Age-Dependent Biphasic Changes in Ischemic Sensitivity in the Striatum of Huntington’s Disease R6/2 Transgenic Mice

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Klapstein, Gloria J. and Michael S. Levine. Age-dependent biphasic changes in ischemic sensitivity in the striatum of Huntington’s disease R6/2 transgenic mice. J Neurophysiol 93: 758–765, 2005. First published September 15, 2004; doi:10.1152/jn.00483.2004. We used the oxygen/glucose deprivation (OGD) model of ischemia in corticostriatal brain slices to test the hypothesis that metabolic deficiencies in R6/2 transgenic Huntington’s disease (HD) mice will impair their recovery from an ischemic challenge. Corticostriatal extracellular field excitatory postsynaptic potentials (fEPSPs) were evoked in transgenic and wild-type (WT) mice in three age groups: 3–4 wk, before the overt behavioral phenotype develops; 5–9 wk, as overt behavioral symptoms begin; and 10–15 wk when symptoms were most severe. OGD for 8 min completely and reversibly inhibited fEPSPs. Although responses of 3–4 wk WTs showed a tolerance to ischemia and recovered rapidly, ischemic sensitivity developed progressively; at 5–9 and 10–15 wk, responses recovered more slowly from OGD. In contrast, although 3–4 wk R6/2 transgenic fEPSPs showed significantly more ischemic sensitivity than their WT counterparts, the R6/2 fEPSPs maintained a relative tolerance to ischemia at 5–9 and 10–15 wk. As a result, a “crossover” point occurred, roughly coinciding with the development of the overt behavioral phenotype (5–9 wk), after which time R6/2 fEPSPs were significantly more resistant to ischemia than WT responses. The increased ischemic sensitivity in 3–4 wk R6/2 responses was not due to excessive glutamate release during OGD as it persisted in the presence of the glutamate receptor antagonist kynurenic acid (1 mM). Although the mechanism for development of ischemic resistance in R6/2 transgenics remains unknown, it correlates with metabolic and biochemical changes described in this model and in HD patients.

INTRODUCTION

Huntington’s disease (HD) is a progressive, autosomal dominant neurodegenerative disorder characterized by motor and cognitive symptomatology (Haddad and Cummings 1997) and is caused by expansion of a polyglutamine (CAG) tract in the huntingtin protein (The Huntington’s Disease Collaborative Research Group 1993). Because the actions of the native protein are not known, the mechanism(s) responsible for the effects of the mutant protein, huntingtin, is (are) not clearly understood. Although huntingtin is expressed in cells throughout the body, many of the primary HD symptoms involve changes in striatal neurons and the cortical neurons that comprise a major excitatory input to the striatum. Alterations in both neuronal morphology, electrophysiology, and biochemistry have been documented in HD patients and animal models of the disorder (Brouillet et al. 1999; Cepeda et al. 2001, 2003; Klapstein et al. 2001; Levine et al. 1999). Of the numerous murine models that have been developed to facilitate the study of HD, the R6/2 transgenic mouse line containing exon 1 with ~150 CAG repeats of the human HD gene (Mangiarini et al. 1996) has been the most thoroughly studied and characterized. This mouse model displays an overt behavioral phenotype beginning at ~5 wk of age that becomes more severe as the animals age, and transgenic mice die between 3 and 4 mo of age of unknown causes.

