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Supporting the Use of MBD-1 Knockout Mice as an Animal Model for Autism

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ABSTRACT:

OBJECTIVES: The prevalence of autism in our society has grown over the past few decades while lack of a central pathophysiological cause for the disease has hindered research and design of an animal model with the disease. Methyl CpG Binding Domain-1 knockout mice are a potential model for autism. The mice are owned solely by Dr. Xinyu Zhao of the University of New Mexico. The mice appear normal, both anatomically and developmentally, however, they exhibit behaviors that are often seen in autistic patients. While there are few specific physiological hallmarks of autism among patients, a few studies have noted increased levels of adrenocorticotropic hormone and lower plasma corticosterone, which these mice exhibit. The project aims to look at the central deregulation of corticosterone releasing factor-hypothalamic-pituitary-adrenal axis by comparing the presence of receptors for corticosterone releasing factor-1 in the hippocampi of knock-out mice and wildtype. METHODS: Six knock out mice and 6 wild type mice were euthanized and the hippocampi removed and homogenized. A western blot was conducted to determine relative amounts of corticosterone releasing factor receptor-1 in wild-type and knock out mice. Amount of the secondary antibody was quantified using the Pierce Supersignal West Pico Chemiluminescent Kit. RESULTS: The knockout mice had significantly less expression of corticosterone releasing factor receptor-1 in the hippocampi than wildtype p<0.03 CONCLUSIONS: The findings show that there is a difference in the central regulation of the corticosterone releasing factor-hypothalamic pituitary adrenal axis between wildtype and Methyl CpG
Binding Domain-1 knockout mice. While additional studies are needed to understand the full-picture of the regulation of this axis in these mice, the project is promising in supporting the use of the methyl CpG Binding Domain-1 knockout mice as models to study the specific physiology of autism.

**INTRODUCTION:**

Autism is defined by the Diagnostic and Statistical Manual for Mental Disorders (DSM-IV) as the presence of qualitative impairment of social interaction and communication and restrictive or repetitive patterns of behavior, interests and activities. Delays or abnormalities in at least one of the areas manifests before the age of three years old, and the condition cannot be better explained by a diagnosis of Rett’s Disorder, or Childhood Disintegrative Disorder (DSM-IV). Autistic patients span a broad range of capabilities and degree of disability ranging from mental retardation with life-long dependence to improvement of social skills and ability to live independently, and occasionally even to marry and have children (Rapin, 2006). There appears to be an increased incidence in autism over the past few decades, which may be partially explained by more sensitive diagnostic methods (Rapin, 2006) but most certainly also points to an increase in environmental exposures that lead to the disorder. Autism is a condition of many known biological causes, both pre-natal and post-natal, but despite some theories, there is not yet an accepted mechanism for its pathogenesis. The presence of common characteristics among patients with autism, despite different disease triggers, points to a common etiology and pathophysiological process.
One physiological trait that is common among autistic patients is an apparent deregulation of the hypothalamic pituitary axis. A recent study based on the influence of the limbic system, the brain center for emotion and social interactions, over the hypothalamus and pituitary gland looked for changes in hormone levels controlled by these glands in autistic individuals. The study found that cortisol concentrations in autistic individuals were significantly lower than those in control individuals, while plasma adreno-corticotrophic hormone (ACTH) concentrations were significantly higher. The change indicates that there is an abnormality in the hypothalamic-pituitary-adrenal axis in autism (Curin, et. al., 2003). ACTH is a peptide hormone that stimulates synthesis and secretion of the adrenal cortical hormones including cortisone, androgens and aldosterone. Negative feedback inhibition of ACTH occurs when plasma levels of cortisol reach a normal biological level. Elevated levels of ACTH accompanied by low levels of cortisol indicate an abnormality in either delivery of ACTH to the adrenal cortex receptors, in biosynthesis of cortisol, or in negative feedback inhibition by cortisol and ACTH within the CNS (Costanzo, Linda S. 1998). Cortisol is vital for survival. It is important for many physiological processes including the ability to deal with both physical and emotional stress—something that children with autism have trouble with (Muller, 2004). While cortisol deficiency can often be associated with adrenal problems, the cortisol deficiency in autism is most likely associated with a central defect since the disease is a psychological problem and autistic patients are not commonly known to exhibit traits of primary adrenal insufficiency (Chamberlain et al., 1990).
Despite extensive characterization and study of patients with autism, further research and understanding of pharmacological treatment is hindered by the lack of a good animal model with which to study autism. A recent study proposed the use of MECP2 knockout mice as an animal model for Rett’s disease, a disorder that is closely related to autism (Vincent JB, 2005 and Moretti P, 2006). MECP2 is a methyl-CpG binding protein from a family of methyl-CpG binding proteins that are located on an area of the X chromosome and are involved in transcriptional repression (Li H, 2005).

