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Neuroanatomical substrates for paroxysmal dyskinesia in lethargic mice

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The paroxysmal dyskinesias are a group of neurological disorders described by intermittent attacks of involuntary abnormal movements superimposed on a relatively normal baseline. The neuroanatomical substrates for these attacks are not fully understood, though available evidence from studies of affected people and animal models points to dysfunction in the basal ganglia or cerebellum. In the current studies, the anatomical basis for paroxysmal dyskinesias in lethargic mice was determined via histochemical methods sensitive to changes in regional brain activity followed by surgical elimination of the suspected source. Cytochrome oxidase histochemistry revealed increased activity in the red nucleus. Surgical removal of the cerebellum worsened ataxia but eliminated paroxysmal dyskinesias. These studies support the hypothesis that abnormal cerebellar output contributes to paroxysmal dyskinesias.

Keywords: Animal model; Dystonia; Ataxia; Channelopathy; Calcium

Introduction

The paroxysmal dyskinesias are a group of clinical neurological disorders characterized by attacks of involuntary abnormal movements superimposed on a normal baseline (Fahn and Marsden, 1994; Demirkiran and Jankovic, 1995; Bhatia, 1999). There are several subtypes that are classified according to a number of factors including the duration of the attacks, the nature of the abnormal movements, and the influences that trigger attacks.

The neurobiological mechanisms underlying the paroxysmal dyskinesias are not well understood. Both genetic and non-genetic causes are known. Inherited causes among humans include mutations affecting the myofibrillogenesis regulator protein and ion channels (Giffin et al., 2002; Ohtsuka et al., 2003; Lee et al., 2004; Rainier et al., 2004; Du et al., 2005). At the neuroanatomical level, there is limited information on the source of the abnormal movements. The types of abnormal movements seen in the paroxysmal dyskinesias are commonly associated with dysfunction of the basal ganglia, implying that these motor pathways are involved (Fahn and Marsden, 1994; Demirkiran and Jankovic, 1995; Bhatia, 1999). In keeping with this suggestion, lesions of the basal ganglia are found in some people with paroxysmal dyskinesias (Blakeley and Jankovic, 2002). However, such lesions are uncommon, and most affected individuals have none. In individuals without apparent brain lesions, functional imaging has been used to identify areas of abnormal metabolic activity. One study found abnormal function of the basal ganglia (Lombroso and Fischman, 1999), while another found abnormal function of the cerebellum (Kluge et al., 1998). These studies implicate specific brain regions, but they do not provide a definitive answer, because the primary source of the movements cannot be distinguished from secondary compensatory changes. The neuroanatomical foundations for paroxysmal dyskinesias in humans therefore remain uncertain.

Animal models can provide valuable tools for more rigorous studies of the responsible brain regions. An inbred line of hamsters with paroxysmal dyskinesias has no apparent neuropathological defects, but biochemical and physiological abnormalities are found in many regions (Loscher et al., 1989; Wahnschaffe et al., 1990; Loscher and Horstermann, 1992; Loscher et al., 1994; Pratt et al., 1995; Gernert et al., 1997; Nobrega et al., 1997; Gernert et al., 1998; Nobrega et al., 1999; Rehders et al., 2000; Bennay et al., 2001; Gernert et al., 2002). Abnormal metabolic activity detected by functional histochemical stains such as cytochrome oxidase (CO) histochemistry or [^{14}C] 2-deoxyglucose (2DG) uptake is found in several areas, including the basal ganglia and cerebellum (Nobrega et al., 1998; Richter et al., 1998). These widespread abnormalities make it difficult to determine the precise anatomical pathways for paroxysmal dyskinesia in these hamsters.

More recently, paroxysmal dyskinesias have been described in association with mutations affecting calcium channels in mouse models (Barclay et al., 2001; Fureman et al., 2002; Khan and Jinnah, 2002; Du et al., 2005). Tottering mice exhibit paroxysmal dyskinesias triggered by stress, caffeine, or alcohol (Fureman et al., 2002). Neuropathological studies of Nissl-stained material reveal no abnormalities, but maps of regional activity derived from c-*fos* in situ hybridization demonstrate selective activation of the

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cerebellum and connected structures such as the red nucleus, thalamus, and cortex (Campbell and Hess, 1998). 2DG autoradiography also reveals the most prominent abnormalities to be in the red nucleus and thalamus (Noebels and Sidman, 1979). The cerebellum is more directly implicated as the source of paroxysmal dyskinesias in tottering mice by observations that eliminating cerebellar output after cross breeding the animals with *pcd* mutants to cause selective degeneration of cerebellar Purkinje cells results in mice with ataxia, but without paroxysmal dyskinesias (Campbell et al., 1999).

Lethargic mice also exhibit paroxysmal dyskinesias (Khan and Jinnah, 2002). Unlike tottering mice, dyskinesias are triggered by procedures that promote motor activity, such as placing them in a large open cage or providing motor stimulants. The goal of the current studies was to delineate the neuroanatomical basis for paroxysmal dyskinesias in lethargic mutant mice, via histochemical methods sensitive to changes in regional brain activity and surgical elimination of the suspected source.

