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Detection of MPTP-induced Substantia Nigra Hyperechogenicity in Rhesus Monkeys by Transcranial Ultrasound

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Abstract

Detection of substantia nigra (SN) hyperechogenicity by transcranial ultrasound has been proposed as a putative biomarker to differentiate between idiopathic Parkinson's disease (PD) and other forms of parkinsonism. In the present study, we evaluated the feasibility of using transcranial ultrasound to detect SN echogenicity in normal and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated Rhesus monkeys, a well-established model of PD. All animals had natural temporal bone windows for transcranial sonography. We could show that it is possible to visualize major brain landmarks including the "butterfly shaped" midbrain, basal cisterns, third and lateral ventricles in all animals by transcranial ultrasound. Blinded assessments showed that all normal monkeys had no SN hyperechogenicity. Bilaterally parkinsonian (overlesioned) monkeys showed hyperechogenicity of both SN, whereas right hemiparkinsonian monkeys only showed left nigral hyperechogenicity. These findings confirm the feasibility of transcranial ultrasound to detect SN hyperechogenicity in MPTP-treated Rhesus monkeys and suggest that this animal model may provide a platform for understanding the pathophysiological basis of nigral hyperechogenicity.

Keywords

basal ganglia; nigrostriatal degeneration; movement disorders; *Macaca mulatta*; non-human primate

Introduction

Detection of hyperechogenicity of the substantia nigra (SN) by transcranial ultrasound is used as a diagnostic marker for the diagnosis and differential diagnosis of Parkinson's disease (PD). Several studies indicate that hyperechogenicity is present with a high degree of sensitivity in the vast majority of patients with PD (Berg et al. 2001b; Berg et al. 2001c; Ruprecht-Dorfler et al. 2003; Berg et al. 2005; Berg 2006a; Berg 2006b; Prestel et al. 2006; Schweitzer et al. 2006; Ruprecht-Dorfler et al. 2007; Schweitzer et al. 2007; Walter et al. 2007a; Walter et al.

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2007b; Berg 2008; Berg et al. 2008; Gaenslen et al. 2008) and that such hyperechogenicity is not or only rarely and to a lesser extent present in other parkinsonian syndromes like MSA-P, PSP and toxin-induced welding-related parkinsonism, indicating a sufficient specificity (Behnke et al. 2005; Walter et al. 2008). Hyperechogenicity of the SN has been proposed as a criterion to differentiate between PD and these other parkinsonian conditions in early disease stages when clinical differentiation is quite difficult (Walter et al. 2003; Prestel et al. 2006; Walter et al. 2007b). Hyperechogenicity of the nigra can also be used in psychiatric patients to predict the risk for parkinsonism following exposure to antipsychotics (Berg et al. 2001b; Berg 2008). More recently, hyperechogenicity in the SN region has also been identified in patients with idiopathic REM sleep behavior disorder (Stockner et al. 2009), a well known precursor to overt manifestation of cardinal motor symptoms of PD. The biological basis of nigral hyperechogenicity is not completely understood. Findings from animal experiments as well as post-mortem examinations strongly suggest that increased tissue iron content is a major factor contributing to the enhanced echosignal (Berg et al. 1999; Berg et al. 2001a; Berg et al. 2002; Berg et al. 2006; Berg 2007).

Understanding the biological basis of nigral hyperechogenicity may provide an opportunity to understand mechanisms related to the pathophysiology of PD and thus aid in discovering new therapies or methods for prevention. Animal models of PD may offer an interesting tool to shed light into some of these mechanisms. We performed transcranial ultrasound studies of the SN in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated Rhesus monkey model of PD. We hypothesized that nigral hyperechogenicity if present will correlate with clinical parkinsonism in these animals and that this method could be potentially used to differentiate nigrostriatal degeneration during various stages of MPTP treatment (stable hemiparkinsonian versus bilateral parkinsonian).

Materials and Methods

Animals

Nine adult female Rhesus (*Macaca mulatta*) monkeys (4–8.5Kg) housed in accordance to NIH guidelines for the ethical treatment of animals in an AALAC accredited facility were used in these experiments. Experiments were in compliance with research protocols approved by the Institutional Animal Care and Use Committee. Four monkeys were clinically normal monkeys with no apparent deficits and five monkeys were rendered parkinsonian by MPTP administration.

