Neuroimaging studies in Rett syndrome


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Abstract

Neuroimaging is a key instrument for determining structural and in vivo functional status of the brain, non-invasively. Multiple approaches can now determine aspects of anatomic and neurochemical changes in brain, and have been utilized effectively in Rett Syndrome patients to understand the biological basis of this neurodevelopmental disorder. Studies performed at our institute include volumetric analyses of MRI, magnetic resonance spectroscopy (MRS), diffusion tensor imaging (DTI), cerebral blood flow measurements with MRI, and positron emission tomography scans (PET). These studies have provided considerable insight into mechanisms underlying the clinical features of this disease. Volumetric analyses suggest that decreased brain volume in RS results from global reductions in both gray and white matter of the brain. A selective vulnerability of the frontal lobes is evidenced by the preferential reduction of blood flow, increased choline and reduced n-acetyl aspartate (NAA) by MRS, and increased glucose uptake in these same regions as shown by (18F)-fluorodeoxyglucose (FDG) PET scans. We hypothesize that the increased glucose uptake relates to increased glutamate cycling in synapses. The resulting neuroexcitotoxic injury to the developing brain contributes to the seizures, behavioral disturbance and respiratory irregularities commonly seen in phases 1 and 2 of this disorder. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Neuroimaging has been a valuable non-invasive tool to assess both the structural and functional status of brain, linking neuronal activity to clinical events. Recent advances promise to correlate gaps in cellular activity, anatomic organization, and cerebral function, to complex behaviors. Various techniques used by investigators internationally in attempts to characterize the in vivo pathology of Rett syndrome (RS), along with studies performed at our Institute, attempt to understand fundamental questions about the biological basis of this disease. These studies in RS will provide essential insights into the anatomic status, altered neurochemical metabolism, and sequential changes, crucial to determining the biological consequences of defects in the MeCP2 gene [1] and future therapy. The studies described in this paper were conducted (with the approval of the Institutional Review Board of Johns Hopkins University School of Medicine) on patients admitted to the Pediatric Clinical Research Unit (PCRU).

2. Morphometric/Volumetric studies

After our earlier volumetric studies that characterized the neuroanatomical phenotype of RS in general terms [2–4], we have begun to apply new quantitative approaches. These new protocols are the result of improvements in MR acquisition and image processing. Scientifically, the studies focus on the detailed characterization of the macroscopic neuroanatomy of RS, including structural-clinical correlates, and the determination of patterns of brain growth. This structural information will also serve for the analysis and interpretation of data obtained by other imaging modalities. The first phase of these new morphometric studies is centered on the assessment of the cerebral cortex, with special emphasis on characterizing regions of selective impairment or preservation as determined by volumetrics. We are also focusing on the growth trajectories of individuals with RS in the 5–12 years age range. We have focused on this period because it corresponds to both a dynamic phase in the evolution of RS, [5] with some ‘regressive’ elements, and to a period of active growth of the cortex as shown by recent longitudinal
studies of normal children [6]. The latter have shown that the peak of frontal and parietal lobe growth occurs at about age 11 years in females.

Our studies are based on an age-sex-matched subject design and include normal controls and individuals with classical and atypical RS. The analyses are designed to determine what is characteristic or distinctive to RS, by comparing both RS groups with normal female controls, and the specific anatomic features of classical RS, by comparing classical and atypical subjects. In terms of growth patterns, we are carrying out cross-sectional analyses in age-paired control-RS groups. The calculation of the differences in the slope of the regression curves will ultimately be complemented by serial analyses (two time points). To date, we have recruited and scanned 66 normal control subjects, and 134 RS girls, of whom 115 are classical and 19 atypical. Longitudinal data are collected on 27 classical RS girls and six atypical cases.

In contrast with the previous investigations that were based on standard T2-weighted MR sequences, our data are currently acquired using volumetric T1 sequences (SPGR/FFE). These are characterized by their higher spatial resolution (1.5 mm slice thickness) and continuous (gapless) nature. The images are first tissue-segmented into the three basic classes: gray matter, white matter, and CSF, and then oriented in a standardized space that uses the anterior and posterior commissures as main landmarks. The latter process, which is based on stereotactic principles, uses a referential system, the Talairach atlas and grid, to subdivide the brain into more than 1,000 sectors [7]. Because of the standardization process, which eliminates individual information about surface and other landmarks, this approach has relatively high predictive value only for large structures such as the cerebral cortex [8]. The particularly high sensitivity and specificity for lobar volumes was demonstrated in our validation studies, in which we used both normal pediatric controls and subjects with RS [9]. Recently, we also developed protocols for the subdivision of all four lobes and the insular cortex [10] (see Fig. 1). The advantage of this semi-automated method is its high output, which allows studies of a large number of subjects, including multiple-group comparisons. Nevertheless, these studies need to be extended into more labor-intensive manual delineation protocols, which take into consideration each individual’s gyral/sulcal anatomy. These latter analyses are able to evaluate discrete cortical and subcortical regions (e.g. dorsolateral prefrontal cortex). Because more precise protocols are only compatible with a relatively small series of subjects, sample selection is an important issue.

