguish between acute antiictogenic effects of a drug and longer-lasting antiepileptogenic effects (Silver et al. 1991). Adenosine-releasing polymers, or corresponding silk-only control rods, were implanted into the lateral brain ventricles of rats after the onset of epilepsy. Compared to naïve controls, hippocampal DNMT activity was elevated in the epileptic controls prior to the adenosine delivery, whereas local silk-based adenosine delivery almost completely abrogated any DNMT activity. Consistent with those findings, DNA in the epileptic controls was hypermethylated vs. healthy controls, however the transient delivery of adenosine for only 10 days reverted the DNA methylation status in the epileptic animals back to normal; Importantly, normal methylation was maintained even weeks after cessation of adenosine release from the polymer. To assess epilepsy progression following silk-polymer implantation, the animals were monitored for an additional 3 months after expiration of active adenosine delivery. Sham treated controls and those that received control silk implants progressed in frequency and severity of seizures. Conversely, animals receiving a transient dose of adenosine for 10 days did not progress further in epilepsy development. Three month after treatment the seizure rate stabilized at ~2 per week, whereas controls progressed to at least 8 seizures per week and some controls died from excessive seizure activity. Consistent with those findings a transient dose of adenosine halted mossy fiber sprouting, a characteristic marker for epileptogenesis. Methylated DNA immunoprecipitation arrays and bisulfite sequencing revealed distinct sets of genes whose methylation status increased during epileptogenesis and was corrected by adenosine therapy. Among the targets with reduced DNA methylation during adenosine therapy several interact with DNA, or play a role in gene transcription or translation (*PolD1, Polr1e, Rps6kl1, Snrpn, Znf524, Znf541, Znf710),* making them candidates for mediating adenosine-dependent changes in major homeostatic functions (Williams-Karnesky et al. 2013). Further research into epigenetically regulated antiepileptogenic mechanisms may reveal transcriptional activators as epigenetic meta-regulators such as those linked to the mTOR pathway (Cho 2011). In further support of an antiepileptogenic role of hypermethylated DNA, we kindled rats in the presence of the DNMT inhibitor 5-Aza2dC (5AZA). Under those conditions, kindling epileptogenesis was suppressed, and when re-stimulated after an 11 day drug-free washout period, rats kindled in the presence of 5AZA showed a robust reduction of the seizure phenotype (stage 3 instead of stage 5 seizures) compared to control animals kindled in the absence of 5AZA. Thus changes in DNA methylation patterns are a key determinant of epilepsy progression and adenosine augmentation may reverse DNA hypermethylation and break the cycle of increasing seizure severity (Williams-Karnesky et al. 2013).

Based on our new findings we propose an amended version of our original "ADK hypothesis of epileptogenesis" (Boison 2008) by including a biphasic response of the DNA methylome in response to an epileptogenesis triggering insult: Acute DNA hypomethylation, also seen within 24 h after TBI and associated with microglial activation (Zhang et al. 2007), may contribute to the initiation of epileptogenesis, whereas chronic DNA hypermethylation associated with astroglial activation and adenosine deficiency (Williams-Karnesky et al. 2013) might be required to maintain the epileptic state and to promote disease progression; this biphasic response may be directly related to biphasic expression changes of ADK during the course of epileptogenesis (Boison 2008, Li *et al.* 2008, Williams-Karnesky et al. 2013).

We acknowledge that at this time our hypothesis is largely based on correlative evidence. Although key data, such as long-term epigenetic and antiepileptogenic effects of a short-term adenosine dose and antiepileptogenic activity of a conventional DNMT inhibitor, strongly support a causal relationship between increased DNA methylation and increased epileptogenesis, more research is needed to identify relevant epigenetic targets and mechanisms.