Intracarotid and systemic injections of MPTP

Initially, left intracarotid infusions of MPTP were performed on 5 monkeys as described previously (Subramanian et al. 1997; Subramanian et al. 2005). Briefly, the left common carotid artery and the bifurcation were exposed under general anesthesia and the external carotid was ligated. Retrograde manual injection of MPTP solution 0.5mg/Kg (Sigma Chemicals, USA) was performed through the left internal carotid artery in each animal using strict aseptic technique. All animals were assessed for their parkinsonism using a behavioral rating scale modeled after the Unified Parkinson's Disease Rating Scale, part III (motor sub scale, mUPDRS). Each animal was observed for a minimum of 3 weeks to determine if there was a clinical effect from the injection before a repeat injection was performed. A maximum of 4 left intracarotid injections was required to obtain the stable hemiparkinsonian state in these 5 animals. These behavioral assessments (mUPDRS) were performed by a blinded rater who was unaware of the number of MPTP injections or their previous behavioral state. All 5 animals developed a stable right hemiparkinsonian state that remained behaviorally unchanged over a minimum period of 3 years prior to transcranial ultrasound.

Additional intravenous injections of MPTP (0.2 mg/Kg/IV) made 2 of these 5 stable hemiparkinsonian monkeys stable bilaterally parkinsonian (overlesioned model) (Oiwa et al. 2003). These 2 animals were also rated for stability using blinded mUPDRS assessments for a minimum of 6 months before transcranial ultrasound examinations. To confirm drug responsive parkinsonism in the MPTP-treated parkinsonian animals, all 5 animals were treated with carbidopa/levodopa, 300mg twice a day orally for 2 weeks. All 5 parkinsonian animals showed consistent response to levodopa treatment. The average improvement in mUPDRS scores was 52.6%. Post-MPTP mUPDRS scores were 12.55 (SEM=2.079) that significantly improved to 5.95 (SEM=1.062) after 2 weeks of levodopa treatment (mUPDRS scores represent mean of 2 independent blinded ratings 3 days apart, Student's t-test: p-value=0.02). Withdrawal of anti-parkinsonian treatments (levodopa washout for 2 weeks) restored the baseline clinical levels of parkinsonism in all 5 animals. All MPTP-treated animals were imaged with high resolution magnetic resonance imaging (MRI) using methods we have previously described (Fowler et al. 2001; Subramanian et al. 2005) that revealed no evidence of any structural lesions in these animals (Fig. 1C). Thus, all animals used in this study were clinically and behaviorally normal (N=4), stable hemiparkinsonian (N=3) or stable bilateral overlesioned parkinsonian (N=2) with excellent response to levodopa treatments and normal brain MRI.

Transcranial Ultrasound

All monkeys were tested by a single blinded investigator (DB) who had no knowledge of the motor and behavioral status of the animals. A Siemens Sequoia high-end ultrasound machine with a 5V2c (Siemens Acuson) pediatric probe operated at a frequency of 5MHz was used for these studies. We chose this probe to correct for the animal head size, as the Rhesus monkey head size approximates the head size of a premature human infant. B-mode echo with black and white imaging was utilized. The B-mode imaging technique we used was the same as the technique we applied in previous human studies to visualize human brain tissue transtemporally. It was agreed on defining hyperechogenicity in relation to the surrounding brainstem parenchyma as either present or absent as there was no ambiguity in this finding in the 3 different groups of animals. Rating for the presence or absence of hyperechogenicity at the anatomical site of the SN was performed in conformity with previous reports on the assessment of SN hyperechogenicity in patients with PD (Berg et al. 2008). Quantitative definition of the size of hyperechogenicity was not attempted in this pilot study. All animals were anesthetized for the brief period of the tests using intramuscular ketamine injections. Animals were placed in the lateral position and the ultrasound probe was applied on the skin just anterior to the external auditory meatus. This location is identical to the approach to the temporal bone window used in human transcranial studies. The term “bone window” is used to denote naturally occurring thin areas in the skull that permit insonation of intracranial structures using transcranial ultrasound. An additional and sometimes better natural bone window was detected in some animals right behind the external auditory meatus. All 9 animals recovered completely from the procedure and had no clinically apparent consequences.