Numerous studies have implicated defects of energy metabolism in HD pathogenesis (Browne et al. 1997). Symptomatic HD patients exhibit increases in striatal and cortical lactate levels (Harms et al. 1997; Jenkins et al. 1993; Koroshetz et al. 1997), indicating a deficiency in mitochondrial oxidative metabolism that correlates with CAG repeat number (Jenkins et al. 1998). Mitochondria from patients with HD as well as from at least one transgenic mouse model exhibit altered membrane potentials and increased sensitivity to Ca2+ challenge (Panov et al. 2002). In addition, the rate of ATP generation in muscle is significantly reduced in HD patients and presymptomatic HD gene carriers, and this too is correlated with CAG repeat length (Lodi et al. 2000). Furthermore, striatal medium-sized spiny neurons in R6/2 transgenic mice exhibit age-related alterations in both their membrane properties and excitatory synaptic inputs (Cepeda et al. 2003; Klapstein et al. 2001), consistent with the hypothesis that energy metabolism is impaired in mutant mice. Because many neuronal homeostatic processes are energy dependent, it would be expected that metabolic alterations caused by the HD mutation may render affected neurons incapable of maintaining homeostasis, especially under conditions of physiological stress, thereby leading to cellular dysfunction or death. Paradoxically, decreased sensitivity to both ischemic and excitotoxic manipulations have been documented in various HD mouse models at symptomatic ages (Hansson et al. 2001a,b; Schieber et al. 2002), and no report has shown a relative susceptibility to ischemia at any age as might be expected given a metabolic deficiency.

As an in vitro model of ischemia, oxygen/glucose deprivation (OGD) produces neuronal damage by preventing cellular energy metabolism and can be used to test ischemic sensitivity at even early ages. The sequence of events caused by OGD has been studied in several brain regions, including the striatum (Calabresi et al. 1995; Tanaka et al. 1997). Within minutes of deprivation, neurons undergo depletion of ATP, leading to rapid and complete membrane depolarization, changes in permeability to K+, Na+, and Ca2+, and inhibition of synaptic transmission. Recovery after short periods of OGD can be

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nearly complete. The faster a neuron is able to restore its ATP supply, the faster it will recover from OGD (Ekholm et al. 1993; Kristian 2004). Thus metabolic efficiency can be reflected in the rate of recovery from OGD. Because there is considerable evidence for energy dysfunction in HD, the present experiments were designed to test the hypothesis that R6/2 transgenic HD mice have impaired recovery from an ischemic challenge.

METHODS

Animals

All mice used in these experiments were obtained from the R6/2 breeding colony maintained at the University of California, Los Angeles. Offspring were obtained from mouse pairings consisting of either male R6/2 hemizygote × female wild type (WT); strain B6CBAF1) or male WT × female WT with ovarian transplant from R6/2 hemizygote (obtained from the Jackson Laboratory, Bar Harbor, ME). All experimental procedures were carried out in accordance with the National Institutes of Health “Guide for Care and Use of Laboratory Animals” and were approved by the Institutional Animal Care and Use Committee at UCLA. Experiments were performed using three age groups of R6/2 transgenic and age-matched WT control mice based on our previous physiological data (Cepeda et al. 2003; Klapstein et al. 2001; Levine et al. 1999) as well as on the development of the overt motor phenotype. The first group was tested before the appearance of the overt behavioral symptoms at 3–4 wk [n = 16 slices from 13 R6/2 mice, 22.9 ± 0.6 (SE) day, range: 19–28 day; n = 14 slices from 13 WT mice, 21.2 ± 0.3 day, range: 19–23 day]. Of these, six slices from six WT mice and six slices from six R6/2 mice were used to test the effect of the glutamate receptor antagonist kynurenic acid. The age of the second group corresponded to the onset of overt motor symptoms and was 5–9 wk ([n = 8 slices from 7 R6/2 mice, 56.1 ± 3.4 day, range: 45–64 day; n = 9 slices from 7 WT mice, 53.6 ± 4.3 day, range: 40–65 day]). The third group displayed the full behavioral phenotype and was 10–15 wk ([n = 6 slices from 6 R6/2 mice, 80.8 ± 0.7 day, range: 79–83 day; n = 6 slices from 5 WT mice, 84.0 ± 2.7 day, range: 75–91 day]).

