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Initial analysis of peripheral lymphocytic extracellular signal related kinase activation in autism

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ABSTRACT

Background: Dysregulation of extracellular signal-related kinase (ERK) activity has been potentially implicated in the pathophysiology of autistic disorder (autism). ERK is part of a central intracellular signaling cascade responsible for a myriad of cellular functions. ERK is expressed in peripheral blood lymphocytes, and measurement of activated (phosphorylated) lymphocytic ERK is commonly executed in many areas of medicine. We sought to conduct the first study of ERK activation in humans with autism by utilizing a lymphocytic ERK activation assay. We hypothesized that ERK activation would be enhanced in peripheral blood lymphocytes from persons with autism compared to those of neurotypical control subjects.

Method: We conducted an initial study of peripheral lymphocyte ERK activation in 45 subjects with autism and 26 age- and gender-matched control subjects (total n = 71). ERK activation was measured using a lymphocyte counting method (primary outcome expressed as lymphocytes staining positive for cytosolic phosphorylated ERK divided by total cells counted) and additional Western blot analysis of whole cell phosphorylated ERK adjusted for total ERK present in the lymphocyte lysate sample.

Results: Cytosolic/nuclear localization of pERK activated cells were increased by almost two-fold in the autism subject group compared to matched neurotypical control subjects (cell count ratio of 0.064 ± 0.044 versus 0.034 ± 0.031; $p = 0.002$). Elevated phosphorylated ERK levels in whole cell lysates also showed increased activated ERK in the autism group compared to controls (n = 54 total) in Western blot analysis.

Conclusions: The results of this first in human ERK activation study are consistent with enhanced peripheral lymphocytic ERK activation in autism, as well as suggesting that cellular compartmentalization of activated ERK may be altered in this disorder. Future work will be required to explore the impact of concomitant medication use and other subject characteristics such as level of cognitive functioning on ERK activation.

Trial Registration: Not applicable.

1. Introduction

Autism spectrum disorder (ASD) is a developmental disorder characterized by deficits in social communication and interaction, and restricted, repetitive patterns of behavior, interests, or activities. Autism-like behaviors are also observed in Fragile X Syndrome (FXS), which is the most common type of inherited cognitive disability (Mariner et al., 1986). Our goal is to investigate the pathobiochemical pathway(s) responsible for these disorders in order to devise rational-based drug targets and develop useful

Abbreviations: ABC, Aberrant Behavior Checklist; ERK, Extracellular signal-related kinase; SCQ, Social communication questionnaire; SRS, Social responsiveness scale; VABS, Vineland adaptive behavior scale.

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biomarker(s). We hypothesize that a common cell signaling pathway takes different routes depending on the disease conditions.

The pathophysiology of autism remains poorly understood. As a behaviorally defined disorder with significant phenotypic heterogeneity, success in understanding the cause of illness has remained elusive. Biomarker development in autism, while the focus of significant research, has been met with limited success to date. In this context, we have previously reported higher levels of secreted amyloid-β precursor protein-alpha form (sAPPα) and lower levels of potentially toxic amyloid-β (Aβ) peptide in plasma and brain tissue of children with severe autism (Sokol et al., 2006; Sokol et al., 2011; Lahiri et al., 2013). How sAPPα mediates cell signaling relevant to ASD remains a major unanswered question, and the present work could shed some lights on this knowledge gap in the field.

Communication deficits and repetitive behaviors are seen in autism along with various symptoms that can vary in severity, including seizures and increased anxiety (Maski et al., 2011; Fung and Hardan, 2014). Considering almost innumerable genetic, environmental, or a combination of both factors may contribute to the etiology of a single case of autism spectrum disorder, approaches examining central points of cellular signaling and activity may hold promise to direct efforts towards unifying elements of cellular dysregulation. With these concepts in mind, we focused on study of extracellular signal-related kinase (ERK; also recognized as a mitogen activated kinase or MAP kinase) regulation in autistic disorder. ERK1 (MAPK3) and ERK2 (MAPK1) are central elements of intracellular signaling governing neuronal development (Samuels et al., 2008; Samuels et al., 2009), synaptic plasticity (Kelleher et al., 2004), and memory formation (Cui et al., 2008) which are all functions that are likely dysregulated in autism. ERK1/2 activation has also been implicated in various seizure models (Merlo et al., 2004; Yamagata et al., 2013). An imbalance in optimal ERK1/2 activation may play a role in cognitive function seen in autism-related disorders (Chevere-Torres et al., 2012). ERK1 and ERK2 isoforms exhibit significant functional redundancy and are thought to have resulted from single gene duplication at the onset of vertebrate evolution (Busca et al., 2015). Both exhibit a similar three dimensional structure and are ubiquitously expressed in mammals with similar specific activity (Robbins et al., 1993; Lefloch et al., 2008). Evidence from genetic studies in idiopathic autism, known genetic syndromes associated with autism, and murine models all point to potential aberrant ERK1/2 activity associated with the disorder. Specifically, copy number variation at the human 16p11.2 locus is a common risk variant associated with autism accounting for up to 1% of all cases (Malhotra and Sebat, 2012). The *MAPK3* gene which encodes for ERK1 is located in this region. Interestingly, reports on the impact of 16p11.2 deletion on ERK1/2 activity have been conflicting with reports of resultant ERK1/2 up (Pucilowska et al., 2015) or down regulation (Tian et al., 2015).

