Role of Serum Brain Derived Neurotrophic Factor (BDNF) and BDNF Gene Mutation in Autism Spectrum Disorders (ASD).

Origin of Proposal
The diagnosis of autism is based solely on behavioral characteristics. There is currently no laboratory test that can be done to identify autism. The etiology of Autism Spectrum disorders (ASD) is not well understood, though it likely involves genetic, immunologic, and environmental factors. The condition was initially described in the U.S. and European medical literature in the mid-1940s; however, references to individuals both fictional and historical who apparently meet the ASD clinical profile go back several centuries. Through the 1980s, ASDs were believed to be rare, with a prevalence of no more than 5 per 10,000 persons (Gillberg et al, 1991) and were considered more of an intriguing clinical dilemma than a major public health problem. The etiology of autism still remains unknown, with many factors implicated in the development of autism phenotype. Therefore the identification of a biological marker of autism would be advantageous.

Today, the prevalence of ASD is understood to be many times greater, with the condition now thought to be second only to mental retardation among the most common serious developmental disabilities, however inadequately studied in Indian population. The dramatic increase in reported prevalence has spurred an intense effort to identify early biological markers. Such markers could allow earlier identification and therapeutic intervention, contributing to improved prognosis of children with ASD. Hence the origin of the proposal lies in studying BDNF levels and mutational studies in varied clinical spectrum of ASDs with behavioral sub groups.

A) Rationale of the project-
Autism as originally described by Kanner, is a syndrome defined by a constellation of behavioral characteristics (Kranner, 1943). The cardinal features of this disorder are deficits in social and communicative abilities, a stereotyped and restricted range of interests and activities (Nelson, 2001). Today, the prevalence of ASDs is understood to be many times greater, with the condition now thought to be second only to mental retardation among the most common serious developmental disabilities in the United States (Yeargin-Allsopp et al, 2003). Most recent reviews of epidemiology estimate a prevalence of one to two cases per 2,000 people for autism, and about six per 1,000 for ASD. ASD averages a 4.3:1 male-to-female ratio.

Most cases of autism present classically, with the affected child failing to develop normal social or language skills, which is termed early onset autism. However in a subset of cases, known as regressive autism, the child appears to develop typically until approximately 18 to 24 months of age after which the child shows neuro-regression. The etiology of autism still remains unknown, with many factors implicated in the development of autism phenotype. Therefore the identification of a biological marker of autism would be advantageous. Several genetic studies link autism with genes that have immune functions. However genetic studies have not yet revealed a specific definitive autism marker which has led to the hypothesis that an environmental trigger could be associated with some cases of autism (Enstrom et al 2008).
Autism Spectrum Disorder (ASD) is a complex neurological disorder of unknown etiology. It is a developmental disability that manifests as problems with social interaction and communication. Symptoms usually start before three years of age and can cause delays or problems in many different skills that develop from infancy to adulthood. Different people with autism can have very different symptoms. The prevalence of Autism has been increased to 6.7 per 100 children in USA and the scenario is similar in our country as well (Bhanumathi et al., 2009). Health care providers think of autism as a “spectrum” disorder, a group of disorders with varied degree of features. One person may have mild symptoms, while another may have serious symptoms. But they both have an autism spectrum disorder. The clinical diagnosis of ASD is by DSMIV Criteria.

B) Hypothesis

Several genetic studies link autism with genes that have immune functions. However genetic studies have not yet revealed a specific definitive autism marker. Brain Derived Neurotrophic Factor (BDNF) is one of the neurotrophins which is important for neurodevelopment, neuronal differentiation, neuronal protection and survival, & recently it has become a hot molecule for exploring its diagnostic and therapeutic value including autism.

Brain Derived Neurotrophic Factor (BDNF) is one of the neurotrophins which is shown as a major regulator of synaptic plasticity, neuronal differentiation and survival. BDNF is important for neurodevelopment, neuronal differentiation and neuronal protection (Enstrom et al, 2008). Activated microglial cells are able to secrete BDNF, which may protect the brain from inflammation induced apoptosis and may alter synaptic properties of neurons (Bessis et al, 2007). BDNF is a 119-amino-acid, 13.6-kDa protein (Mowla et al, 2001), belonging to the neurotrophin family of signaling proteins that appears to be involved in the central regulation of energy homeostasis. BDNF is found in neurons of the central nervous system. It is expressed predominantly in hippocampus, cortex, and synapses of the basal forebrain. BDNF selectively supports the survival of primary sensory neurons and retinal ganglia.

