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Adenosine and Autism - Recent Research and a New Perspective

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1. Introduction

Autism Spectrum Disorders (ASD) are associated with atypical social, behavioral and physiological characteristics. Here we outline an emerging connection among the increased incidence of epilepsy, disrupted sleep and perseverative behaviors exhibited and sought by persons with autism. Specifically, we propose that persons with autism can benefit from increased levels of adenosine, a powerful inhibitory neuromodulator and the core molecule of adenosine triphosphate (ATP). We review the literature and present recent data obtained via a customized questionnaire administered to parents of children with a confirmed autism diagnosis. This customized questionnaire demonstrates that symptoms of autism are reduced subsequent to stimuli predicted to increase adenosine. In addition, we present evidence from the literature and pilot data from a retrospective study of children with epilepsy or epilepsy and autistic behavior who were treated with a ketogenic diet, a long-established anticonvulsant therapy that recently has been shown to suppress seizures via the adenosine A₁ receptor (A₁R) subtype. Our discussion focuses on the actions of adenosine in the central nervous system, with multiple implications for ASD, and the potential for developing new evidence-based therapies. Taken together, published peer-reviewed research and recent preliminary research suggest that adenosine could help resolve multiple physiological and behavioral symptoms of ASD.

2. Adenosine - role in physiology and behavior

Adenosine is a purine molecule present throughout the body. In the central nervous system it is increasingly appreciated as a homeostatic bioenergetic network regulator (Boison et al., 2011). Fundamental to its homeostatic influences on neuronal networks are adenosine's integral roles in metabolism and cell signaling, and its regulatory influence as an obligate

by-product of transmethylation reactions. In terms of metabolism, adenosine is the core of arguably the most important purine, adenosine triphosphate (ATP), the pre-eminent cell energy molecule, and also of related adenine nucleotides adenosine diphosphate (ADP) and adenosine monophosphate (AMP); these purines determine overall cell energy charge (Dunwiddie & Masino, 2001). In terms of signaling, adenosine binds to a family of membrane-bound G protein-coupled cell-surface receptors (A_1 , A_{2A} , A_{2B} , A_3) to influence membrane potential, synaptic transmission and second messenger signaling (Ralevic & Burnstock, 1998; Dunwiddie & Masino, 2001). Its ubiquitous presence in every cell, widespread receptor distribution, and tonic levels throughout the extracellular space - sufficient to activate a subset of these receptors - make adenosine a key player in neuron-glia interactions (Fields & Burnstock, 2006), and able to exert a dynamic and broad regulatory influence.

Adenosine A_1 receptors (A_1 Rs) and adenosine A_{2A} receptors (A_{2A} Rs) have the highest affinity for adenosine and the most predominant ongoing functional effects in the central nervous system (Fredholm et al., 2005). Generally, A_1 Rs serve to decrease synaptic transmission and decrease cAMP, whereas A_{2A} Rs facilitate transmission and increase cAMP (Schulte & Fredholm, 2003). A_1 Rs are expressed widely throughout the brain (Goodman et al., 1982), and A_{2A} Rs are highly concentrated in the basal ganglia and olfactory tubercle (Rosin et al., 2003). Thus, many effects of adenosine are location- and subtype-specific. These two receptor subtypes are both targeted by caffeine, a non-selective A_1 R/ A_2 R antagonist and the most widely used psychoactive drug world-wide (Fredholm et al., 1999).

A_1 Rs exert a tonic inhibition on excitatory transmission via pre- and postsynaptic actions (Ralevic & Burnstock, 1998; Dunwiddie & Masino, 2001). The inhibitory effects of A_1 Rs are well-known to prevent and stop seizures (Dunwiddie & Worth, 1982), reduce anxiety (Florio et al., 1998; Johansson et al., 2001), and promote neuronal survival during times of severe cell stress (Fredholm, 1997). Blocking the ongoing influence of A_1 Rs can precipitate or prolong seizures and reduce neuronal survival, and enhancing their actions offers seizure protection (Dunwiddie, 1999) and neuroprotection (Fredholm, 1997). Events such as strokes, seizures, or other metabolic dysfunctions are associated with a net dephosphorylation of ATP and a large increase in extracellular adenosine (Latini & Pedata, 2001) and its inhibitory influence via A_1 Rs can reduce excitotoxicity and short-circuit further metabolic demand. Thus adenosine was initially regarded as a “retaliatory metabolite,” as it acts to maintain metabolic and network homeostasis during cell stress (Newby, 1984).