Intriguingly, genetic variants of ADK were associated with the development of posttraumatic epilepsy in humans (Diamond *et al.* 2015). Therefore, changes in adenosine metabolism, such as those triggered by pathological overexpression of ADK or by genetic mutations, emerge as an attractive biomarker for the prediction of epileptogenesis and a therapeutic target to prevent posttraumatic epilepsy.

Rehabilitating Urate — the Maligned and Forgotten Purine

Urate's Generally Bad Reputation

Urate (a.k.a. uric acid) is often referred to as a waste product of purine metabolism (Johnson et al., 2009; Rock et al., 2013). It circulates at high levels in humans and other hominoids due to mutations in the gene encoding the urate-catabolizing enzyme urate oxidase (*UOx*) during primate evolution (Wu et al. 1992, Oda et al. 2002). In our species its circulating concentrations are so high they approach the limits of solubility. Urate is best known clinically for the pain and damage that results when these limits are exceeded and urate crystallizes. When this occurs in joints it results in gout, a form of inflammatory arthritis triggered by urate crystals. Similarly, when urate (or more typically its acid form, uric acid) crystallizes in the urine then it can cause kidney stones.

In addition to its direct, causal contributions to these crystallopathic disorders, higher blood levels of urate have been found to carry an increased risk of heart disease, hypertension, kidney disease and diabetes (Feig et al., 2008; Edwards, 2009; Johnson et al., 2013). Although these adverse associations are partially explained by other co-morbidities of elevated urate such as obesity (Palmer et al., 2013), they have fostered concerns that higher urate may mediate as well as mark major classes of human disease. The advent of multiple new urate-lowering therapies may be adding to the unfavorable image of urate even in the absence of gout or stones (Gaffo & Saag, 2012; Bach et al., 2014; Borghi et al., 2014).

Urate's Protective Potential for Parkinson's and other Neurodegenerative Diseases

Despite these known and theoretical adverse effects of higher urate levels, the evolutionary biology and biochemistry of urate have suggested that its salubrious actions may offset and possibly outweigh is detrimental effects (Álvarez-Lario & Macarrón-Vicente, 2010). Because the urate-elevating inactivation of the urate oxidase enzyme in chimpanzees, gorillas and humans can be attributed to multiple independent mutations in *UOx* during the speciation of primates (Wu et al. 1992, Oda et al. 2002), it is reasonably presumed that urate elevation conveyed a critical survival advantage to our ancestors. The discovery that urate possesses strong antioxidant properties, with a comparable or greater activity than ascorbate at their physiological concentrations in humans (Ames et al., 1981), suggested potential benefits of protection against oxidative stress.

The findings for urate's antioxidant actions converged with evidence that neurodegenerative diseases like Parkinson's disease (PD) result from excessive oxidative damage to neurons (Jenner, 2003). They hypothesized that higher levels of urate may help protect the brain from PD and prompted epidemiologists to investigate the relationship between blood urate levels and the risk of PD. Studies of prospectively followed healthy cohorts have repeatedly demonstrated that higher but 'normal' blood urate among healthy individuals conveys a reduced risk for developing PD later in life in men (Davis et al., 1996; de Lau et al., 2005; Weisskopf et al., 2007; Chen et al., 2009), with findings less consistent in women (O'Reilly et al., 2010; Jain et al., 2011) who have lower serum urate levels on average. For example, men in the top quartile of plasma urate levels had a significantly (55%) lower risk of later developing PD than men in the bottom quartile in a rigorously followed cohort of health professionals (Weisskopf et al., 2007). The decrease in risk was even greater (with an 80% PD risk reduction in highest compared to the lowest quartile; $p<0.01$ for trend) in those with blood collected at least four years before diagnosis, suggesting that the lower urate in those who develop PD precedes symptom onset and is thus unlikely to be a consequence of changes in diet, activity or medical treatment early in the clinical course of PD. Complementary epidemiological findings that a urate-elevating diet (Gao et al., 2008) and gout (Alonso et al., 2007; De Vera et al., 2008) are also associated with a lower risk of PD strengthen the link. Similarly, many cross-sectional studies have reported lower urate levels (Bakshi et al., 2015b) or urate-lowering genotypes (Gonzalez-Aramburu et al., 2013) are more likely in PD than controls. The link appears robust as it has been demonstrated across nationalities and races (Jesus et al., 2012; Sun et al., 2012), and in community (Winquist et al., 2010) as well as academic center-based (Cipriani et al., 2010) cohorts.