Multiple lines of evidence suggest that BDNF plays a critical role in serotonergic functions. In rat brain BDNF has been found to promote the survival and sprouting of serotonergic axons and the axonal growth of injured serotonergic neurons (Grider et al, 2005 & Mamaounas et al, 2000). In-vitro and in-vivo studies support a regulatory role of BDNF in the survival and maturation of serotonergic neurons (Djalali et al, 2005). BDNF has also been shown to modulate serotonergic neurotransmission in-vitro. In addition BDNF administration has been found to increase the synthesis and/or turn over of serotonin in-vivo (Goggi et al, 2002).

Brain-derived neurotrophic factor (BDNF) is a small protein found throughout the central nervous system (CNS) and peripheral blood. BDNF is involved in the survival and differentiation of dopaminergic neurons in the developing brain (Hyman et al. 1991), and plays an important role in the formation and plasticity of synaptic connections (Binder and Scharfman 2004). BDNF is trophic for serotonergic neurons, and abnormalities in serotonin levels are the most common biochemical findings in autism (Anderson 2002; Tsai 2005).

The human brain derived neurotrophic factor (BDNF) gene (MIM: 113505) is located at 11p13. It spans 70 kb and contains 11 exons (Pruunsild et al., 2007). BDNF is composed of, at least, four 5’ nontranslated exons and a single 3’coding exon (Ribases et al, 2003). Since mRNA transcripts
were different at their 5’ ends, we determined the existence of four 5’ untranslated exons that give rise to four distinct mRNA isoforms (named A, B, C and D).

A non synonymous G to A single nucleotide polymorphism (SNP) exists at position 196 of exon 2 (rs6265), which results in valine (val) to methionine (met) substitution at codon 66 (val66met), changing the 5’ pro-region of the human BDNF protein. This polymorphism affects intracellular packaging of pro-BDNF, its axonal transport and, in turn, activity-dependent secretion of BDNF at the synapse (Egan et al., 2003; Chen et al., 2004). BDNF gene transcription is driven by at least nine different promoters, each of which drives transcription of a short, 5’ non-coding exon, which is spliced to a 3’ common coding exon.

Several DNA variants mapping within the BDNF genomic region have been associated with a number of human traits, such as performance on intelligence tests, various cognitive functions, personality, and memory [Eagan et al, 2003; Rybakowski et al, 2003; Sen et al, 2003; Itoh et al, 2004; Tsai et al, 2004]. Notably, there are many evidences for BDNF contribution to the pathogenesis of several neuropsychiatric disorders. To date BDNF has been reported to be associated with schizophrenia [Hawi et al, 1998; Wassink et al, 1999; Krebs et al, 2000; Nanko et al, 2003], Parkinson’s disease [Momose et al, 2002; Hakansson et al, 2003; Hong et al, 2003; Toda et al, 2003], addictive substance use or dependence [Uhle et al, 2001], Alzheimer’s disease [Kunugi et al, 2001; Tsai et al, 2004], bipolar disorder or depression [Neves-Pereira et al, 2002; Sklar et al, 2002; Nakata et al, 2003; Tsai et al, 2003] and obsessive compulsive disorder [Hall et al, 2003; ]. In particular, the common non-conservative single nucleotide polymorphism (SNP) rs6265 (G.A), resulting in a Valine to Methionine amino acid change at codon 66 in the pro-domain of BDNF protein (pro-BDNF), has been extensively analyzed in several neuropsychiatric disorder through linkage and association studies leading to conflicting results [Gratacos et al, 2007; Ribases et al, 2003; Lohoff et al, 2005; Rybakowski et al, 2006; Rosa et al, 2006]. This functional polymorphism was shown to affect the ability to perform verbal episodic memory tasks and hippocampal function [4], to influence BDNF mRNA localization, putatively impairing dendritic targeting of BDNF transcript [Chiaruttini et al, 2009] and to alter the intracellular distribution and activity-dependent secretion of the BDNF protein.

Brain Derived Neurotrophic Factor (BDNF) is one of the neurotrophins which is important for neurodevelopment, neuronal differentiation, neuronal protection and survival, & recently it has become a hot molecule for exploring its diagnostic and therapeutic value including autism.

C) Key Questions
1. Do the protein levels of BDNF in the serum of patients with autism vary from those in the normal population?
2. Is the Secretion of BDNF in Autistic children increased or decreased as compared to normal children?
3. Is the mutation Val66Met prevalent in Indian Autistic population?
4. Does Val66Met mutation affect the serum concentration of BDNF in Autistic population?