More recently we have recognized that the homeostatic impact of adenosine is regulated dynamically under non-pathological conditions, and regulated largely by astrocytes (Halassa et al., 2009). ATP and adenosine are released directly from astrocytes (Pascual et al., 2005), and ATP is dephosphorylated rapidly to adenosine in the extracellular space (Dunwiddie et al., 1997; Cunha et al., 1998); the level of extracellular adenosine is regulated primarily by intracellular astrocyte-based adenosine kinase (Gouder et al., 2004; Boison, 2006).

3. Purines and autism

Adenosine affects general arousal, and increased adenosine at A_1 Rs reduces anxiety; conversely, decreased A_1 R activation can increase anxiety (Florio et al., 1998; Johansson et al., 2001). Both A_1 Rs and A_{2A} Rs may be involved in adenosine’s ability to promote sleep

(Rainnie et al., 1994; Porkka-Heiskanen, 1999; Huang et al., 2005). Notably, increased seizure propensity, disturbed sleep and anxiety are all found commonly in persons with ASD (Malow, 2004; Polimeni et al., 2005; Oswald & Sonenklar, 2007), suggesting that an increased influence of adenosine could reduce all three co-morbidities.

In dopamine-containing areas, A_{2A}Rs are expressed at higher levels than in other regions, and A_{2A}Rs have an opposing relationship with dopamine D₂ receptors. Increased adenosine receptor activation reduces D₂ receptor activation (Fuxe et al., 2007), and the two receptors form a functional heteromer (Ferre et al., 2007). This complex also seems to be under the control of A₁Rs, and adenosine agonists have been shown to reduce repetitive behaviors (Poleszak & Malec, 2000; Tanimura et al., 2010). Therefore, in addition to reducing general excitability through increased A₁R activation, adenosine can influence dopamine-related behaviors through multiple adenosine receptor subtypes and reduce behavioral pathologies - such as repetitive behaviors, which are particularly relevant for ASDs - through these interactions. At this time the research evidence linking adenosine to repetitive behaviors is growing, but not as strong as the link between adenosine and epilepsy. It is important to note that dopamine is involved in many aspects of behavior, and increasing adenosine in dopamine-containing areas could impact motivation (Salamone & Correa, 2009); the full complement of positive and negative effects and their interactions in ASD would be difficult to predict.

Abnormalities in purine metabolism have been observed in autism (Nyhan et al., 1969; Page & Coleman, 2000; Bottini et al., 2001; Marie et al., 2002; Page & Moseley, 2002), but have not been linked directly to core symptoms of the disorder or receptor-mediated effects on neuronal function, and purinergic strategies have not been exploited for practical clinical benefits. Whereas some symptoms and behaviors are likely due to aberrant neuroanatomical development and other genetically-determined substrates, increasing the inhibitory influence of adenosine could help significantly with multiple behavioral and physiological sequelae.

3.1 Symptoms of autism and regulation of adenosine

Recent work suggests adenosine dysregulation may be associated with psychiatric disorders such as schizophrenia (Boison et al., 2011). In general it is not surprising that a brain-wide metabolic/neuronal regulator such as adenosine could influence multiple co-morbidities associated with complex psychiatric disorders. The co-existence of neurological/psychiatric disorders is also becoming more appreciated, and observed in adults as well as children and adolescents (Jones et al., 2008). As just a few examples, there are well-documented comorbidities between epilepsy and depression/anxiety (Kanner, 2004, 2008; LaFrance et al., 2008; Ekinici et al., 2009), schizophrenia (Hyde & Weinberger, 1997), and, more broadly, sleep disorders and psychiatric disorders (Reeves et al., 2010), including autism (Spence & Schneider, 2009; Jeste, 2011). As noted above, based on behavioral and physiological characteristics of ASD, including impaired sleep (Malow, 2004; Polimeni et al., 2005), increased seizures (Malow, 2004; Canitano, 2007; Spence & Schneider, 2009), anxiety (Chalfant et al., 2007), and perseverative behaviors (Ridley, 1994; Liss et al., 2006), an insufficient influence of adenosine might underlie some symptoms. These parallels are summarized in **Figure 1**. Alternatively, even if levels are normal, increased adenosine may still be beneficial, offering short term improvements and potentially facilitating more long-term changes.