Mice were decapitated under deep halothane anesthesia. Brains were removed into ice-cold low-Ca2+ oxygenated artificial cerebrospinal fluid [ACSF; composition (in mM): 130 NaCl, 5 MgCl2, 1 CaCl2, 3 KCl, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose] and sliced coronally at 350 μm using a DSK microslicer (Ted Pella, Redding, CA). Slices containing striatum and overlying cortex were maintained at room temperature in an incubation chamber filled with ACSF and connected to an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Stimulus response curves were constructed and stimulus intensities were chosen to produce responses from the linear portion of the curve. Only those preparations exhibiting a stable baseline response amplitude for ≥20 min prior to each experiment were included in the data set.

ODG

ODG was induced by substituting N2 for O2 in the atmosphere of the slice during recording (using a 95% N2-5% CO2 mixture) and perfusing the slice with ACSF that had an equimolar substitution of sucrose for glucose and that was also equilibrated with 95% N2-5% CO2 (Klapstein et al. 1998). Care was taken to ensure that the speed of gas flow over the slice chamber remained constant during the switch. Concentrated stock solutions of drugs were diluted in ACSF immediately prior to use and were applied via the bath.

Data were collected and analyzed off-line using commercially available software (pClamp 8.0, Axon Instruments, Foster City, CA). The fEPSP amplitude was measured from the positive peak immediately after the stimulus artifact to the following negative peak, with care to exclude the presynaptic volley, when it could be distinguished. Amplitudes were normalized to the average amplitude of 10 responses immediately preceding the OGD period and were subsequently expressed as percentage of baseline fEPSP amplitude. The rate of recovery of synaptic potentials following the return to control conditions was measured by fitting with a Boltzmann sigmoidal function [y = A1 − A2/1 + e(−(x−x0)/dx)] using Origin 6.0 software (Microcal Software, Northampton, MA), where A1 is the amplitude at the beginning of the recovery period (arbitrarily set to 0), A2 is the maximal percentage recovery, x0 is the time required to achieve 50% of maximal recovery, and dx is the recovery time constant. The rate of reduction of the fEPSP during OGD was also measured this way. Linear regression of age effects was performed using Origin 6.0 software and compared between genotypes (Zar 1984). For illustrative purposes and analysis, the fEPSP amplitudes were binned into 5-min epochs (average of 20 data points each) and plotted as means ± SE. Data were analyzed with appropriate ANOVAs for independent and repeated measures using SigmaStat software (SPSS, San Rafael, CA). For post hoc evaluations after ANOVAs, the Bonferroni t-test was used because this is one of the more conservative tests for multiple comparisons. Differences were considered statistically significant when P < 0.05.

RESULTS

Average fEPSP amplitudes in 3–4 wk R6/2 and WTs were similar (1.16 ± 0.22 and 1.00 ± 0.14 mV, respectively; P = 0.576). These values corresponded to 67.1 ± 5.1 and 69.3 ± 4.6% of the maximum amplitude evoked response, respectively (P = 0.764). Average fEPSP amplitudes in 5–9 wk R6/2 and WTs were also similar (1.01 ± 0.09 and 1.31 ± 0.12 mV, respectively; P = 0.194) and corresponded to 60.3 ± 5.3 and 52.1 ± 1.9% of the maximum amplitude evoked response, respectively (P = 0.149). Average fEPSP amplitudes in 10–15 wk R6/2 and WTs were also similar (1.10 ± 0.13 and 0.87 ± 0.12 mV (P = 0.234), respectively, corresponding to 65.3 ± 5.4 and 66.8 ± 8.2% of the maximum amplitude evoked response, respectively (P = 0.883). There were also no consistent differences in the intensity of the stimulus necessary to evoke the response among the three age groups or genotypes. Mean intensities ranged from 0.20 to 0.83 mA. For each experiment, the intensity chosen was ~50% of the intensity to induce a maximum response. These values ranged from means of 46–
OGD resulted in complete inhibition of fEPSPs in all slices (Fig. 1). Inhibition began within a few minutes of OGD application and proceeded rapidly over the course of 1–2 min. The times required to reach 50% inhibition of fEPSP amplitude after the switch to OGD conditions in 3–4, 5–9, and 10–15 wk R6/2 transgenics were 3.22, respectively. The times required to reach 50% inhibition of fEPSP amplitude were significantly longer to reach 50% inhibition in group, responses from transgenics took significantly longer to reach 50% inhibition in 3–4, 5–9, and 10–15 wk after the switch to OGD conditions in 3–4, 5–9, and 10–15 wk R6/2 transgenics were 3.22, 2.04 ± 0.15, and 1.86 ± 0.22 min, respectively. The times required to reach 50% inhibition in 3–4, 5–9, and 10–15 wk R6/2 transgenics were 3.22, 2.53 ± 0.34, and 2.36 ± 0.19 min, respectively. In each age group, responses from transgenics took significantly longer to reach this 50% point (P = 0.022). In addition, there was a significant decrease over age regardless of genotype (P = 0.003).

