Dysregulation of Ascorbate Release in the Striatum of Behaving Mice Expressing the Huntington's Disease Gene

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The extracellular fluid of the striatum contains a high level of ascorbate, an antioxidant vitamin known to play a key role in behavioral activation. We assessed the extracellular dynamics of ascorbate in R6/2 mice engineered to express the gene for Huntington's disease (HD), an autosomal dominant condition characterized by the loss of striatal neurons. Slow-scan voltammetry was used to measure striatal ascorbate during anesthesia and subsequent behavioral recovery. Although both the HD mice and their littermate controls had comparable ascorbate levels during anesthesia, the gradual return of behavioral activation over the next 120 min led to dramatically different ascorbate responses: a progressive increase in controls and a 25–50% decline in HD mice. In contrast, 3,4-dihydroxyphenylacetic acid, a major dopamine metabolite, showed no group differences. Behaviorally, HD mice were less active overall than controls and showed a relatively restricted range of spontaneous movements. Both the ascorbate and behavioral deficits were evident in 6-week-old HD mice and persisted in all subsequent test sessions through 10 weeks of age. Collectively, although these results are consistent with inadequate antioxidant protection in the HD striatum, they indicate that the ascorbate deficit is confined to periods of behavioral activation.

Key words: ascorbate; basal ganglia; dopamine; glutamate; Huntington's disease; motor control; striatum; voltammetry

Huntington's disease (HD), a progressive neurodegenerative disorder of the striatum, is characterized by striking behavioral changes, most notably deficits in cognition and motor control (Vonsattel and DiFiglia, 1998). Although HD is caused by an expanded polyglutamine repeat within the coding region of the HD gene (Huntington's Disease Collaborative Research Group, 1993), pharmacological and other efforts to reverse HD symptoms have been largely ineffective. Such outcomes have led to the development of transgenic mouse models that mimic the progressive behavioral and neurological phenotype of HD to provide a basis for testing new therapies. One of these models, the R6/2 line, develops many of the motor-control deficits that are characteristic of HD (Carter et al., 1999). In this report, we evaluated the behavioral phenotype of these mice and assessed potential deficits in ascorbate, the physiological form of vitamin C found throughout the extracellular fluid of the striatum (Basse-Tomusk and Rebec, 1991).

Although the precise role of ascorbate in striatal function is unclear, increasing evidence indicates that it is important to monitor the HD striatum for a possible ascorbate imbalance. At the cellular level, ascorbate offers antioxidant protection against highly reactive oxygen species (Rice, 2000). The accumulation of these substances leads to oxidative stress and excitotoxicity, both of which have been implicated in HD pathogenesis (Browne et al., 1999; Sayre et al., 2001). It also is interesting that a loss of striatal ascorbate impairs motor behavior (Rebec and Wang, 2001), a prototypical feature of HD (Penney and Young, 1998).

To examine a possible ascorbate deficit in the HD striatum, we used slow-scan voltammetry to measure extracellular ascorbate in both R6/2 mice expressing exon 1 of the human HD gene and their littermate controls. Because ascorbate release is linked to behavioral activation (Rebec and Pierce, 1994), we monitored the change in striatal ascorbate release as animals recovered from general anesthesia. Our recordings also provided data on 3,4-dihydroxyphenylacetic acid (DOPAC), a major dopamine metabolite.

MATERIALS AND METHODS

Animals. R6/2 mice (B6CBA-TgN[HDexon1]62Gpb) were obtained from The Jackson Laboratory (Bar Harbor, ME) at 5 weeks of age. Data were collected from male hemizygous HD mice and littermate controls. All mice were housed individually in the departmental animal colony under standard conditions (12 hr light/dark cycle with lights on at 7:30 A.M.) with access to food and water *ad libitum*. Both the housing and experimental use of the animals followed National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

Preparation for voltammetric recording. Shortly after arrival in the colony, animals were anesthetized with chloropent (0.4 ml/100 gm, i.p.) and mounted in a stereotaxic frame. The skull was exposed and bilateral holes were drilled over the striatum (0.5 mm anterior and 2.0 mm lateral to bregma). A plastic hub was secured over each hole at a 5° angle to allow for adjacent placement of both working and reference electrodes (Rebec and Wang, 2000). Each hub was sealed with a plastic cap, and the animals were returned to the colony for at least 4 d of recovery.