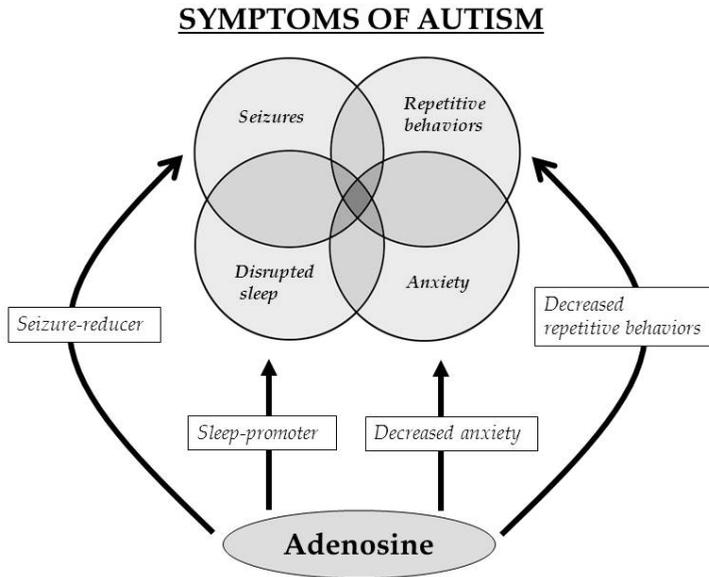


Fig. 1. Key actions of adenosine in the central nervous system can target multiple symptoms of autism. Increased adenosine at the cellular level alters physiology and behavior (promoting sleep, and decreasing seizures, anxiety and repetitive behaviors). This cohort of outcomes can target overlapping co-morbidities and symptoms of autism (sleep disruption and increased seizure propensity, repetitive behaviors and anxiety).

Research has shown that diverse stimuli such as mechanical pressure or sudden physical impact (Franke et al., 2006), seizures (Whitcomb et al., 1990), intense exercise (Dworak et al., 2007), increased temperature (Masino & Dunwiddie, 1999), decreased pH (Dulla et al., 2005; Dulla et al., 2009) and reduced glucose (Kawamura et al., 2010) can all increase brain adenosine directly (or indirectly via ATP dephosphorylation: Dunwiddie et al., 1997; Cunha, 2001; Zhang et al., 2003; Pascual et al., 2005) and thus modulate synaptic transmission within minutes. Most of these effects appear to reverse rapidly; others are relatively long-lasting (up to hours) or related to chronic changes in physiology. For example, acupuncture has been shown to increase local adenosine, perhaps related to the mechanical stimulation (Franke et al., 2006; Goldman et al., 2010). Other recent work suggests that a ketogenic diet, an effective treatment for pediatric epilepsy, might act via A_1 Rs (Masino & Geiger, 2008; Kawamura et al., 2010; Masino et al., 2011b). Unfortunately, it is impractical to measure brain adenosine in humans (necessarily an invasive procedure), and adenosine levels outside the central nervous system (e.g., in plasma or urine) are not informative.

In compiling a list of stimuli known to increase adenosine it becomes apparent that many behaviors exhibited by persons with ASDs or shown to improve symptoms could be related to one of these physiological changes – e.g. mechanical pressure, including rocking or spinning and Grandin’s “hug machine” (Edelson et al., 1999; Escalona et al., 2001), fever

(Curran et al., 2007), etc. Therefore, some behaviors expressed by persons with autism could be attempts at metabolic and neuronal homeostasis via increased adenosine. Engaging in adenosine-increasing activities could thus lead immediately to decreased symptoms of autism.

Ongoing work testing the relationship between adenosine and autism is proceeding in animal models. However, because no animal model represents the human condition entirely (which itself is a broad spectrum of complex behaviors), we felt it was important to test initially for evidence for a link between adenosine and ASD in humans. As noted, direct tests in humans to assay or manipulate adenosine in a meaningful way would involve highly invasive measurements or manipulations, or, potentially, drugs which are not approved in children. Initial work, described below, and coupled with evidence in animals, will be building blocks for pursuing this further.

3.2 Predictions and outcomes - adenosine and autism spectrum disorders symptomatology

Based on animal research suggesting non-pathological stimuli that could increase adenosine, we explored the possibility of a beneficial relationship between autism and adenosine via a customized behavioral questionnaire focused on behaviors typical of ASD (Masino et al., 2011a). Subjects were recruited with the assistance of the Interactive Autism Network (IAN) Research Database at the Kennedy Krieger Institute and Johns Hopkins Medicine - Baltimore, sponsored by the Autism Speaks Foundation. Participants had received a formal autism diagnosis according to DSM-IV criteria made by a qualified professional (e.g., psychologist, psychiatrist) and were above a threshold score of 12 on the Social Communication Questionnaire (SCQ) parent-report measure (Rutter et al., 2003). Autism diagnosis was confirmed by IAN staff; fully 98% of participants ascertained as on the spectrum according to standard IAN phenotyping procedures were ASD-positive according to clinicians' best estimate (Lee et al., 2010).