Examination of responses from the three age groups of WT mice revealed a significant effect of decreasing tolerance or increasing sensitivity to ischemia with age (Fig. 2A). Responses from 3–4 wk WT mice exhibited remarkable tolerance to an 8-min OGD episode, recovering completely (98.0 ± 4.1% of control amplitude) and reaching half-maximal recovery by 13.8 ± 1.7 min. Tolerance to ischemia decreased markedly in the older WT mice, however, and responses from 5–9 and 10–15 wk mice recovered progressively slower and incompletely, requiring 38.1 ± 5.6 and 39.3 ± 3.2 min to reach half-maximal recovery (P < 0.001 for comparison with responses from 3–4 wk WT). During the OGD period, we have calculated correlation coefficients for age versus percentage recovery and found significant differences between the age groups or genotype. The times required to reach 50% inhibition of fEPSP amplitude and reaching half-maximal recovery by 13.8 ± 3.4 min with the responses of R6/2 transgenics displaying greater recovery (Fig. 2C, asterisks). At 10–15 wk, R6/2 transgenics had clearly developed ischemic tolerance compared with WT mice, being inhibited more slowly and requiring only 21.2 ± 1.8 min to reach half-maximal recovery, compared with 39.3 ± 3.2 for WTs (P < 0.001, Fig. 2C). The amount of maximal recovery between the transgenic and WT groups was not significantly different (77.5 ± 7.9 and 66.6 ± 10.8%, respectively). At this age, there were significant differences in the percent recovery from 40 to 60 min with the responses of R6/2 transgenics displaying greater recovery (Fig. 2C, asterisks).

To test whether a possible increase in glutamate release in 3- to 10–15 wk R6/2 transgenics and WT mice during the OGD period might contribute to the alterations in recovery, we applied OGD in the presence of the broad-spectrum glutamate receptor antagonist kynurenic acid (1 mM) in a group of additional slices. In WT mice, the presence of kynurenic acid prior to and during the OGD period had no effect on the recovery of the fEPSP (maximum recovery to 94.5 ± 4.9% of control; 17.7 ± 3.6 min to reach half-maximal recovery; Fig. 4). Likewise, the impaired recovery of 3–4 wk R6/2 transgen-
ics (*n* = 6 slices) was not altered in the presence of kynurenic acid, and they still recovered significantly slower than age-matched WTs (50% recovery time of 27.9 ± 1.9 min; *P* = 0.030). To try to confirm this finding, OGD was also carried out in the combined presence of the more selective glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) and 2-amino-5-phosphono-valerate (APV, 100 μM) to specifically block ionotropic non-N-methyl-D-aspartate (NMDA) and NMDA receptors, respectively. However, the time required for washout of these drugs exceeded the OGD recovery period, and it was not possible to detect differences in recovery rates (data not shown).