This epidemiological association between urate and PD *risk* in healthy populations prompted investigatation of whether urate might also be linked to PD *progression* among those already diagnosed with PD. In multiple prospectively followed PD cohorts, higher blood (or CSF) urate was strongly associated with a slower clinical progression (Schwarzschild et al., 2008; Ascherio et al., 2009; Moccia et al., 2015). A similarly significant, inverse association between serum urate and subsequent rates radiographic progression was measured by serial measurement of dopamine transporter (DAT) binding sites from the striatum using DAT brain scan imaging (Schwarzschild et al., 2008). Similarly, lower urate levels have been linked to the development or more rapid progression of other neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and Huntington's disease (Bakshi et al., 2015b).

Preclinical studies of pharmacological (Gong et al., 2012) or genetic (Chen et al., 2013) strategies to elevated brain urate levels in animal models of PD provided biological evidence of protection by urate against the dopaminergic neuron degeneration characteristic of PD. Interestingly, an indirect antioxidant effect of urate, via its activation of the Nrf2 antioxidant response pathway in astrocytes (Zhang et al., 2014; Bakshi et al., 2015a), may account for much of urate's protection potential in PD.

Together these epidemiological, clinical and neurobiological data identified urate as a promising molecular biomarker and possible mediator of favorable clinical progression of PD. They prompted an initial clinical trial of the urate precursor inosine as a potential urate-

elevating strategy for disease modification in PD. The Safety of Urate Elevation in PD (SURE-PD) study (Parkinson Study Group, 2014) assessed three primary outcomes for safety, tolerability and urate-elevating ability of oral inosine in early PD. Despite known risks of gout and uric acid kidney stones, inosine demonstrated overall good safety and tolerability and significant elevation of both CSF and serum urate, supporting further clinical development of inosine for PD.

Prospect for protection against acute neuronal injury: from stroke to TBI

Urate elevation has emerged as a neuroprotective strategy in acute neuronal injury, as well as in chronic neuronal degeneration. As with targeting urate in PD, that in stroke has undergone rapid translation to phase 2/3 clinical trials based on a combination of laboratory and clinical data. Building on its well-established antioxidant properties (Ames et al., 1981), stroke biologists administered urate just before or during transient unilateral cerebral ischemia and found reduced striatal or cortical damage as well as preserved neurological function (Yu et al., 1998; Romanos et al., 2007). Clinical epidemiology studies found that people with higher serum urate levels when presenting with an ischemic stroke had better clinical outcomes upon hospital discharge ~two weeks later (Chamorro et al., 2002).

Based on these human and animal data Charmorro and colleagues conducted a phase 2b/3 trial of intravenous urate in acute ischemic stroke (Charmorro et al. 2014). Although it did not demonstrate a statistically significant overall benefit of urate, it was sufficiently suggestive to warrant for fuller phase 3 clinical testing. Interestingly, post hoc analysis stratifying by gender indicated significantly better anatomical and clinical outcomes after urate treatment in women, who at baseline have substantially lower serum urate levels than do men (Llull et al., 2015). Interestingly, this sex difference appeared to be mirrored for PD in the SURE-PD study (Parkinson Study Group SURE-PD Investigators, 2014; Schwarzschild et al. 2014) and warrants further attention in future studies.