Along with basic information such as age, diagnosis and verbal level, we queried behavioral changes following activities identified as expected to increase, decrease or have no effect on adenosine. Our final data set included responses from 155 parents of children with a confirmed ASD diagnosis. Parents were naive to all study hypotheses.

Results from this study revealed a significant relationship between engaging in stimuli pre-assigned as expected to increase adenosine and parental report of decreased severity of ASD symptoms (Masino et al., 2011a). We found a trend for persons diagnosed with Asperger's to show a stronger behavioral effect of adenosine-increasing activities, a finding which deserves additional exploration. In general, the significant effects of adenosine-increasing activities were unrelated to participant age, gender, or verbal level. These results suggest that, if validated, increasing adenosine could have broad applicability to ASD.

Interestingly, we found a significant relationship between reported caffeine intake and reduced symptoms of autism. Because the dose of caffeine was not controlled, and because the effect of caffeine on the nervous system is determined by both the dose and the frequency, it is not possible to make strong interpretations based on this finding. However, akin to the trend for more beneficial effects on persons with Asperger's, the relationship between caffeine and autism deserves further exploration; along these lines, recently others have also posited a relationship between caffeine and autism (Ghanizadeh, 2010). Because of its well-established safety profile and limited side effects, vast epidemiological database,

and known actions in the nervous system, caffeine is being considered as a low-cost adjuvant or for its own therapeutic potential in multiple neurological disorders (Arendash & Cao, 2010; Cunha & Agostino, 2010; Prediger, 2010; Chen & Chern, 2011).

4. Metabolic regulation of adenosine - ketogenic diet

The ketogenic diet is a restrictive diet high in fat and low in carbohydrate that significantly reduces the frequency of seizures. Epilepsy is characterized as a disorder of unprovoked spontaneous seizures, which affects approximately 1% to 3% of population (Shneker & Fountain, 2003); rates of epilepsy in ASD are substantially higher – with estimates ranging from 5% up to 40% (Canitano, 2007). The most common treatment for epilepsy is antiepileptic drug therapy; the ketogenic diet is a metabolic treatment whereby limiting carbohydrates restricts available glucose and initiates ketone-based (ketogenic) metabolism. This dietary approach forces the use of ketones for energy, a metabolic shift which also occurs during fasting (Aoki, 1981; Hartman & Vining, 2007). Although the exact neural mechanisms of the ketogenic diet are currently unknown (although under active investigation by a number of laboratories), its efficacy in reduction of seizures has been described extensively. In particular, studies have demonstrated that the ketogenic diet can decrease seizure frequency by more than 50% in one-third to one-half of children, and eliminate seizures in 10-15% of children in 6-12 months (Vining et al., 1998; Keene, 2006).

Despite its high success rate, the ketogenic diet is typically offered very infrequently. Notable exceptions include proper diagnosis of a very few metabolic conditions where it is becoming known as the preferred therapy, and others where it is gaining ground as a potential first-line therapy (e.g. GLUT-1 deficiency syndrome). In parallel, it is specifically contraindicated for a small set of metabolic conditions (Kossoff et al., 2009). When a ketogenic diet is offered, it is typically a “last resort” therapy - after attempting at least two (and often more) antiepileptic drugs. The major problems associated with the diet are the inconvenience - preparation of special meals - and negative side effects in a subset of patients including vomiting, weight loss, increase in serum lipids, acidosis, gastric problems or renal stones (Groesbeck et al., 2006; Keene, 2006; Freeman et al., 2007). Although most of these are rare or short-term, the diet is usually only prescribed to patients with multiple seizures per day, unacceptable drug-related side effects, or seizures that are not sufficiently responsive to anti-epileptic drugs.

Despite a clear link between energy metabolism and brain activity established by historical observations and clinical and basic research, the first randomized, prospective, clinically controlled study using a ketogenic diet has been published only recently (Neal et al., 2008). At last, this much-needed clinical research report proved (at a higher scientific standard – prospective, randomized) what had been reported in many other research studies and observed clinically for many decades: the ketogenic diet is an effective treatment for pediatric epilepsy, and it can be successful even in cases of medically-intractable epilepsy (Kinsman et al., 1992; Vining et al., 1998). In this study, the number of seizures observed in children placed on the diet decreased by 38% in three months. In contrast, the number of seizures observed in a matched group of children who continued their current treatment increased by 37% in 3 months. In some children who started the ketogenic diet seizures stopped entirely, whereas this was never observed in the control group. This report and an accompanying commentary

(Wiznitzer, 2008) note that the critical mechanisms underlying the success of the diet remain unknown, and this knowledge gap has thus far stymied efforts to develop treatments based on the metabolic changes precipitated by the ketogenic diet.