Results

In line with published human studies using the same B-mode echo black and white imaging, transcranial ultrasound was able to image and delineate major brain landmarks including the “butterfly shaped” midbrain, basal cisterns, third and lateral ventricles in all 9 monkeys. The red nucleus and basal cisterns were easily noted in most animals at the level of the mesencephalon. Tilting the ultrasound probe approximately 15 degrees upward from the mesencephalic scanning plane allowed visualization of the hyperechogenic ependyma of the third ventricle and the hypoechogenic basal ganglia.

Blinded assessments showed that 4 out of the 4 normal monkeys had no change of echogenicity at the anatomical site of the SN in comparison to the surrounding brain stem (Fig. 1B). Two out of 2 MPTP-treated animals that had initially received left intracarotid MPTP injections to cause stable hemiparkinsonism and later received multiple doses of systemic MPTP injections to cause bilateral parkinsonism (overlesioned model) showed increased echogenicity (hyperechogenicity) of both SN. Three stable hemiparkinsonian monkeys showed left nigral hyperechogenicity (Fig 1A). Therefore, it can be stated that in both MPTP models, a distinctly visible hyperechogenic comma shaped structure was visible at the anatomical site of the SN, which allowed the classification into the group of MPTP-treated monkeys by the blinded sonographer. Fisher's exact test revealed statistical significance for the observed results (p value=0.007).

Discussion

Our results demonstrate for the first time the ability of transcranial ultrasound to visualize parenchymal structures *in vivo* in monkeys. Moreover, it seems suitable to detect hyperechogenicity at the anatomical site of the SN in MPTP-treated Rhesus monkeys. The presence of nigral hyperechogenicity unilaterally in the unilaterally affected animals and bilaterally in the bilaterally affected animals suggests that the Rhesus monkey model may provide a platform to understand the mechanisms leading to the pathophysiological basis of nigral hyperechogenicity in PD. This finding is quite different from what has been reported in PD patients, where hyperechogenicity appears bilaterally even in early PD patients with clinically apparent minimal symptoms. Still, in PD patients hyperechogenicity is more pronounced on the SN contralateral to the side that was clinically affected earlier and the side that is more severely affected (Berg et al. 2008). One possible reason for our ability to detect distinct unilateral and bilateral hyperechogenicity in the unilaterally affected and bilaterally affected monkeys could be related to the severity of the MPTP-induced lesion in the SN. Intracarotid MPTP injections are highly potent in their toxicity. Such a potent unilateral exposure to a neurotoxin dramatically differs from the more gradual bilateral, but, asymmetric loss of dopaminergic neurons in patients with idiopathic PD. Moreover, nigral degeneration in PD occurs much more gradually than in the MPTP-induced model of PD. These differences may explain why staged, side specific hyperechogenicity of the SN is not readily apparent in PD patients with varying degrees of parkinsonism.

In humans, quantification of the grades of hyperechogenic signal at the SN is not possible – most likely because of the scattering of ultrasound waves at the bone window. Therefore, after several years of only rating the SN as either “hyperechogenic” or “normal”, the areas of hyperechogenicity at the anatomical site of the SN are now being quantified by encircling which enables a planimetric measurement of the structure (Berg et al. 2008). To obtain such a cut off value for the area of “hyperechogenicity at the level of the SN”, a large group of healthy subjects as well as PD patients needs to be examined in every lab using the method. In the present pilot study designed to demonstrate feasibility, we could determine the “presence” or “absence” of hyperechogenicity at the anatomical site of the SN. A more detailed characterization to determine cut off values could not be attempted due to the small sample size. Future studies in larger groups of monkeys may permit determination of quantitative cut off values for the area of SN hyperechogenicity in monkeys. Such studies combined with histopathological analysis at each stage may help to understand aspects of the pathophysiological basis of nigral hyperechogenicity in PD.

Although the pathophysiological basis of nigral hyperechogenicity is not fully understood, some studies suggest that iron content in the nigra may be closely correlated with the presence of nigral hyperechogenicity (Berg et al. 1999; Zecca et al. 2005; Berg 2006a; Berg et al. 2006). A recent investigation further supports this hypothesis of iron content by correlating