**DISCUSSION**

It has long been known that immature brains exhibit a relative resistance to ischemic damage (Schurr and Rigor 1987; Yager et al. 1996; Young et al. 1992). In the present study, we showed that although corticostriatal responses in brain slice preparations from 3–4 wk WT mice show a tolerance to ischemia, ischemic sensitivity develops progressively over age. On the other hand, while responses of 3–4 wk R6/2 transgenic preparations show significantly more ischemic sensitivity than do their 3–4 wk WT counterparts, the R6/2 preparations do not become more sensitive over age but rather maintain a relative tolerance to ischemia. As a result, a crossover point occurs, roughly coinciding with the appearance of the overt behavioral symptoms, after which time responses of R6/2 transgenics are more resistant to ischemia than those of WTs.

There are a number of potential explanations for the differential changes observed in response to OGD challenge between R6/2 transgenics and WTs. It has been reported that striatal aspiny neurons are more resistant to ischemia than are medium...
spiny neurons (Centonze et al. 2001). An increase in the proportion of aspiny neurons would tend to confer more ischemic resistance on the population. This is unlikely to explain our results, however, because wide-scale loss of spiny neurons does not occur in this model except at a very late stage (Jenkins et al. 2000; Turmaine et al. 2000), and changes in the relative population densities of different neuronal subtypes have not been reported in this model at any age.

The use of alternate energy substrates may also play a role in the differential sensitivity to ischemia. Lactate has been shown to serve as an obligatory energy source during the early recovery period after OGD (Schurr et al. 1997), and hyperglycemia has been shown to raise the ischemic accumulation of lactate by as much as 10-fold in adult animals of various species subjected to cerebral ischemia while having little effect on immature animals (Young et al. 1992). However, the diabetes reported in R6/2 mice (Hurlbert et al. 1999) is unlikely to play a role in the ischemic susceptibility of the 3–4 wk R6/2 cohort as blood glucose levels become significantly elevated in only ~25% of transgenics, and even then not until ~9 wk of age (Luesse et al. 2001). Furthermore, although lactate levels are increased in human HD patients (Jenkins et al. 1993; Koroshetz et al. 1997), there is no evidence for increased striatal lactate levels in R6/2 mice under normal conditions at 12 wk of age (Jenkins et al. 2000).

It is well established that a brief episode of ischemia confers protection against subsequent ischemic events (Schurr et al. 1986) This phenomenon is known as ischemic preconditioning, and it shares several features with the pathophysiology of HD. For example, a brief ischemic episode has been found to induce an elevation of ubiquitin conjugate levels that lasts longer as mice age (Vannucci et al. 1998) and is thought to be a neuroprotective compensatory mechanism. In the R6/2 model, neuronal intranuclear inclusions which are present in the cortex and striatum become ubiquitinated by 5–6 wk (Davies et al. 1997), about which time ischemic tolerance becomes apparent in the R6/2 model. As a second example, the succinate dehydrogenase inhibitor 3-nitropropionic acid, which is known to produce HD-like phenotypes in rodents (Beal et al. 1993) in an age-dependent manner (Brouillet et al. 1993), has also been shown to induce tolerance to OGD in rat cortex (Weih et al. 1999).

What is the origin of the apparent preconditioning found in symptomatic mutants? Perhaps even minor fluctuations in local blood flow, which are common in a normal brain (Yager et al. 2001). An increase in the proportion of aspiny neurons would tend to confer more ischemic resistance on the population. This is unlikely to explain our results, however, because wide-scale loss of spiny neurons does not occur in this model except at a very late stage (Jenkins et al. 2000; Turmaine et al. 2000), and changes in the relative population densities of different neuronal subtypes have not been reported in this model at any age.

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1996) and have no lasting effect, may produce damage in a brain already burdened with metabolic insufficiency and contribute a sufficient catalyst for reactive neuroprotective processes. The additive effects over time of such minor incidents would then help to explain the slow onset and progressive nature of the disease.