Recent research suggests that the ketogenic diet may act by increasing the influence of adenosine acting at A₁Rs (Masino & Geiger, 2008; Kawamura et al., 2010; Masino et al., 2011b). Other research suggests that the ketogenic diet acts by reducing reactive oxygen species and oxidative stress (Sullivan et al., 2004; Davis et al., 2008), direct actions of ketone bodies on ion channels (Ma et al., 2007) or transporters (Juge et al., 2010), or increased GABA (Yudkoff et al., 2007). Any of these mechanisms could be highly beneficial in ASD, which is associated with disinhibition (Hussman, 2001; Fatemi et al., 2009a,b) and oxidative stress (James et al., 2004; McGinnis, 2005; James et al., 2006). While the link between the efficacy of the ketogenic diet and increased adenosine is still emerging, there are at least 3 strong reasons why – based on well-established evidence – a ketogenic diet could be helpful in autism:

1. The ketogenic diet treats epilepsy. A high percentage of children with autism have epilepsy. Importantly, these numbers might be higher with the routine ability to measure electrographic (non-behavioral) seizures and altered subcortical activity.
2. The ketogenic diet reduces reactive oxygen species and oxidative stress. Autism is associated with increased oxidative stress.
3. Most importantly, an established, medically-supervised diet-based strategy is cost-effective and available to translate immediately into practical benefits.

It is important to note that both adenosine and a ketogenic diet can stop drug-resistant epilepsy, suggesting mechanisms other than those targeted by existing drugs. Both mobilize a broad homeostatic bioenergetic regulation (Boison et al., 2011) and thus may be best suited to address a broad spectrum of symptoms with limited side effects.

Since we published a hypothesis that a key therapeutic aspect of a ketogenic diet is to increase adenosine acting via A₁Rs (Masino & Geiger, 2008) this hypothesis has been tested in three ways: (1) we explored an *in vitro* model of ketogenic diet electrophysiologically in individual neurons, (2) we administered a ketogenic diet *in vivo* to three types of transgenic mice, all with spontaneous electrographic seizures due to decreased A₁R signaling, and (3) we tested the ability of a ketogenic diet to reduce pain and inflammation, a prediction of this hypothesis. Respectively, we found that (1) metabolic conditions designed to mimic a ketogenic diet in brain slices mobilized a novel autocrine regulation of neuronal activity via adenosine acting at A₁Rs (Kawamura et al., 2010), (2) a ketogenic diet reduced electrographic seizures in mice with intact A₁Rs, but did not in mice lacking A₁Rs (Masino et al., 2011b), and (3) a ketogenic diet reduced pain and inflammation, as predicted (Ruskin et al., 2009).

While data continue to emerge, and multiple mechanisms could play a role, together these studies suggest that a ketogenic diet reduces seizures by increasing adenosine-mediated inhibition and offer insight into therapies for other clinical conditions where adenosine is known or hypothesized to offer clinical benefits. Using a retrospective analysis, we explored the effects of the ketogenic diet on behavior and temperament of children with epilepsy with or without hallmark symptoms of autism.

4.1 Ketogenic diet - retrospective analysis in children

To evaluate the effects of a ketogenic diet on symptoms of autism, alongside changes in seizure frequency, behavior and mood, we identified children who began dietary therapy

because of their epilepsy diagnosis. Based on decades of research and consistent clinical findings, we expected diet therapy to reduce seizures significantly in the majority of these children. At the same time, because of the high incidence of epilepsy in children with autism, a subset of these children with pediatric epilepsy exhibited co-morbid symptoms of ASD. Thus, we were able to assess changes in behavior in children with or without symptoms of ASD in parallel with other physiological and neurological outcomes of diet therapy. In this way we examined the outcomes based on treatment with a standard diet protocol, available currently to any clinician and in use at many centers nationally and globally.

Using a retrospective analysis we examined clinical and behavioral features of children with epilepsy between 18-months and 12-years of age who initiated the ketogenic diet at Connecticut Children’s Medical Center between January 2004 and January 2009. All aspects of the study were approved by the Institutional Review Board. Potential participants in this retrospective study were identified using the ketogenic diet database at the Connecticut Children’s Medical Center. Because some measures used in this study are only available currently in English, children with non-English speaking parents were excluded.