Another population of knockout mice that is related to the MECP2 population is a population of MBDI knockout mice, which were developed by Xinyu Zhao (Department of Neurosciences, University of New Mexico). They have no detectable developmental defects and appear grossly healthy, however neurological studies show that they have reduced neuronal stem cell differentiation and increased genomic instability (Zhao, 2003). Recent studies with the MBDI knockouts found them to have behavioral characteristics similar to autism—poor learning and increased distractibility, poor social interaction with other mice, increased helpless behavior, and poor sensory gaiting (Allan, 2008). The MBDI knock-out mice also have physiological abnormalities, most notably, elevated ACTH and decreased corticosterone levels, just like the patients in the studies by Curin et. al. (2003); Tani et al. (2005); and Tordjaman et al.,(1997). The behavioral and physiological abnormalities similar to autism in humans are presumably the result of a central physiological deregulation and defining the central abnormalities in these mice will most certainly provide further insight to the pathophysiology of autism.
Central to the physiological abnormalities of elevated ACTH and decreased cortisol is the HPA axis and possible dysregulation of the axis. This study sought to better define the point of this dysregulation in the MBD1 -/- mice; specifically to answer the question of whether or not there is a difference in expression level of corticotrophin-releasing factor (CRF) receptors in the two groups of mice. CRF-1 receptors are widely distributed in the brain, including the hippocampus, and activation of the receptors has been shown to play an important role in regulation of the HPA axis and in anxiety related behavior (Groote, 2005). While other areas of the brain express the CRF-1 receptor, including the pituitary gland, our currently available methods would not have allowed us to use the very small pituitary glands of these mice for individual analysis. The hippocampus, which is large enough to provide individual samples for our experiments, is known to be one of the areas of the brain to which CRF-1 receptors are distributed. The hippocampus provides neural input to the paraventricular nucleus of the hypothalamus, thereby exerting positive and negative control to the HPA axis. There is a negative feedback loop mediated by glucocorticoid receptors through which concentration of glucocorticoids not only affect the activity of the hypothalamus and anterior pituitary glands, but in which their effects extend even more centrally to the hippocampus to down-regulate neural input from the hippocampus to the hypothalamus. Comparing the expression of CRF-1 receptors in the hippocampi of the MBD1 -/- mice to wild-type mice was meant to help determine the level of deregulation of the HPA axis in these mice. Thus, it was predicted that the MBD1 -/- mice would exhibit a down-regulation in hippocampal CRF-1 receptors because low levels of corticosterone in the
mice will lead to a perpetual increase in CRF release from the hypothalamus and subsequent down-regulation of receptors.

**METHODS:**

**Animals**

All procedures involving animals were approved by the University of New Mexico Laboratory Animal Care and Use Committee and were conducted by a trained laboratory technician. The laboratory holds currently approved protocols for these studies.

Six MBD-1 knock out mice and 6 wild type mice were euthanized and their brains dissected on ice. The hippocampi were removed and placed in 0.5 ml ice cold HB Buffer and homogenized in preparation for the anti-CRF Receptor 1 Immunoblot Protocol. Each hippocampus was homogenized in a 1ml Dounce tissue grinder by 5 strokes with a loose fitting pestle and 5 strokes of the tight fitting pestle. Homogenate was centrifuged and the supernate aliquoted into fresh tubes. One aliquot from each sample was run in a Protein Assay (using Bio Rad) to determine the protein concentration and the rest of the aliquots were frozen at -80C until use.
A western blot was conducted to determine the relative amounts of CRF-1 in wild-type and MBD1 -/- mice. At the time of the experiment, aliquots were removed from the freezer and diluted to standard concentration with a sample buffer and reducing agent and transferred to and run on Tris-Acetate Polyacrylamide Gels. After running the gels, the membranes were blocked and incubated overnight with a primary Rabbit Anti-Corticotropin-Releasing Factor Receptor 1 polyclonal antibody made by Chemicon International. After incubation the membranes were washed, incubated for an hour and a half with the secondary antibody, washed once more and then detected for immunolabeled CRF-1 proteins with the Pierce Supersignal West Pico Chemiluminescent Kit.