basal ganglia hyperechogenicity (SN and lenticular nucleus) to changes in T2-weighted MRI hypointensity (Walter et al. 2009). Other studies in MPTP-treated monkeys suggest significant increase in the iron content in the MPTP lesioned SN post MPTP treatment, and the increase in nigral iron was significantly correlated to the extent of dopaminergic cell loss (He et al. 2003). Histological studies provide evidence that in this MPTP monkey model of PD, unilateral intracarotid injections of MPTP causes a predominantly unilateral loss of SN neurons in the mesencephalon ipsilateral to the intracarotid injections and relative sparing of the contralateral nigra. In animals that receive subsequent sequential systemic injections of MPTP (overlesioned model), there is bilateral substantial asymmetric loss of dopamine in the striatum and associated SN cell loss (Bankiewicz et al. 1986; Eberling et al. 1998; Oiwa et al. 2003). Histological assessments of Rhesus monkeys treated with MPTP unilaterally to cause stable hemiparkinsonism and overlesioned to cause bilateral parkinsonism in our laboratory also reproduce these results consistently (Subramanian et al. 1997; Subramanian et al. 2005). Therefore, the unilateral occurrence of hyperechogenicity in hemiparkinsonian animals in the corresponding nigra suggest that critical thresholds of pathophysiological changes in the SN are necessary before hyperechogenicity of the SN can be detected by transcranial ultrasound. In humans, no such threshold has been detected yet. Rather, the area of SN hyperechogenicity does not change in the advancement of PD progression as seen in a 5-year longitudinal follow up study (Berg et al. 2005; Berg et al. 2008). SN hyperechogenicity has been found in clinically healthy subjects with a vulnerability to nigrostriatal impairment. In humans it is known that the premotor period may last years to decades (Berg 2008). During this time, about 60% of the dopaminergic neurons are lost until first motor symptoms allow the clinical diagnosis. Presumably, the pathophysiological process has already led to major alterations in the SN including accumulation of iron and protein aggregation, which potentially lead to increased echogenicity of the SN on ultrasound in such clinically unaffected individuals (Berg et al. 2008). Further changes in the process of the disease do not appear to affect the size of echogenic signal at the anatomical site of the SN in humans, possibly because of mutually neutralizing effects (i.e. reduction in cell count but increase in iron) that occurs over a lengthy period of time. In animal models, however, the lesion to the SN is made artificially in a shorter time span. Here, the staged induction of SN degeneration appears to be more amenable to be monitored via sequential transcranial ultrasound examinations and correlated to motor deficits. Although the pathological mechanisms of MPTP-induced parkinsonism differ from that of idiopathic PD, the findings of the present study indicate that the MPTP-treated Rhesus monkey may provide important information about the underlying pathophysiological basis of SN hyperechogenicity. This well-characterized animal model of PD may be utilized in future studies for repeated and longitudinal investigations that monitor changes of SN hyperechogenicity as well as correlating these changes to long-term and post-mortem histological, biochemical and imaging analysis studies. Thus, this model may help verify the underlying reasons of nigral hyperechogenicity that are difficult to evaluate in the PD patient population.

The use of 5MHz probe was necessitated by the smaller size of the brain in the monkey as opposed to the 2MHz probe that is utilized for human studies with a larger brain. This change in frequency does not appear to have influenced our findings at the level of the SN or other basal ganglia structures that were visualized. The echogenicity and visualization of the aqueduct, basal cisterns, lateral ventricles and the striatum were exactly as noted in previously published human studies. Therefore, it is very likely that the hyperechogenicity we note in MPTP-treated monkeys is the appropriate correlate to the hyperechogenicity noted in SN in PD patients. We do acknowledge that the mechanisms involved in creating the animal model (neurotoxin induced) is different from the gradual neurodegeneration noted in idiopathic PD. However, the MPTP-treated monkey has been a great animal model in the past 3 decades and led to numerous insights into the pathophysiology of PD and helped develop multitudinous treatment strategies to alleviate human suffering in PD. Therefore, future studies that correlate

the histopathology and biochemistry in the MPTP-treated monkey to SN hyperechogenicity is likely to yield important clinically relevant pathophysiological and therapeutic insights.

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Fig 1.

A. Transcranial ultrasound obtained through a preauricular bone window from a right hemiparkinsonian monkey showing hyperechogenicity of the left SN (red arrow). **B.** Midbrain ultrasound through a preauricular transcranial window showing bilateral symmetric absence of hyperechogenicity. The ultrasound examination was performed by an investigator blinded to the type of MPTP lesion in the monkeys as well as the mUPDRS. **C.** Brain MRI of monkey in 1A showing midbrain structures. M-midbrain, b-bone window, Sk-skull, A-aqueduct, BC-basal cisterns, L-left, R-right