The atlas-based morphometric approach is currently being applied to RS subjects in order to re-examine the issue of selective vulnerability and abnormal brain growth, as initially suggested by head circumference measurements [4,5]. A preliminary report [12] confirmed the ~25% decrease in brain volume shown earlier by neuropathologic studies [13]. It also demonstrated global reduction in cortical volume with gray and white matter being similarly involved [12]. Our study of lobar volumes in RS during childhood will be followed by sublobar analyses in an expanded sample that includes adolescents as well. Subsequent phases of this research program will include high precision manual delineation-based evaluations of the cerebral cortex and basal ganglia, as well as the sequential studies mentioned above.
3. Diffusion tensor imaging studies

Volumetric studies in RS indicate that cortical gray and white matter are involved to a similar degree [2–4,12]. These data are puzzling since neuropathologic studies demonstrate that in RS there is a marked reduction in neuronal size and dendritic arborizations, but no major qualitative changes in axons or myelin [4,10,14]. Moreover, in a recent magnetic resonance spectroscopic study described below we showed that, with the exception of the frontal lobe, the neuronal marker N-acetyl-aspartate (NAA) was more markedly decreased in the cortical white matter than in gray matter [15]. These data suggest that the decrease in cortical white matter volume in RS may not only be secondary to changes in neuronal somata, but also the consequence of a primary axonal disturbance. Elucidation of this issue requires the application of complementary in vivo imaging techniques, such as diffusion tensor imaging (DTI). DTI is a technique based on the restricted manner in which water diffuses in the white matter, as a consequence of the axonal fiber architecture of this region [16]. Two types of information can be obtained by DTI: quantitative parameters of water diffusion, which reflect axonal preservation, and imaging correlates of global white matter organization. The latter include maps of fiber bundle orientation (plane, direction) and higher resolution delineation of specific fiber pathways [17,18].

In order to evaluate axonal pathology, and to obtain a global view of white matter architecture in RS, we have begun to apply a relatively shorter (15 min) segmented-EPI-based DTI acquisition sequence. This axial plane protocol yields gapless 5-mm thick slices of the entire brain. We will evaluate axonal preservation by measuring DTI parameters such as fractional anisotropy. We will also determine any major change in regional fiber bundle orientation and size, as well as in certain large tracts such as the corpus callosum and cingulum by using color-coded DTI (anisotropy) maps as previously reported [17,19]. In this color-coded map, the two types of information obtained from DTI (diffusion anisotropy and fiber orientation) are displayed simultaneously; image intensity reflects anisotropy and fiber orientation is represented by color. We have successfully scanned sedated RS subjects, as shown in Fig. 2, and we are currently developing strategies for reducing motion-related artifacts in non-sedated age- and sex-matched normal controls.

Although color-coded maps may provide insight into selective involvement of fiber pathways in RS, a more precise delineation of these abnormalities requires fiber tracking techniques [18,20]. The latter are based on the identification of tracts using the color maps. Fiber bundles are then delineated by software that traces the trajectory of the vectors representing water diffusion. This process requires a higher spatial resolution than the aforementioned protocol (e.g. 3-mm thick slices), requiring longer scanning time up to 50 min [18]. These fiber delineation/reconstruction studies will focus on specific pathways, which have been postulated as involved by anatomical or neurochemical data [4,10,15,21–23]. An example of particular importance is the study of fronto-striatal circuits, since earlier volumetric studies have demonstrated that there is a selective reduction in the volume of the caudate nucleus in RS [2–4]. These studies will be correlated with functional imaging studies described below.