The developmental syndromes known as RASopathies include neurofibromatosis type 1 (NF1), Noonan syndrome (NS), Costello syndrome (CS), and cardio-facio-cutaneous syndrome (CFC) which are all associated with enhanced Ras/MAPK activity resulting in excessive ERK1/2 activation (phosphorylation; pERK1/2). There are several clinical features that overlap among each syndrome including dysmorphic facial features, short stature, and increased cancer risk (Cizmarova et al., 2015). Recently, a systematic phenotype assessment of the RASopathies noted increased autistic traits in those with a RASopathy compared to non-affected siblings (Adviento et al., 2014).

FXS is a well-established single gene disorder and the leading genetic cause of autism. In both brain samples from patients with FXS and in brain tissue from *Fmr1* knockout mice, pERK1/2 is elevated (Michalon et al., 2012; Wang et al., 2012). In *Fmr1* knockout mice, treatment with a MEK1/2 inhibitor or lovastatin reduces ERK1/2 phosphorylation and has been associated with phenotypic rescue including reduction in audiogenic seizures (Wang et al., 2012). Lovastatin, an HMG-CoA reductase inhibitor, inhibits Ras-ERK1/2 and prevents the development of seizure-like symptoms in *Fmr1*$^{-/y}$ mice (Osterweil et al., 2013). Tuberous sclerosis complex (TSC) is an autosomal dominant neurocutaneous and neurodevelopmental disorder caused by the loss of TSC1 or TSC2 suppressor genes which results in enhanced activation of the mammalian target of rapamycin (mTOR) signaling cascade. It is estimated that 50% of persons with TSC meet criteria for autism and/or developmental disability (Curatolo et al., 2008; Jeste et al., 2008). Constituents of the ERK1/2 pathway are overactive in TSC cell lines and in TSC-associated brain lesions further implicating this central signaling cascade in the pathophysiology of autism related disorders (Govindarajan et al., 2003; Ma et al., 2007).

In the BTBR inbred mouse model of autism, pERK1/2 levels were shown to be increased in the prefrontal cortex (Faridar et al., 2014). Additionally, in this BTBR report pERK1/2 was elevated in lymphocytes which correlated with the cortex findings. In mouse models, there may be a critical developmental period when ERK1/2 dysregulation may result in autistic features. Phospho-blockade of ERK1/2 at postnatal day 6 (P6), but not at P14 leads to the development of autistic-like behaviors in adult mice (Yufune et al., 2015). A conditional ERK2 knockout mouse expresses a phenotype marked by aggressive behavior, reduced social behaviors, and learning deficits(Satoh et al., 2011), which are findings potentially consistent with an autism-like phenotype.

ERK1/2 is expressed in peripheral blood cells including lymphocytes. Analysis of ERK1/2 activation in lymphocytes is well established in the leukemia literature (Balakrishnan et al., 2014; Naci and Aoudjit, 2014; Uzan et al., 2014). Parsing ERK1 and ERK2 activation apart in human biological samples has not to date been reported. ERK requires phosphorylation for full activity and employs phosphatases to regulate signal transduction cascades (Caunt and Keyse, 2013). Activation and inactivation of ERK is influenced by the subcellular localization of the phosphatase (cytoplasm and nuclear compartments) (Owens and Keyse, 2007). Phosphorylation of ERK indicates the translocation of activated ERK into the cytosolic compartment. Given the feasibility to analyze ERK (ERK1 and ERK2 combined) activation in peripheral blood combined with the above evidence indirectly implicating ERK dysregulation in the pathophysiology of autism, we undertook the first known human study to date of ERK activation in autism using peripheral lymphocyte assays. It has been shown that inflammatory responses may lead to homing of lymphocytes to the CNS (Weller, 1996). The brain pathology of children diagnosed with ASD suggests ongoing neuroinflammation in various regions of the brain (Morgan 2010; Tetreault et al., 2012). This connection between neuroinflammation, lymphocyte migration, and the CNS may link the activation of ERK1/2 in the peripheral blood to that of the CNS in many neurological disorders including ASD. We hypothesized that ERK activation visualized as its translocation from the nucleus to the cytoplasm, would be increased in the peripheral lymphocytes of persons with autism compared to age- and gender-matched neurotypical control subjects. Our working hypothesis is based on the findings of potentially enhanced ERK activation in the RASopathies, the BTBR mouse model of ASD, and in FXS.

2. Methods

All subjects were recruited and enrolled at the Christian Sarkine Autism Treatment Center at Riley Hospital for Children between February and June 2012. The project was approved by the Indiana University Institutional Review Board (IRB). Inclusion criteria for