One of the key mediators of ischemic damage is the large associated influx of Ca\(^{2+}\). In addition to Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels during OGD, neurons release neurotransmitters, including glutamate, as they depolarize completely. Activation of NMDA receptors has been shown to impair the recovery from hypoxia in area CA1 of the hippocampus (Sebastiao et al. 2001), and mitochondrial sensitivity to NMDA-induced Ca\(^{2+}\) influx has been shown to increase during postnatal maturation (Marks et al. 2000). Not surprisingly, glutamate-induced Ca\(^{2+}\)-mediated excitotoxicity has also been hypothesized as a mechanism underlying cellular dysfunction and degeneration in HD. For example, populations of striatal medium-sized spiny neurons of HD mutant mice show increased sensitivity to NMDA (Cepeda et al. 2003; Klapstein et al. 2001). Although R6/2 striatal neurons are more sensitive to NMDA application than WT neurons (Cepeda et al. 2001; Levine et al. 1999), with fewer glutamatergic terminals in the symptomatic R6/2 striatum, less glutamate would be released during population depolarization and less postsynaptic NMDA receptor activation would occur in the striatal neurons, thus reducing any subsequent cellular damage that might occur. Resistance to excitotoxicity has also been previously described in vivo in the R6/2 and R6/1 HD mouse models (Hansson et al. 1999; Morton and Leavens 2000; Petersen et al. 2001). However, it has been shown that excitotoxin-induced pathology in vivo requires intact synaptic circuitry (Jenkins et al. 1996; Kim and Chan 2002). Thus, the resistance seen in vivo may be due, at least partially, to a decrease in the density of excitatory spines and possible disconnection from excitatory inputs (Cepeda et al. 2003; Klapstein et al. 2001). Furthermore, given that acute blockade of glutamatergic transmission in the present study did not alter the time course of recovery from OGD in either WT or R6/2 in the 3–4 wk group, it seems unlikely that NMDA receptor activation during the ischemic insult contributes greatly to the observation of differential ischemic sensitivity in this model at this age.

An alternate excitotoxic hypothesis suggests that enhanced recovery in older transgens may result from epileptiform activity evident in this model. At 5–7 wk, coinciding with the age at which transgens recover more quickly than WTs, a significant proportion of striatal neurons in transgens exhibit abnormally large spontaneous glutamatergic synaptic events (Cepeda et al. 2003). Although seizure activity can result in epileptiform preconditioning, reducing damage from subsequent ischemic events (Plamondon et al. 1999), it has also been observed that ischemic tolerance can be induced by even transient neuronal depolarization (Taga et al. 1997).

These phenomena may help to explain why 5–9 and 10–15 wk R6/2 transgens are less sensitive to ischemia than WTs. However, they are insufficient to explain the increased striatal ischemic susceptibility in young transgens, as recovery from OGD remained significantly slower in the presence of a blockade of glutamatergic synaptic transmission. Other factors may contribute to the differences in the young group. The pattern of ischemic sensitivity in the R6/2 model correlates with expression of neuronal nitric oxide synthetase, which is higher in 3-wk-old R6/2 mice and reduced at 6 and 11 wk (Deckel et al. 2002). Nitric oxide combines with superoxide to produce the potent neurotoxin peroxynitrite (Schulz et al. 1997). The tri-carboxylic acid cycle enzyme aconitase is particularly sensitive to inhibition by both peroxynitrite and superoxide radicals, and, indeed, its activity is severely decreased in HD caudate, putamen, and cortex (Tabrizi and Schapira 1999). A reduction in ATP levels is a probable consequence of this, which, in addition to having acute effects, may produce a secondary effect similar to ischemic preconditioning, which would then account for the age-dependent ischemic resistance seen in the present study. This would furthermore suggest that a rational treatment strategy might include antioxidant pharmacotherapy.

In conclusion, the present study demonstrates that the response to ischemic challenge in the R6/2 HD model is complex and may depend on different factors according to the age of the mouse and the degree of progression of the phenotype. It is likely that metabolic deficiencies underlie an increased sensitivity to ischemia that can be seen in presymptomatic mice, but that neuronal responses to these deficiencies invoke compensatory mechanisms, culminating in ischemic tolerance that develops as the disease progresses.

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