TBI like stroke entails a sudden profound metabolic (as well as mechanical) injury of neurons, and thus may trigger a common cascades of excitotoxic, inflammatory and oxidative factors that contribute to functional disability. Thus the rapid clinical translation and potential of urate as a therapeutic target in stroke as well as neurodegenerative disease warrants consideration of its ‘lateral translation’ to TBI. Although urate itself has not been systematically investigated in TBI models, its precursor inosine (which rapidly metabolized to urate, and is currently in clinical development for PD) has been found to improve outcomes in rodent models of TBI (Dachir et al., 2014) and spinal cord injury (SCI) (Kim et al., 2013). Evidence that astrocytic Nrf2 pathway activation confers protection against neuronal cell death in TBI/SCI (Mao et al., 2012; Miller et al., 2014) and neurodegeneration, and that urate confers protection in PD models via this pathway (Zhang et al., 2014; Bakshi et al., 2015a), lends support to the rationale for investigating urate in TBI and SCI.

Alternatively, inosine may have direct beneficial effects independent of its metabolism to urate (Cipriani et al., 2014) as it can also protect via extracellular engagement of adenosine receptors (Gomez & Sitkovsky, 2003; Shen et al., 2005) and intracellular activation of the Mst3b signaling cascade (Kim et al., 2013). However, the metabolism of inosine to urate by way of peripheral xanthine oxidase could in theory generate deleterious oxidative stress via

its hydrogen peroxide byproduct (Kelley et al., 2009), potentially offsetting some of its putative benefits. Thus in traumatic CNS injury, as in acute stroke, the intravenous administration of urate itself may be the most effective as well as expedient strategy to take advantage of its benefits. Urate elevation represents a readily testable candidate neuroprotective strategy across disorders of neurodegeneration and acute neuronal injury.

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Abbreviations

2′,3′-cNMPs	nucleoside 2′,3′-cyclic monophosphates
ADK	adenosine kinase
CNPase	2′,3′-cyclic nucleotide 3′phosphodiesterase
DNMTs	DNA methyltransferases
KO	knockout
LC-MS/MS	liquid chromatography-tandem mass spectrometry
mPTPs	mitochondrial permeability transition pores
m/z	mass-to-charge ratio
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SRM	selected reaction monitoring
TNAP	tissue non-specific alkaline phosphatase
TBI	traumatic brain injury

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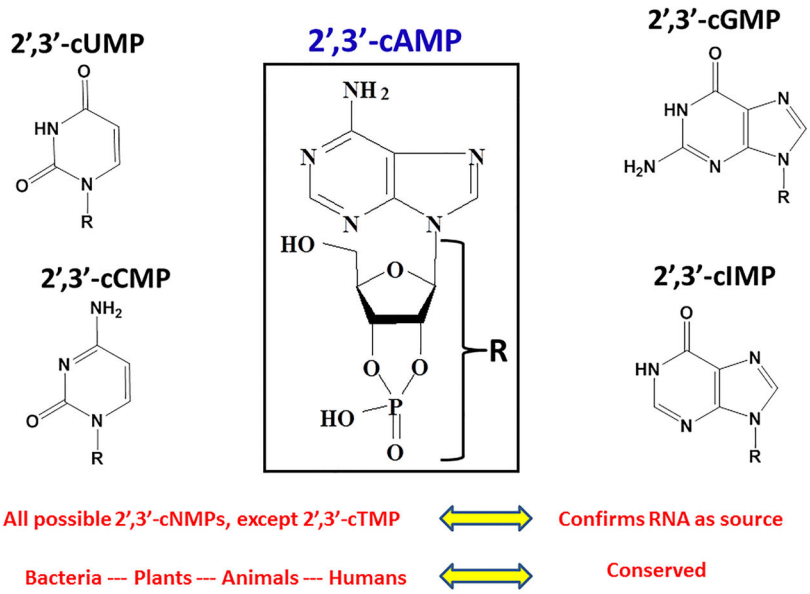

Figure 1. Chemical Structures of Nucleoside 2′, 3′-Cyclic Monophosphates (2′,3′-cNMPs)