Parents of selected children were invited to participate in the study with a phone call. After obtaining a verbal consent, questionnaires were mailed to the parents. The participating children, whose parents filled out the questionnaires, were divided into two groups: subjects currently on the ketogenic diet (KD group) and subjects who discontinued the ketogenic diet (non-KD group). Children in the KD group had stayed on the diet for at least 6 months, and children in the non-KD group stopped the diet at least 2 months before the testing. Demographic and clinical data were collected from chart review.

Statistical analyses (Student *t*-test, Pearson test, and chi-square test) were performed to compare the differences between the KD group and the non-KD group. To dissociate differences related to either autism or cognitive impairment, subjects who were assessed as autistic based on their Child Autism Rating Scale score (filled out by parent) and subjects who were evaluated as cognitively impaired by a neurologist were analyzed separately.

In this initial retrospective analysis there was no difference in the number of boys versus girls (10 each) and no substantial difference in the number of children who were still on the ketogenic diet versus those who continued the diet (11 and 9, respectively) or the average age of the KD versus non-KD group (6.4 ± 0.8 and 8.3 ± 1.1 , respectively, $P=0.18$). We expected to find a significant difference in seizure frequency, and although there was a trend in the average number of seizures per week in each group (KD group, 16 ± 8 seizures per week; non-KD group, 99 ± 52 seizures per week) there was high variability (and thus the lack of significance) due primarily to two clinically-expected outcomes: (1) some children in the non-KD group became seizure-free after being on the diet, and went off the diet with a current score of zero seizures per week; (2) children who did not comply and stay on the diet, or for whom the diet didn’t work, had more than 100 seizures per week. Both of these types of cases were included in the non-KD group. These data are summarized in **Table 1**.

	KD Group	Non-KD group	P value
Number	11 (6 females)	9 (4 females)	1.0
Age	6.4 ± 0.8	8.3 ± 1.1	0.18
Number of Seizures/week	16 ± 8	99 ± 52	0.10

Table 1. Summary statistics of children who were on the ketogenic diet (KD-group) or children who started but were not currently on the ketogenic diet (non-KD group) at Connecticut Children’s Medical Center from January 2004-January 2009. See text for additional details.

We also considered whether the children were autistic only, cognitively impaired only, or autistic and cognitively impaired. There was no difference in the number of children who were autistic and cognitively impaired in each group (KD group: 5/11; non-KD group: 4/9); the numbers in other categories were too small for statistical comparison.

Statistical analysis of data from the Child Behavior Checklist showed that subjects who were still on the ketogenic diet (KD group, n=11) had significantly fewer behavioral problems than subjects who were no longer on the ketogenic diet at the time of testing (non-KD group, n=9; χ^2 (1), $P < 0.05$). When analyzing separately cognitively impaired children and autistic-like children, we found that in both of these subgroups, the subjects who were currently on the ketogenic diet (KD group) had better behavioral traits than subjects who had already stopped ketogenic diet treatment (non-KD group); $P < 0.001$ and $P < 0.01$, respectively; (Svedova et al., 2010). Effects of the ketogenic diet on behavior of children will continue in ongoing retrospective and prospective studies.

Although these data are preliminary, they add to positive outcomes discussed below in previous prospective studies (Pulsifer et al., 2001; Evangelidou et al., 2003) and anecdotal reports. Along with evidence that a ketogenic diet increases A₁R activation, evidence that A₁R_s decrease anxiety, and evidence that either a ketogenic diet or A₁R activation decreases seizures, a proven metabolic approach such as a ketogenic diet should be considered for ASDs.

4.2 Ketogenic diet, adenosine and autism

Previous studies have shown that autism is caused by one or more gene defects, which can affect brain development, cell signaling, cell transport or structure (Santangelo & Tsatsanis, 2005; Zhao et al., 2007). It has been suggested that some of these gene defects may result in metabolic dysfunctions that can eventually cause behavioral abnormalities associated with autism, and several metabolic disorders present symptoms similar to autism, underscoring a link between autism and metabolism (Zecavati & Spence, 2009).

Based on these findings, there have been numerous attempts to treat autism with different types of restrictive diets, and dietary therapies remain popular in the autism community. Here we have focused our discussion primarily on the ketogenic diet, based on (1) the proven clinical success of the ketogenic diet for epilepsy, and preliminary positive results with autism, (2) *in vivo* and *in vitro* research evidence linking the ketogenic diet to adenosine, and (3) a recent retrospective study of the classic ketogenic diet in children with epilepsy and autism, discussed above.