Current Research
Association of BDNF is found with changes in behavior, such as hyperactivity, increased
depression and psychiatric disorders including schizophrenia and bipolar disorder (Montegia et al 2007). Multiple studies have shown increase in BDNF in both blood and brain tissue from autistic subjects (Miyazaki et al, 2004). Since BDNF readily crosses the Blood-Brain-Barrier, the serum concentrations correlate directly to brain concentration, therefore plasma studies of BDNF are thought to accurately reflect CNS concentration. A detrimental effect of BDNF on the aforementioned processes has been implicated in the pathogenesis of neuro-developmental disorders like autism. Specifically elevated BDNF expression has been observed in the brain, blood (Nelson et al, 2001) and serum (Miyazaki et al, 2004) of autistic individuals compared to healthy controls. Recently, it was conversely reported that the serum levels of BDNF in patients with autism were significantly lower than those of normal controls.

Depletion of brain and serum BDNF is also been reported in neurodegenerative disorders like Alzheimer, Parkinson and Huntington diseases. As pre-clinical results have linked BDNF depletion with autism, various laboratories are exploring the therapeutic significance of BDNF in autism and also in neurodegenerative disorders. It is recently well reviewed by Zuccato and Cattaneo (2009). Implications of serum BDNF levels have been investigated in children with mental retardation and ASD, however, with equivocal findings. Moreover, it has been challenging, both to classify typical autism (by DSM-IV), and associate a suitable biomarker with clinical phenotype spectrum. BDNF levels are also known to be different in autistic children based on patterns of early onset, and severity of autism behavior. To the best of our knowledge, reports on such studies in Indian subjects are lacking. Role of BDNF as a putative biological marker in autism spectrum disorders (ASD) is gaining significance to evaluate the severity of cognitive and behavioral deficits. The above mentioned research makes BDNF an appropriate candidate for investigation in studies related to the development of the brain dysfunction and autism.

Skewed expression of BDNF has been linked to neurologic and psychiatric disorders including Fragile X syndrome, epilepsy, Parkinson’s disease, Alzheimer’s disease, schizophrenia and depression (Pezet and Malcangio 2004; Binder and Scharfman 2004; Hashimoto et al.2004;Angelucci et al. 2005). Elevated levels of BDNF were found in postmortem brain tissue from adults with autism (Perry et al. 2001) and concentrations different from those in controls have been found in peripheral blood of adults and children diagnosed with autism (Miyazaki et al. 2004; Connolly et al. 2006; Hashimoto et al. 2006;Enstrom et al. 2008). The two studies that have examined BDNF levels in neonatal specimens from individuals later diagnosed with autism have yielded inconsistent results (Nelson et al. 2001; Nelson et al. 2006).

As of 2008, Val66Met is probably the most investigated SNP of the BDNF gene, but, besides this variant, other SNPs in the gene are C270T, rs7103411, rs2030324, rs2203877, rs2049045 and rs7124442. The current research is being focused on novel sequence variations in the BDNF gene and its associations with neurodegenerative and neuro-psychiatric disorders (Licinio,et al,2009; Zaccato et al, 2001) Interestingly BDNF polymorphism has been suggested as the genetic modifier of disease severity in Rett Syndrome indicating a significant role of BDNF function in the pathogenesis of Rett Syndrome.(Zeev et al., 2009). The altered BDNF levels in amniotic fluid were shown to be associated with central Nervous system (CNS)
abnormalities of the fetus, suggesting that amniotic fluid (AF) BDNF levels could be indicative of Fetal CAS development (Marx et al, 1999). Recently, the Val 66 Met genotype might influence BDNF protein levels in AF has been shown, supporting the involvement of this polymorphism in behavioral and functional brain individual differences (Cattaneo et al, 2010).

The relevance and expected outcome of the proposed study
The diagnosis of autism is based solely on clinical & behavioral characteristics. There is currently no laboratory test that can be done to identify autism. In this study, brain derived neurotrophic factor (BDNF) will be investigated from the point of its serum levels as well as at the level of mutation as a possible early biologic marker for autism.

As cited above there have been studies carried out in various countries to assess the role of BDNF in Rett syndrome and other neurodegenerative disorders. However the present study has a focus on exploring BDNF as a marker in “Clinical Spectrum of Autism” and its involvement in manifestation of genotype and phenotype in severity of the disease condition.