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Voltammetry. A glass-insulated carbon fiber (10 μm diameter) served as the working electrode for voltammetric recording. The active surface area extended beyond the glass by ~150 μm. The electrode was electrochemically pretreated, as in previous work (Pierce and Rebec, 1993), to separate the oxidation signals for ascorbate, DOPAC, and other easily oxidized endogenous compounds (Gonon et al., 1981b). Ample evidence has confirmed the identity of the ascorbate and DOPAC signals in rodent striatum (Rebec and Wang, 2001; Kamata et al., 1986), and applications of exogenous ascorbate have confirmed the ascorbate signal in mice (Rebec and Barton, 2001). Generation of waveforms for staircase voltammetry and storage of sampled current was performed using a computer interfaced to a locally constructed potentiostat operating in two-electrode mode. A potential was applied in 6 mV steps from −200 to +600 mV versus reference to ensure ascorbate and DOPAC oxidation. The scan rate was set at 20 mV/sec; scans were obtained at 60 sec intervals.

On the recording day, the working electrode was first tested in citrate–phosphate buffer containing 100 μM ascorbate and 20 μM DOPAC to ensure adequate sensitivity and peak separation. Both the working electrode and a Ag/AgCl reference electrode were fitted into separate miniaturized microdrives (total weight of <2 gm) designed to mate with the head-mounted hubs (Rebec and Wang, 2000). To install the microdrives, we anesthetized each mouse (half the anesthetic dose for surgery) and removed the cap over each hub. The working electrode was lowered manually into the left or right striatum (~3.2 mm ventral to skull surface), whereas the reference electrode was positioned in the contralateral hub and lowered onto the brain surface. A lightweight, shielded cable attached to each microdrive was connected to the potentiostat via an electric swivel to permit complete freedom of movement. Each animal was placed in a circular, glass arena (15 cm diameter) housed inside a sound-attenuating cubicle for recording. After 10–20 min to allow the voltammetric signal to stabilize, scans were obtained for the next 2.5 hr, which included the initial period of anesthesia (30–60 min) and subsequent behavioral activation. After completion of the recording session, most electrodes were removed and tested for sensitivity in a postcalibration step, but some were used to mark the recording site (see below).

In most cases, we were able to record from the opposite striatum 2–3 weeks later. For the second recording session, new working and reference electrodes were prepared, and their left–right placement was reversed. This strategy allowed us to record twice from undisturbed striatal tissue in the same animal. The second session followed the same protocol as the first (i.e., recording during and after a period of light anesthesia).

Ethological assessment. All behavioral tests were run at least 1 d before or 2 d after voltammetric recording. No behavioral data were obtained after an electrode-marking lesion. Mice were individually placed in an open-field arena (45 × 26 cm) with clear, Plexiglas walls (20 cm) in an isolated observation room equipped with videotaping facilities. A wire-mesh cylinder for climbing (7 cm tall and 4 cm diameter) was placed near the center of the arena along with a rubber ball (~2 cm diameter); inert wood shavings covered the floor. Each mouse remained in the arena for 15 min of videotaping and was then returned to the home cage. Testing was performed weekly for at least 5 weeks. All sessions occurred between 10:30 A.M. and 12:30 P.M.

Videotapes were analyzed by an independent observer who recorded 10 operationally defined behavioral categories: climbing the cylinder, crawling along the rim of the arena, digging in or pushing the bedding, grooming (either forepaw or hindpaw), jumping, forward locomotion, pushing the ball, rearing, sniffing (head or whisker movement), and quiet rest. An ethogram was compiled in which the total number of behaviors and the percentage of time spent exhibiting each behavior were noted for each session.