In general, gastrointestinal problems and metabolic disturbances appear to be closely related to ASD, and there are several known metabolic impairments that are associated with autistic symptoms. These include phenylketonuria, adenylysuccinate lyase deficiency, histidinemia, or hyperuricosuric autism (Page & Coleman, 2000). The prevalence of food allergies (Gurney et al., 2006) and the incidence of at least 25-30% of children with autism suffering from chronic diarrhea, constipation or food issues (Page & Coleman, 2000; Ibrahim et al., 2009), suggest that some types of ASD may be treated with dietary restrictions. In the past few decades, several diets have been described in terms of their effects on symptoms of autism, including gluten-free, casein-free (GFCF) diet, specific carbohydrate diet, body ecology diet, or ketogenic diet (Srinivasan, 2009). A survey by Witwer and Lecavalier (2005) estimated that 15.5% of autistic children or adolescents were on modified diets for treatment of autism. It should be noted that that in several studies,

including a double-blind placebo-controlled intervention, the GFCF diet was found to have no benefits for ASD (Elder et al., 2006; Millward et al., 2008), and has been associated with protein malnutrition (Arnold et al., 2003). Thus, the specificity of GFCF approaches to symptoms of ASD should be viewed with caution.

Direct evidence supporting a beneficial relationship between a ketogenic diet and autism comes from one published report – a prospective study – which tested the effects of a ketogenic diet in children with autism (Evangelidou et al., 2003). However, there are multiple unpublished anecdotal reports (Beth Zupiec-Kania, personal communication), including reports of dramatic success with a ketogenic diet in autism. In the prospective study, Evangelidou et al. applied a ketogenic diet protocol to children with autism and found that 60% of diet-compliant children aged 4-10 who participated for 6 months showed an improvement in their symptoms. Significant improvement (> 12 points on the Childhood Autism Rating Scale) was recorded in 2/18 patients, average improvement (> 8-12 points) in 8/18 and minor improvement (> 2-8 points) recorded in 8/18. Notably, the diet protocol included 4 weeks on and 2 weeks off the ketogenic diet – a different regimen than administered typically for treatment of epilepsy. Still, these results are promising and provide preliminary data supporting a link between a ketogenic diet and autism, and potentially among autism, adenosine and a ketogenic diet. The biggest improvements were quantified in those patients who showed only mild autistic behavior, similar to our findings for a trend for the biggest benefit for adenosine-increasing activities in individuals with Asperger's (Masino et al., 2011a).

In addition to a reduction of their seizures, children on the ketogenic diet can enjoy other benefits – including reduced drug intake and general improvements in cognition, alertness, development, attention and social life (Kinsman et al., 1992; Sirven et al., 1999; Pulsifer et al., 2001; Hallböök et al., 2007; Hallböök et al., 2007), and – as we found in our retrospective study – fewer behavioral problems (Svedova et al., 2010). However, the number of studies researching the effects of the ketogenic diet on behavior and cognition of children remains surprisingly limited. A single prospective study conducted by Pulsifer et al. (2001) focused on the effects of the ketogenic diet on behavior and development of children with intractable epilepsy. The children were evaluated at the diet initiation and after one year using three parental report measures: Developmental Profile-2nd edition, Child Behavior Checklist, and Parenting Stress Index-Short Form. The results demonstrated an overall significant improvement in developmental functioning. In addition, the Child Behavior Checklist showed a significant improvement in attention and social problems. Interestingly, the authors commented that the developmental and behavioral improvements were not statistically related to better seizure control or to reduced intake of antiepileptic drugs.

A recent study examining the efficacy of writing parental goal letters prior to initiating the ketogenic diet showed that improvements in cognition and alertness were stated as the third most common goal (Farasat et al., 2006). Interestingly, the study suggested that cognition and alertness improvements were more likely to lead to long-term adherence to the diet – more so than improved seizure control or a decreased intake of antiepileptic drugs. From this observation, it is evident that potential improvements in cognition and behavior are crucial for many parents. Unfortunately, as noted above, and despite many decades of clinical use, there is a limited literature exploring the effects of the ketogenic diet on behavior and cognition of children. However, a wealth of resources about the ketogenic diet can be found on-line at the Charlie Foundation (www.charliefoundation.org).

5. Conclusions

Here we propose adenosine as a beneficial neuromodulator for ASD symptoms based on published research, behavioral and physiological symptoms of autism, and recent indirect evidence obtained in children (parent report via a customized adenosine questionnaire and retrospective analysis of children with epilepsy and with or without hallmark symptoms of autism). Obtaining direct evidence (measuring adenosine accurately in humans) is too invasive, and drugs that may increase adenosine have not been specifically established as such, would need to be used "off-label," and are not approved for use in children. Gathering correlated evidence regarding changes in adenosine in animal models can proceed in parallel with the type of "proof-of-principle" work gathered in humans and presented herein.