![Fig. 2. Color-coded anisotropy maps displaying axial slices, at the level of the dorsal basal ganglia, in RS (B) and age/sex-matched normal control (A). The white arrowheads show longitudinally-oriented (in green) dorso-lateral frontal fibers. The yellow arrowheads indicate laterally-oriented (in red) insular/posterior frontal fibers. Fibers running preferentially in the dorso-ventral plane are shown in blue.](image-url)
4. Magnetic resonance spectroscopic imaging

In vivo magnetic resonance spectroscopic imaging (MRSI) can detect important cerebral metabolites of interest in RS: N-acetyl aspartate (NAA), total choline (Cho), total creatine (Cr), and glutamate/glutamine. Since the underlying biochemical defect of RS is unknown, MRSI has the potential to reveal impairment of regional cerebral metabolism in RS non-invasively. We carried out high resolution, multi-slice $^1$H MRSI in 17 girls with RS. The control group consisted of nine healthy children. As shown in Fig. 3, average Cho concentration was 12% higher ($P < 0.005$) and average NAA concentration 11% lower ($P < 0.0001$) in RS compared to the control group. Regional metabolic differences included significantly lower NAA concentration in the frontal gray and white matter, insula, and hippocampus in RS, while no differences in regional Cho and Cr concentrations were found. A 20–38% higher Cho/NAA ratio in frontal and parietal gray and white matter, insular gray matter, and hippocampus ($P < 0.05$) and 14–47% lower NAA/Cr in frontal cortical gray matter, parietal and temporal white matter, insula and putamen ($P < 0.05$) were found in RS subjects compared to controls. Patients with seizures had higher average concentrations of Cho, Cr, and NAA compared to those without seizures (8–19%, $P < 0.05$). In conclusion, metabolic impairment in RS involves both gray and white matter, and particularly involves frontal and parietal lobes, and the insular cortex. Loss of NAA most likely reflects reduced neuronal and dendritic tree size, while increased Cho may be due to gliosis.

5. CBF measurements with MRI

Cerebral blood flow (CBF) has been evaluated by PET [22] and SPECT scans [24–27]. All studies have demonstrated reduced global blood flow but more so in the frontal regions. Nielsen et al. [24] postulated that it resembled an immature pattern, whereas Lappalainen et al. [25] argue that this may not be the case as some of their younger patients did not demonstrate changes in blood flow. Uvebrandt et al. [26] noted that in addition to frontal regions there was reduced mean perfusion in midbrain and upper brainstem, corresponding to the pathological and radiological changes in frontal, midbrain, and caudate regions. We performed PET studies in four adult patients using O-15 labeled water, and observed low normal levels of blood flow in the frontal regions, similar to Yoshikawa et al. [22] observations in older RS patients. Therefore, CBF in the older RS patient may be improved relative to younger RS patients. The biological basis for the improvement in frontal blood flow is uncertain.

While PET and SPECT are excellent methods for determination of CBF and metabolism, it is difficult to justify their use in young children (particularly young control subjects) because of the radioactivity associated with these procedures. However, recent developments in MRI make it a good alternative to PET for the measurement of CBF, using radio-frequency (RF) labeling of the arterial water protons as an intrinsic free diffusible tracer. We used in this study the recently-published Transfer Insensitive Labeling Technique (TILT) sequence [28] to measure the CBF.
without exposure to radioactivity, that can therefore be used serially to document progressive changes or effects of therapeutic interventions. For this reason, and following our preliminary PET studies using O-15 labeled water on four adults with RS, we conducted a series of measurements of CBF using MRI in a completely non-invasive way. This non-invasive TILT measurement of perfusion has been used in 15 RS patients so far. This method is based on the concept of arterial spin labeling (ASL) [29], in which the magnetization of arterial blood is inverted proximally to a particular slice of interest about 1–2 s before the acquisition of the actual slice. During the delay between labeling and acquisition, the tagged blood has time to reach the slice of interest and exchange with the surrounding brain tissue. A subtraction of images acquired with and without labeling provides information on the exchanged water spins between arteries and tissue, and therefore on perfusion. In order to obtain qualitative measurement of the perfusion, a single axial slice parallel to the AC–PC line just above the ventricles was acquired in all patients, with the following parameters: slice thickness = 8 mm, matrix = 64 x 64, FOV = 240 mm, single shot SE-EPI, 50 averages. Absolute T1 measurements were also performed in the same slice of interest to gain information on absolute quantification of CBF by using the model originally developed by Williams et al. [29]. Preliminary results show a lower perfusion of the frontal lobe compared to other brain areas, and are in agreement with previous studies in the literature; however, no comparison was performed with an aged-match group of volunteers so far. Fig. 4 shows this finding in a 7-year-old patient. Efforts are underway to recruit age-matched control subjects. Further developments of the technique include improved quantification, labeling efficiency, and whole brain coverage. These advances are critical for better comparison with absolute blood flow studies of the whole brain performed with PET or SPECT.