Methods

Animals

Because homozygous male lethargics do not reproduce, homozygous females (*cachb4*$^{lh/lh}$) and normal F1 hybrid (C3 H × C57BL/6J) males obtained from The Jackson Laboratories (Bar Harbor, ME) were bred to generate heterozygotes. The heterozygotes were then crossed to generate homozygous lethargics. Their normal and heterozygous littermates served as controls. Mice were weaned at 4 weeks of age and housed in groups of 2–8 with a 14:10-h light:dark cycle and free access to food and water. Animals were 8–14 weeks of age at the time of testing. All animal procedures were conducted in accordance with guidelines established by the Johns Hopkins University Animal Care and Use Committee and National Institutes of Health.

Experimental design

We used a two-step approach to delineate the neuroanatomical substrates for paroxysmal dyskinesias. The first step involved mapping regions of abnormal brain activity with two well-established and complementary functional histochemical methods. Cytochrome oxidase (CO) is a mitochondrial enzyme that is regulated in part by metabolic demands. Multiple studies have shown that its activity is increased or decreased in parallel with changes in regional brain activity (Wong-Riley, 1989). Alterations in CO activity generally require chronic changes in regional activity. Acute or transient changes often have little influence. In comparison, the immediate early genes are less sensitive to chronic changes but instead respond to acute sustained increases in neural activity associated with calcium influx (Sharp et al., 1993). These two functional histochemical methods are sensitive to different metabolic influences, so they often provide overlapping but complementary results.

Studies using these methods often compare more than 40 brain regions, but such broad surveys increase the chances of finding spurious differences unless stringent statistical corrections are applied. Because the goal of the study was to address the substrates for the motor disorder, we focused on 10 regions relevant to motor control rather than a broad survey of multiple regions not relevant to the question under consideration. Though multiple sites within each region were sampled, the results for each region were combined because of the lack of any significant subregional differences between experimental and control groups.

Both histological methods are useful for correlating changes in regional brain activity with a specific neurological disorder, but their correlational nature precludes using them for establishing a definitive causal link between identified brain regions and the disorder. To establish a causal link, the second step of our experimental design involved an attempt to eliminate the paroxysmal dyskinesias by surgical removal of the suspected source. The histochemical studies of lethargic mice revealed evidence for abnormal activity of the red nucleus, and prior studies of paroxysmal dyskinesias in tottering mice implicated abnormal cerebellar activity (Campbell and Hess, 1998; Campbell et al., 1999). Together these findings suggest the hypothesis that abnormal cerebellar output might be responsible for paroxysmal dyskinesias in lethargic mice. To assess the role of the cerebellum, paroxysmal dyskinesias were evaluated in lethargic mice before and after cerebellectomy.

c-fos in situ hybridization

The method for in-situ hybridization followed previous descriptions (Campbell and Hess, 1998). Briefly, brains were collected after decapitation, rapidly frozen in isopentane cooled on dry ice, and stored at −70 °C. Twenty-micrometer coronal sections were cut at 200 μm intervals using a cryostat through the entire brain, thaw-mounted onto Fisher SuperFrost Plus microscope slides (Fisher, Newark, DE), and stored at −70 °C. In situ hybridization for c-*fos* mRNA was conducted with [^{35}S]-labeled riboprobes. Processed sections were apposed to X-ray film for 2–4 days, and labeled regions were identified and quantified from the films with an MCID image analysis system (Imaging Research, St. Catherines, Ontario). Digital images were obtained from the films at a 640 × 480 pixel resolution and optical density was determined for predefined regions.

Cytochrome oxidase histochemistry

The CO histochemistry was modified from previous methods (Strazielle et al., 1998). Brains were rapidly removed, frozen in isopentane cooled on dry ice, and stored at −70 °C. Brains were sectioned as described above. Prior to staining, sections were fixed with 0.5% glutaraldehyde and 10% sucrose in 0.1 M phosphate buffer at pH 7.6 for 5 min. They were then washed 4 times for 5 min with 10% sucrose in 0.1 M phosphate buffer. Sections were incubated for 1 h in the dark with constant stirring at 37 °C in a solution consisting of 0.05% diaminobenzidine, 0.02% reduced cytochrome *c* from horse heart, 4% sucrose, and 0.02% catalase in 0.1 M phosphate buffer, pH 7.4. They were then washed for 5 min with 4% sucrose in 0.1 M phosphate buffer, followed by immersion in 10% buffered formalin with 10% sucrose for 30 min. The 4% sucrose wash was repeated 3 × 5 min, sections were dehydrated in graded ethanol and xylenes, and slides were coverslipped with Permount. The CO histochemical reaction was quantified with sections of whole homogenized brains at varying thicknesses to construct a standard curve as previously described (Gonzalez-Lima and Jones, 1994). To make these standards, whole brains were homogenized using a tissue homogenizer with a Teflon-glass tube and pestle in Tris buffer to a final concentration of 50% w/v. The homogenate solution was aliquotted into 2 ml centrifuge tubes. Tubes were centrifuged for 5 min at 5000×*g* and the supernatant was removed. The remaining tissue was frozen in isopentane on dry ice.