Histology. To verify electrode placement for voltammetry, some animals were deeply anesthetized and current was passed through the working electrode to mark the recording site. Subsequent transcardial perfusion with formosaline was followed by histological analysis to confirm recording location.

Data analysis. For voltammetry, ascorbate and DOPAC peak heights were measured at the apex in millimeters. Estimates of mean ± SEM concentration were based on postcalibration data as described previously (Basse-Tomusk and Rebec, 1991). Changes in extracellular ascorbate and DOPAC levels during wakefulness were expressed as percentage of anesthesia baseline. Ethological data were analyzed for the total number of behavioral items as well as age differences between HD and control mice. Statistical comparisons were made by ANOVA or Student's *t* tests for paired samples.

RESULTS

Both groups of animals gained weight over the course of the experiment, typically progressing from 20 to 25 gm, arguing against a HD-related nutritional deficit. A total of 13 HD and 15 control mice were used for voltammetry, and most (8 HD mice and 11 control mice) were included in two recording sessions. The first session occurred when animals were between 6 and 8 weeks of age, and the second session occurred 2–3 weeks later. Subsets of these animals (7 HD mice and 7 control mice) were run in weekly ethological assessments of behavior.

Voltammetry

All voltammetric scans obtained from all mice in all sessions revealed distinct oxidation peaks between −70 and −50 mV and between +80 and +100 mV versus reference. Thorough characterization of these peaks *in vivo* (Gonon et al., 1981a) as well as *in vitro* testing of each electrode indicated that these peaks represented ascorbate and DOPAC, respectively. Sample scans, which also depict a third peak thought to represent serotonin, uric acid, and other oxidizables, are shown in Figure 1*A*.

At the beginning of each recording session, all mice were anesthetized to allow placement of the working electrode in the striatum. Under these conditions, there were no group differences in estimated concentrations of either ascorbate or DOPAC. No age differences emerged, and the data are summarized for all recordings in control and HD mice in Table 1.

After 30–60 min, both groups of animals awoke and showed increased behavioral activation. In HD mice, this behavioral change was accompanied by a progressive decline in the amplitude of the ascorbate signal, in some cases by up to 50% (Fig. 1*A*). Controls, in contrast, showed an ascorbate increase as they became behaviorally active. The time course of these opposing ascorbate changes is shown in Figure 1*B* for one animal in each group at 6 weeks of age. Note the beginning of the sharp divergence in the ascorbate response at 60 min, when behavioral activation is imminent. HD mice that emerged from anesthesia sooner showed a similar ascorbate decline but a correspondingly sooner onset. Group data on the ascorbate and DOPAC responses from the last recording of each session when animals were most behaviorally active are presented in Figure 1*C*. The ascorbate difference between HD mice and controls is statistically significant ($p < 0.01$). Note also that the DOPAC response, although variable, is comparable in both groups.

The ascorbate decline in the striatum of awake HD mice persisted across all weeks of testing. No differences emerged between the first or the second recording session. Controls showed variable increases with age.

Ethological assessment

All mice were active when placed in a relatively natural environment with many behavioral choices, but clear group differences emerged. As shown in Figure 2, HD mice allocated a significantly smaller percentage of their behavioral responses to rearing, digging, sniffing, and climbing than controls. In fact, only two of seven HD mice showed climbing behavior, but this behavior was observed for these two mice in every session, even as late as 10 weeks. In contrast, all controls climbed in every session. Two behaviors (crawling on the rim of the cage and pushing the ball) were never expressed by HD mice and were rare even in controls, accounting for <2% of all responses. The only behavior never expressed by controls, however, was quiet rest, which accounted for ~5% of behavior in HD mice. Although HD mice spent more