6. Neurotransmitter receptors and energy metabolism

Several specific cerebral functions of individuals with RS can be evaluated with positron emission tomography (PET) and SPECT studies. Specifically the dopamine and benzodiazpine receptors have been studied with PET and SPECT analyses [30–32]. Our PET scan studies involved individual thermoplastic face masks constructed to fit the facial features of all subjects to facilitate repositioning and realignment for multiple scans, that are coregistered with the analogous MRI or CT images for accurate localization of the anatomic landmarks. This approach capitalizes on the strengths of functional and structural neuroimaging techniques. All studies are conducted under the strict supervision of a pediatric anesthesiologist.

Dopamine receptors and transporters in RS: We specifically focussed on the dopaminergic system initially because of the abnormal hand movements, and progressive rigidity seen in RS patients. To determine the pre-and post-synaptic sites we performed PET studies to determine the number of D2 dopamine receptors in caudate and putamen, and dopamine transporters, which correlate with the concentration of dopaminergic terminals projecting from dopaminergic neurons to caudate and putamen.

Dopamine D2: We studied 12 adult patients (15–39 years), using [33] N-methylspiperone, who showed low normal levels of D2 dopamine receptors. Although this was in contrast to the observations of Chiron et al. [31], their patients were younger (4–15 years). These findings, combined with the autoradiography studies in RS brain

Fig. 4. (A) Axial slice parallel to the AC-PC line showing low blood flow in the frontal area (arrows) of the brain in a 7-year old RS patient. CBF is color-encoded from black (low) to red (medium) to yellow (high blood flow). (B) Corresponding absolute T1 map calculated using images from multiple Inversion-Recovery sequences in the same anatomical location.
tissues by Blue et al. [34] showing significant increases in the glutamate/NMDA receptors in frontal regions of younger patients, suggest age-specific changes in RS. The increases in dopamine D_2 and glutamate/NMDA receptors in younger patients, parallel the clinical worsening in Stage 2 of the disease. The biological basis for the abnormal receptor proliferation in younger RS subjects remains speculative, but corresponds to the period of synaptic amplification in normal subjects. Viewed in light of the function of MeCP2 gene that is defective in a majority of RS cases, the aberrant, age-sensitive neurotransmitter receptor increases must reflect the collective effect of conflicting genetic influences which disrupt brain growth during the most dynamic phase of brain development [5].

**Dopamine transporters:** The compound [11C] WIN-35, 428 was utilized to study dopamine transporters to estimate the density of DA-containing neuron terminals in caudate and putamen [33] of 16 patients (aged 18–40 years) with RS. Dopamine transporters in the caudate and putamen were in the low-normal range in these older patients when compared to controls. To ensure that a reduction in caudate volume would not confound the results, a rigorous partial volume correction of the caudate time-activity curve was performed. This is the first in vivo study that demonstrates density of dopaminergic neurons in RS, and surprisingly does not show a significant reduction. This is in contrast to Lesch–Nyhan disease (LND) in which it is drastically reduced, compared to normals (NOR) [35] as shown in Fig. 5 (with permission from PNAS, 1996, Vol. 93, p. 5541).

**Brain glucose metabolism in RS:** In normal children cerebral glucose metabolic rates are low at birth, reach adult values by 2 years of age, and then progressively increase up to twice the adult values until 9 years of age. Then there is a progressive decline in cerebral glucose metabolic rates to the normal adult values at the end of the second decade of life [36] similar to the synaptic growth profile [37]. We conducted studies utilizing (18F)-fluorodeoxyglucose (FDG) PET scans initially to determine whether seizure foci localized anteriorly as shown by their electroencephalograms (EEG) correlated with changes in glucose metabolism. Surprisingly six RS patients studied demonstrated a relative increase in uptake of glucose in the frontal regions (Fig. 6). This is in contrast to the CBF studies, which shows reduced blood flow to the frontal regions. The occipital/whole brain ratio of cerebral glucose metabolic activity is reduced in RS in contrast to normal control subjects [30]. On the other hand, the cerebellum/whole brain ratio is increased in RS patients in contrast to normal control subjects. In addition, cerebral glucose metabolism is increased in the left hemisphere of subjects with RS in contrast to the right (Roa Villemagne et al., in preparation).