Beyond clinical endpoints indicating that increased adenosine would be beneficial in autism - e.g., reduced seizures - adenosine would be predicted to be increased by the atypical behaviors sought by persons with autism and offer short term benefits. Similarly, a ketogenic diet would be predicted to reduce symptoms of autism. Our customized questionnaire demonstrated highly significant changes - ASD symptoms were reduced after stimuli predicted to increase adenosine. These initial results should be followed up with more detailed controlled and prospective studies, and include stimuli that have been verified to increase adenosine. In this respect, two conditions that might increase adenosine (in different ways) include acupuncture and a ketogenic diet. Published research (Evangelidou et al., 2003) and our retrospective study, described in more detail here, suggest that a ketogenic diet does indeed offer multiple benefits for children with ASD.

Ketogenic diet is an existing treatment option at many institutions treating pediatric epilepsy, and diet therapy appears to be increasing in popularity. Diet therapy works typically within two weeks and certainly within three months, and thus clinical efficacy is determined relatively rapidly after starting treatment. Even though the ketogenic diet is now established as a clinically effective treatment in pediatric epilepsy, it has not been explored adequately in other developmental disorders - such as autism - that may benefit from seizure control and relief from diverse co-morbid symptoms. A more comprehensive overview of the diverse potential benefits of a relationship between a ketogenic diet and adenosine, including autism, is outlined in Masino et al. (2009).

It is important to remember that ketogenic diet therapy can result in a permanent reduction in epileptic seizures, and some children remain seizure-free even after tapering off the diet. The implication that permanent benefits could be achieved in ASD is incredibly enticing - although there is no evidence at this time. Notably, however, the ketogenic diet is often effective in treating seizures which are medically-refractory. In parallel with ongoing use of the classic ketogenic diet, related dietary strategies such as low glycemic index therapy (LGIT; Pfeifer & Thiele, 2005; Muzykewicz et al., 2009) and the modified Atkins diet (MAD; Kossoff et al., 2003; Kossoff et al., 2008) are actively investigated for efficacy in epilepsy. Because these diets are less restrictive than the standard ketogenic diet, they offer more popular appeal and may represent different "doses" of a ketogenic diet. The classical ketogenic diet is the most strict - highest "dose" and in some cases can offer seizure control in patients who did not achieve sufficient control with the MAD (Kossoff et al., 2010).

Because there is neither a proprietary drug nor drug development required, clinically-proven dietary strategies are translatable immediately to another pediatric population such as autism. Although used less often, the ketogenic diet is effective in adults with epilepsy, and thus could be considered for adults with ASD. There are currently no established

effective treatments for ASD in children or adults, and no approved psychiatric drugs for children, making the risks relatively small and the potential benefits quite large. A drug-based strategy would be easier to administer and appeal to a wider patient population; a “ketogenic diet in a pill” remains a hotly pursued therapeutic target (Rho & Sankar, 2008).

In general, a metabolic approach to increase adenosine could offer positive reinforcement, including improved sleep and learning, and reduced seizures and anxiety. The combined evidence of abnormal purine metabolism, stereotyped behaviors, increased seizures and disrupted sleep suggests a general dysregulation of this modulator and thus potential new insight into therapies for ASD. Success with metabolic adenosine-increasing approaches could also provide an impetus for exploring adenosine-enhancing drugs in autism, and establishing their safety and efficacy in children.

This work linking adenosine and autism integrates behavioral and neurobiological data, puts forth a new theory regarding ongoing symptoms of autism, and - if translated to clinical benefits - could address several areas of critical need. First, it targets co-morbidities in the form of anxiety, sleep, and seizure disorders. It is well known that sufficient quality and quantity of sleep is crucial for general mental health and optimizing learning and memory. Second, it proposes a specific and novel neurobiological target for reducing the ongoing symptoms of autism spectrum disorder - increased extracellular adenosine. Third, it targets metabolic mechanisms that alter adenosine, rather than receptor-based strategies. For decades researchers have attempted to develop adenosine-receptor based therapeutics but side effects, particularly due to peripheral actions of adenosine, remain a roadblock (Dunwiddie, 1999; Boison et al., 2011). This physiologic/metabolic approach, which shifts the dynamic level of adenosine itself, is likely to have far fewer side effects when translated into humans. Thus, some stimuli for increasing adenosine levels and decreasing ASD symptoms may be immediately accessible environmental interventions involving changes in diet or behavior, in parallel with pursuing more traditional pharmaceutical interventions.

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