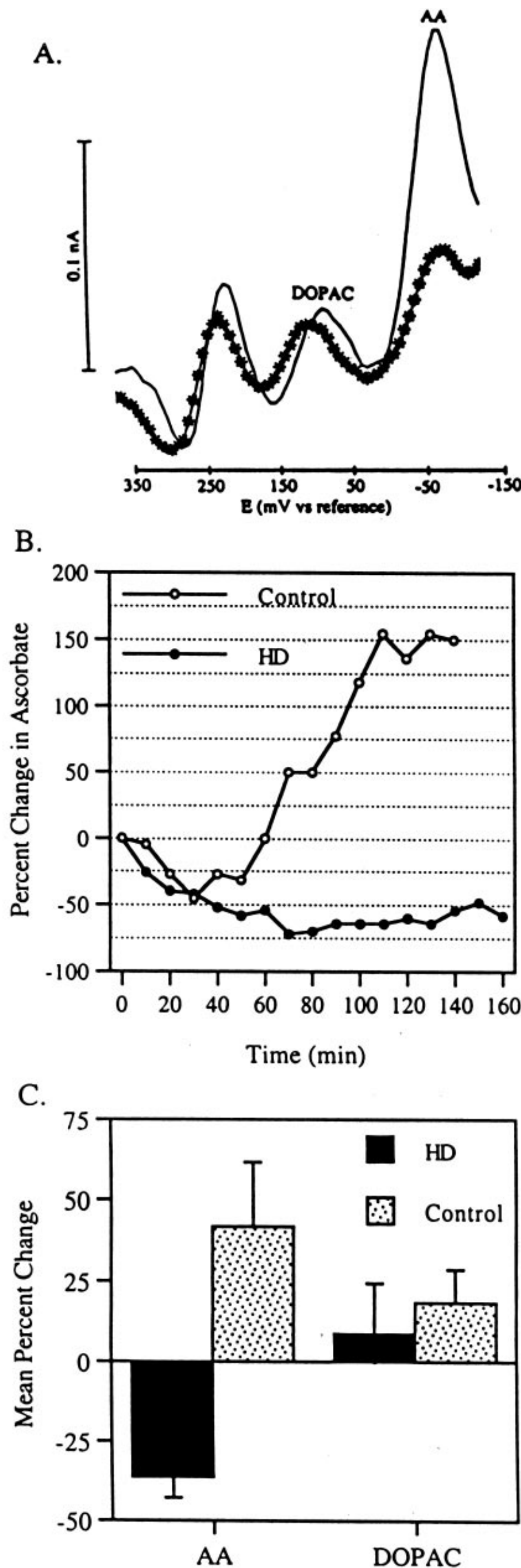

Figure 1. Voltammetric data from the striatum. *A*, Representative voltammograms obtained from a 6-week-old HD mouse during anesthesia (*solid line*) and after behavioral recovery ~120 min later (*asterisks*). Note that the ascorbate (*AA*) peak, which occurs between −70 and −50 mV, shows a behavior-related decline. No such change occurs in the DOPAC peak (between +70 and +90 mV). Several oxidized species are represented in a third, unanalyzed peak (more than +200 mV). *B*, Time course of the change in magnitude of the ascorbate peak recorded during the first recording session for one control and one HD mouse (see above). Both animals are 6 weeks of age. Data are based on individual scans obtained at 5 min intervals and are presented as percentage of change from the first scan obtained 15 min after injection of anesthetic (time 0) when the animals are completely inactive. In both cases, signs of behavioral activation (crawling, head movement, twitching, etc.) begin between time 50 and time 60. *C*, Mean ± SEM percentage of change in magnitude from anesthesia baseline of the ascorbate and DOPAC signals for all HD and all control mice during postanesthesia behavioral activity (see above). Data, which were averaged across all recording sessions in all animals, are plotted for the last 5 min of each session, when behavioral activation was greatest. The HD ascorbate response is significantly different from the control response ($p < 0.01$); there is no difference in the DOPAC response.

Table 1. Estimated mean ± SEM extracellular concentration of ascorbate and DOPAC in the striatum during anesthesia baseline

Group	Ascorbate (μM)	DOPAC (μM)
HD ($n = 21$)	383 ± 64	12.6 ± 2.4
Control ($n = 26$)	365 ± 60	14.6 ± 1.6

Data include cases in which recordings were obtained in two separate sessions; n indicates total recordings. Estimates are based on postcalibration data.

time grooming and locomoting than controls, this difference primarily reflected a stereotyped flick of the hindpaw toward the ipsilateral ear, a behavior unique to these animals (Carter et al., 1999).