The aforementioned references provide the fact that numerous studies have been carried out for the evaluation of BDNF and its role in brain development. However none have been carried out in India. So the aim of the project will be to study the concentrations of BDNF levels in children with ASD and healthy controls. Additionally, the mutational study, specific for val66 met will be conducted to find the frequency of this mutation in Autistic Indian population. Once the mutational characterization is achieved, further setup to investigate BDNF in amniotic fluid can be followed with a preventive approach.

Preliminary work done so far
So far twenty children with autism spectrum disorder and mental retardation have been enrolled for the study. These children were categorized into various groups of ASD using the DSM-IV criteria. Detailed clinical history; including prenatal, birth and family history was obtained; with emphasis on high risk genetic factors like consanguinity, spontaneous abortions, history of sibling or neonatal death, history of MR and Autism in the family, etc. The genetic history was recorded using a pedigree chart.

Ten age matched controls were also enrolled in this study. Informed written consent was obtained from the parent/guardian of each child enrolled in this study. The study was carried out as per the Ethical Guidelines for Biomedical Research on Human Participants, Indian Council of Medical Research (ICMR), 2006; which are based on the Declaration of Helsinki (Reference). Two milliliters of venous blood was collected from each participant. Serum was separated immediately and stored at -20ºC till further use. Serum BDNF concentration was estimated by a sandwich ELISA method using the Human BDNF ELISA kit. The significantly reduced serum BDNF levels were found in typical autistic females possibly indicating the severity of this X-linked condition, eg. Rett Syndrome. Significantly increased serum BDNF levels were observed in atypical autistic subject.

Specific objectives
i) To investigate the role of BDNF as a biological marker in children with ASD
ii) To study genotype of the BDNF gene in the autistic population in India
iii) To investigate for correlation between the serum BDNF concentrations of children with autism and the genotype of the BDNF gene

BDNF serum concentrations would be evaluated in children with autism using commercially available BDNF ELISA kits, which are only research based. These values will be compared with those of controls with similar age groups. Additionally the BDNF gene will be investigated for mutational study through ARMS PCR method and correlated with behavioral phenotype of ASD. This analysis will be targeted for finding out the VAL66MET mutation in the autistic population, and if possible de-novomutations in Indian population.

**Work plan (methodology/experimental design to accomplish the stated aim)**

**Study Design:-**

1. **Selection of patients with ASDs.**

Total of 50 children with autism spectrum disorder neurologically and psychologically diagnosed by DSM IV *Diagnostic Criteria for Autistic Disorder* will be selected for the study. The DSM IV criteria labels a child as autistic on stringent guidelines. There are 3 sections A, B and C, each containing 6 or more items. A child can be labelled as autistic only when it satisfies at least two items from A and one each from B and C, and thus referring as “Definite /Typical Autism” and the rest falling short of criteria are grouped as “Atypical Autism”. These children would be further divided into 2 groups:

Group A - Children with Definite Autism.

Group B - Children with Developmental Disability and Autistic features satisfying some of DSM IV Criteria.

Each Group would be further subdivided into of subgroups children:

1. With high risk genetic factors (e.g. Congenital birth defects, anomalies, dysmorphism and familial history)

2. Without Genetic/Congenital anomalies (idiopathic autism).

Complete clinical history including birth, family history with pedigree charting of each patient will be duly recorded. These will later be presented in detail through a master chart.

The research proposal has been sent to Institutional Ethics Committee this year and awaiting for ethical approval. However, the same study has got Institutional Ethics Committee, SRL
approval where the PI (Dr. Dave) has been working as a Principal Scientist at R& D SRL & the help for laboratory work to do mutational study will be conducted in collaboration.

Method:-

**BDNF Activity**
The serum samples will be subjected to analysis by the Human BDNF ELISA kit which are only commercially available kits for research purpose. The principle is as follows:

The Human BDNF ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human BDNF in serum, plasma, cell culture supernatants and urine. This assay employs an antibody specific for human BDNF coated on a 96-well plate. Standards and samples are pipetted into the wells and BDNF present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated antihuman BDNF antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of BDNF bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

**BDNF Gene Study**
The whole blood samples of the above mentioned subjects and controls will be subjected to molecular diagnostic methods to understand the genotype. ARMS PCR analysis for the Val66met mutation will be carried out (Bath & Lee, 2006).
References


10. Chen ZY, Patel PD, Sant G, Meng CX, Teng KK, et al. ,(2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-
dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons, *J Neurosci*; 5: 24.