Analytical methods, and power calculation:

Power calculation assuming an alpha of 0.05 and an effect size of 30% difference with less than a 10% error rate indicates that an n of 6 per group gives 87.5% power. The data was run in triplicate and quantified using densitometry program (Bioquant) and analyzed using a one way ANOVA with the aid of SPSS v14.

RESULTS:

The results showed that the Hippocampi of the MBD1 knockout mice had significantly less (p<0.03) expression of Corticotropin-Releasing Factor Receptor 1 than the Hippocampi of the wildtype mice. The expression was quantified by individual
immuno-reactivity units, relative to beta-actin, which has previously been shown to be expressed equally in the MBD1 knockout mice and wildtype mice.

Lanes 1-6 MBD1 KO; Lanes 7-12 Wildtype

\[ t(10)=2.5, \ p<0.03 \]

**DISCUSSION:**

Several studies have suggested a dysregulation of the HPA axis among autistic children. Poor adaptive responding to stressful situations has long been noted among autistic children. Abnormal diurnal rhythm and abnormal dexamethasone stress test response has been reported for both autistic children and infants (Hoshino et al., 1987). Recently a comparison of salivary cortisol response to ACTH stimulation has reported that autistic individuals had a slower cortisol response compared to healthy controls.
These findings suggest that the negative feedback mechanism of the HPA-axis may be disrupted in autistic children. A goal of the present study was to evaluate a mouse model for its similarity to the HPA axis dysregulation seen in human autism.

The results of the present study show that the MBD1 knock out mice have less CRF-1 than the wildtype mice. CRFR-1 is known to mediate behavioral responses to stress (Heinrichs and Koob, 2004) and antagonists of CRFR1 are associated with moderating depression and stress in patients (Zobel et al., 2000). Our findings are counterintuitive, as the increased levels of anxiety in these mice in behavioral testing originally led us to hypothesize that an increase in stress-hormone receptors (CRF-1 in particular) may be involved. Thus, it was unexpected that the mice displaying greater depression would have lower levels of CRFR1. It is possible that the lower levels of CRFR1 in the MBD1 KO mice represent a compensatory down regulation as an effort to mitigate the degree of unregulated CRF activation produced by MBD1 knockout. It is possible that we are seeing a down-regulation of the receptors in a physiological attempt to decrease the impact of excess stress hormones on the mice. The fact that the mice continue to have elevated levels of anxiety in light of this indicates that there is likely another mechanism involved in producing these heightened levels of anxiety that deems this down-regulation ineffective.

Several studies indicate functional interactions between CRF and the serotonergic system and have linked these interactions to depression. In addition to CRF effects on the raphe nucleus (Sakanaka et al, 1987; Swanson et al, 1983; Ruggiero et al, 1999; Kirby
et al., 2000; Lowry et al., 2000; Valentino et al., 2001) CRF receptors influence hippocampal serotonin (5HT) levels (Linthorst et al., 2002; Peñalva et al., 2002; Oshima et al., 2003). CRF receptor antagonists reduce 5HT levels in rat hippocampal formation (Isogawa et al., 2000; Linthorst et al., 2002). Homozygous CRFR1 knockout mice showed enhanced hippocampal 5-hydroxyindoleacetic acid (5-HIAAA) under basal conditions and a greater rise in hippocampal 5HT during a forced swim task compared to wild type mice (Peñalva et al., 2002). The most abundant 5HT receptor in the hippocampus is the 5HT1A receptor and its expression is regulated by HPA axis activity (Lopez et al., 1998); with expression being elevated during low corticosteroid conditions and decreased with high corticosteroid levels.

Current research with the MBD-1 mice and the explanation for increased levels of anxiety in these mice is being directed toward serotonin and the role it may play in increased levels of anxiety in autism spectrum disorders. MBD1 protein is known to regulate the expression of serotonin receptors in the brain, and indeed, the MBD1 knockout mice have been found to have increased levels of Serotonin 2c receptors (Allan, 2008). Future studies will seek to test this link between the HPA axis and serotonin regulation of the stress axis.

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