Of the 10 distinct items of behavior recorded in each ethological session, control mice ($n = 7$) at all ages displayed all items at least once, compiling a mean ± SEM of 8.2 ± 0.21 items per session, whereas the mean ± SEM of 6.5 ± 0.24 items in HD mice ($n = 7$) was significantly lower ($p < 0.001$). Approximately the same degree of difference was evident at each of the ages tested.

HD mice consistently engaged in less behavior overall. The mean ± SEM total behavior count per session analyzed across all sessions was 689 ± 22 in controls but only 240 ± 21 in HD mice ($p < 0.001$). Data for weekly sessions showed diverging linear trends with a progressive increase in controls and a progressive decline in HD mice. Controls, for example, scored 666 ± 47 and 731 ± 124 counts/session at 6 and 10 weeks of age, respectively, whereas HD mice went from 280 ± 50 to 160 ± 30 counts/session over this same period. An overall ANOVA on weekly session data revealed a significant difference ($p < 0.001$). Subsequent *post hoc* analysis revealed a significant difference at each week ($p < 0.01$).

DISCUSSION

Our results support the emerging concept of striatal ascorbate release during behavioral activation and indicate a disruption of this process in the striatum of HD mice. In fact, HD mice, which display a wide array of motor deficits, show a pronounced and sustained loss of ascorbate in striatal extracellular fluid that appears only during periods of behavioral activation. Thus, the problem in these animals is not an ascorbate deficit per se, but rather a failure to maintain adequate extracellular levels under behaviorally active conditions.

A low level of striatal ascorbate is consistent with inadequate antioxidant protection as a basis for HD pathogenesis (Coyle and Puttfarcken, 1993). In fact, immunohistochemical studies of HD brain show increased levels of nitrotyrosine and malondialdehyde adducts, both of which suggest oxidative damage (Browne et al., 1999). The HD brain also shows elevated levels of the hydroxy

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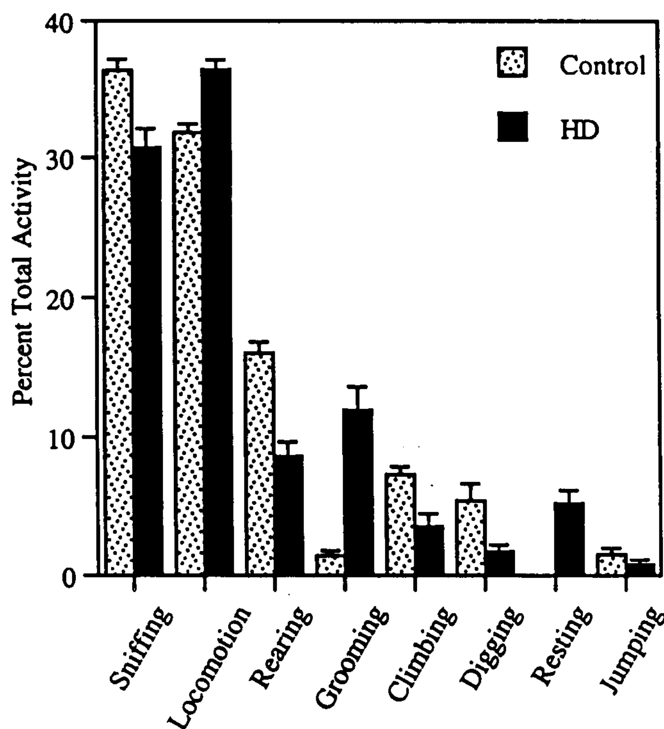

Figure 2. Frequency of expression of key behavioral categories for control and HD mice across all videotaping sessions. Data are presented as mean ± SEM percentage of total. Note that in HD mice behavior was primarily confined to locomotion and grooming, with ample periods of rest. HD and control mice are significantly different for all behaviors except jumping.

radical, a cellular marker of oxidative stress (Sayre et al., 2001). It is not clear, however, whether oxidative mechanisms are a cause or a consequence of HD (Alam et al., 2000; Perez-Severiano et al., 2000). The same uncertainty applies to the loss of ascorbate in HD mice, but if the loss indicates increased vulnerability to oxidative stress, then our results suggest that the vulnerability is confined to periods of behavioral activity.