2′, 3′-cAMP (center) was discovered to exist in biological systems in 2009. Subsequently, a number of other 2′, 3′-cNMPs were discovered including 2′, 3′-cUPM, 2′, 3′-cCMP, 2′, 3′-cGMP, 2′, 3′-cIMP. The absence of 2′, 3′-cTMP is consistent with the concept that all 2′, 3′-cNMPs derive from RNA, rather than DNA, degradation. That 2′, 3′-cNMPs exist in bacteria, plants, animals, and humans indicate that these molecules are ancient and conserved.

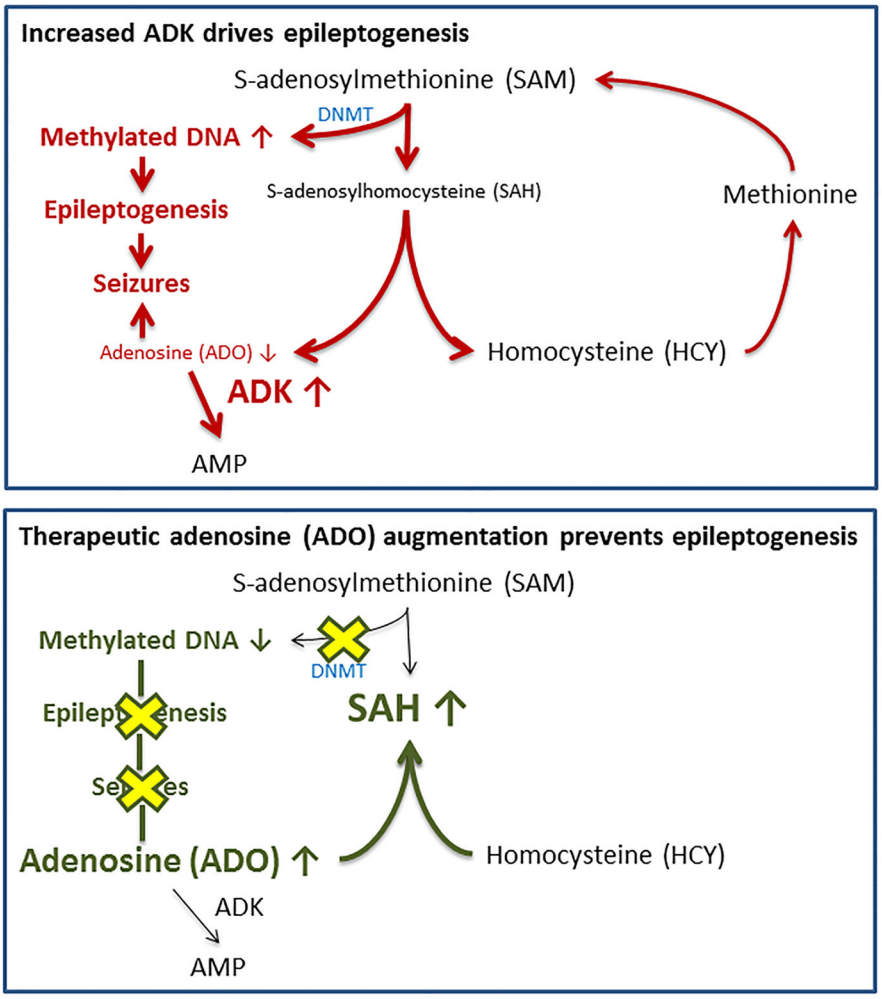

Figure 2. The epigenetics of epileptogenesis

Increased ADK expression (top) drives increased DNA methylation as a prerequisite for progressive epileptogenesis and seizure generation. Conversely, adenosine therapy (bottom) restores normal DNA methylation preventing epileptogenesis. ADO: adenosine; ADK: adenosine kinase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; DNMT: DNA-methyltransferase.