This dichotomy between CBF and energy metabolism needs to be explained and is postulated in light of the increased NMDA glutamate receptors in prefrontal cortex in RS [21].

Neuronal activity is tightly linked to energy metabolism that is derived from glucose in brain. The FDG PET scans detect brain energy derived from glucose metabolism. Glutamate (Glut) is the excitatory neurotransmitter released by the majority of neurons during excitation and is recognized by its post-synaptic receptors. Glutamate is removed from the synaptic cleft by transporters-EAAT (shown to be increased in RS by Johnston et al. in this volume) utilizing the electrochemical gradient of Na\(^+\). A tightly coupled glutamate and Na\(^+\) are taken up by astrocytes that are present at every glutamatergic synapse [39]. The astrocytes maintain the Na\(^+\) gradient by the use of Na\(^+\),K\(^+\)-dependent ATPase (adenosine triphosphatase). Glutamate is converted to glutamine (Gln) within the astrocyte and recycled back to the neuron to replenish the neurotransmitter pool, as shown in Fig. 7. Activation of the glutamate transporter stimulates astrocytes to increase glucose uptake to provide the required ATP, which is achieved through their end feet placed strategically on capillaries [38,40]. The stoichiometry between
oxidative glucose metabolism and glutamate neurotransmitter cycling in the cortex is close to 1:1, implying that cortical glucose metabolism is a measure of synaptic glutamatergic neuronal activity [41,42]. Using $^{13}$C glucose NMR, the rate of glutamate-glutamine neurotransmitter cycle has been shown to be high within the cortex of rats and humans, and it increases during brain activity [43,44] in a 1:1 molar ratio with oxidative glucose metabolism. Therefore, in RS, the increased levels of glutamate in CSF and MRS studies [45–48], and increased glutamate/NMDA receptors in postmortem studies [21,34], suggest that there must be increased glutamate-glutamine neurotransmitter cycling at the synapses, accounting for the increased glucose over the frontal regions as seen in our PET studies. In contrast, the reduced CBF in the same regions of brain in RS is possibly due to poor post-synaptic neuronal response to the excitatory neurotransmitter as there is a lack of pathological evidence for ischemic injury. This apparent uncoupling of CBF to glucose utilization deserves further study to improve understanding of the pathophysiology of RS.

In summary, our in vivo imaging studies confirm diffuse brain involvement with areas of selective vulnerability. A decrease in brain volume is documented, but the growth trajectories of gray and white matter may occur at a normal pace. Regional brain involvement especially affecting the basal ganglia is relevant to our PET studies, and rigorous partial volume corrections are undertaken for these tests. Reduced NAA and increased choline in frontal regions which correspond to areas of reduced CBF suggests increased gliosis as shown in pathological studies as well.

Fig. 6. Note increased cerebellar and frontal glucose metabolism with significant reductions in the posterior brain regions in this 8-year-old RS girl.
[10]. The involvement of frontal lobes with low normal levels of D\textsubscript{2} receptors and low dopamine transporters in older RS patients would suggest susceptibility of frontal cortical-striatal projections. Our studies of DTI propose to identify axonal injury and such pathways that may be involved in RS.

Taken together with the neuropathology, receptor autoradiography, neurotransmitter assays, and gene microarray observations, it appears that the developing RS brain is subject to maximum insult during the phase of rapid synaptic proliferation. Increased CSF glutamate, glutamate/glutamine by MRSI, and increased glucose in FDG PET studies indicate enhanced release of glutamate from presynaptic terminals. The cycling of excessive glutamate through the synaptic cleft, astrocytes, and its return to the pre-synaptic terminals makes synapses also a special target for this disorder, as the developing brain is especially vulnerable to excitotoxic injury [49]. The excessive glutamate release could result from abnormalities in expression of genes involved in pre-synaptic terminals due to the defective MeCP2 gene, including those noted to be abnormal in gene microarray analysis of RS tissue (Johnston et al. in this volume).

Increased synaptic cycling of glutamate combined with increases in glutamate/NMDA in younger RS girls provides a plausible explanation for the seizures, EEG abnormalities, and respiratory irregularities, presenting as signs of encephalopathy in Stages 2 and 3 of RS. Some in vivo studies will be performed serially by us to study evolution of the disease process, and better explain the age-dependent changes in clinical manifestations. Such information will be valuable in identifying biologically-based therapies and effects of interventions.

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