Striatal ascorbate release depends on the activation of glutamate-releasing afferents from the cerebral cortex (Basse-Tomusk and Rebec, 1990), most likely involving heteroexchange with glutamate during glutamate uptake (O'Neill, 1995). Thus, a change in ascorbate release reflects a change in glutamate transmission (Fillenz et al., 1986). Although some cortical damage occurs in HD (Vonsattel and DiFiglia, 1998), corticostriatal degeneration cannot explain an ascorbate deficit that depends on behavioral state. It seems more likely that HD impairs the normal operation of corticostriatal neurons. Consistent with this view, HD patients show context-dependent deficits in striatal function that suggest abnormally low corticostriatal activity (Lawrence et al., 2000). Under these conditions, ascorbate release would also be low. Alternatively, however, low ascorbate may result not from a decline in glutamate transmission, but, if the heteroexchange model is correct, from a failure of glutamate uptake. In this case, because ascorbate normally protects against glutamate-evoked oxidative damage (Rice, 2000), low ascorbate in the face of high glutamate could trigger the degeneration characteristic of HD (Beal, 1998; Levine et al., 1999; Meade et al., 2000; Perez-Severiano et al., 2000). In either case, our results suggest a need for further assessment of glutamate function in HD striatum.

Although we found DOPAC to be relatively normal in HD mice, this metabolite primarily represents intracellular dopamine metabolism (Kuczenski and Segal, 1989). Thus, we cannot rule out a postsynaptic abnormality. In fact, HD mice show a deficiency of dopamine-regulated phosphoprotein markers as well as deficits in dopamine-regulated ion channels and the D1 dopamine receptor signaling cascade (Bibb et al., 2000). Therefore, if a dopamine malfunction plays a role in HD pathogenesis, postsynaptic rather than presynaptic events are likely to be involved.

Although systemic injections of ascorbate can either enhance or suppress motor activity depending on dose (Rebec and Pierce, 1994), the release of endogenous ascorbate in the striatum is correlated with behavioral activation (O'Neill and Fillenz, 1985; Boutelle et al., 1989; Mueller, 1989; Pierce and Rebec, 1990). We saw a similar relationship in our control mice. In contrast, our HD mice showed behavioral deficits similar to those reported for rats treated with intrastriatal infusions of ascorbate oxidase, which deplete extracellular ascorbate by at least 50% (Rebec and Wang, 2001). Like HD mice, these rats become hypoactive and fail to interact with environmental stimuli. Although HD mice spent more time than controls in locomotion, this appeared to be a default response to the decline in other behaviors rather than a preference for forward movement. Overall, HD mice engaged in fewer categories of behavior and spent more time resting than controls. In an interesting parallel with evidence that environmental enrichment delays the onset of motor symptoms in HD mice (van Dellen et al., 2000), our two HD mice that climbed at 6 weeks of age maintained this behavior throughout testing. Early expression of a behavior seems to be crucial for its maintenance, because our other HD mice never showed a climbing response at any age tested.

In sum, our results both extend the behavioral phenotype of R6/2 mice and identify a profound deficit in the control of extracellular ascorbate in the striatum. Although the behavioral data can be useful in developing sensitive assays for testing new therapeutic strategies, the loss of striatal ascorbate points to neuronal deficits that may underlie the disease process, including abnormalities in striatal glutamate transmission. It is also significant that the ascorbate deficit emerges when HD mice are behaviorally active. Although brain ascorbate has not been assessed in HD patients, our results, given the comparable distribution of brain ascorbate in rodents (Milby et al., 1982) and humans (Mefford et al., 1981), suggest a deficiency in the regulation of striatal ascorbate that may hold the key to identifying the mechanism underlying HD.

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