Using Analytical Chemistry to Unravel Disease State Mechanisms: Application to Huntington's Disease

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In today's research environment, productive collaborations, either local, regional, or global, are essential for maximizing the impact of research efforts. This sentiment is especially true in many areas of neuroscience, one of the most rapidly advancing scientific fields. Given the inherent complexity of the central nervous system and the analytical advances that are currently being made in order to resolve these intricacies, it is becoming increasingly difficult to maintain of clear grasp of all of the relevant neuroscience concepts and also develop and employ cutting edge technologies important for investigating these concepts. Thus, it is, perhaps, more important than ever for neuroscientists and analytical scientists to establish symbiotic collaborations that will enhance the importance of the research. In this paper, I discuss ongoing research in my laboratory in which we applied sensitive measurement techniques, both in vivo and ex vivo, in order to gain a more complete understanding of Huntington's disease. An important aspect of this discussion is that productive collaborations have been a positive force in enhancing our ability to resolve some of the underlying neurochemical mechanisms of this disorder.

Modeling Huntington's disease in rodents

Huntington's disease is a fatal, genetic neurological disorder caused by an expansion of CAG repeats on the gene encoding huntington (Htt), resulting in an expanded chain of glutamine residues at the N-terminus of the expressed protein (Huntington's Disease Collaborative Research Group, 1993). A sequence of 40 or more CAG repeats results in 100 percent disease penetrance. Moreover, there is a direct correlation between increasing repeat number and decreasing age of onset. HD results in a debilitating behavioral syndrome that includes both psychological and motor disturbances. The hallmark motor feature of HD is chorea, defined recently as "random, abrupt movements superimposed over purposeful acts" (Bates et al. 2002).

The discovery of the HD mutation in 1993 by the Huntington's disease collaborative research group opened the way for the development of genetically-engineered animals that model the neurological and motor phenotype of human HD. The R6 line of transgenic mice was developed in 1996 (Mangiarini et al. 1996) and represents the first genetic rodent model of HD. Within this line the R6/2 mouse, which possess the gene required for the expression of exon I of the human Htt protein

with a CAG repeat length of about 150, is among the most used HD model rodents. This strain develops a motor syndrome at 9 to 11 weeks of age that roughly approximates HD in humans. Since the development of the R6 line, many other mouse lines have been developed, including knock-in and full-length, that are thought by many to more faithfully replicate the neurological phenotype of HD (reviewed in Levine et al. 2004 and Menelled 2005). Additionally, a transgenic HD (HDtg) rat strain that possesses a fragment of the human HD gene with 51 CAG repeats has been developed (von Hörsten et al. 2003). Although the HDtg rat possesses a mutation similar to that of the R6/2 mouse, it has the advantage of developing the disease syndrome over a longer period of time (20-24 months compared to 9-11 weeks), providing a richer array of behavioral abnormalities over the life span of the animal. Additionally, the larger size of the HDtg rat facilitates the conduction more intricate neurochemical and behavioral experiments, including those that involve in vivo microdialysis and fast-scan cyclic voltammetry measurements.

Our collaborative approach toward studying CNS function

Broadly stated, the mission of our laboratory is to develop and apply analytical methods for the study of biological systems. Current methods that we employ include fast-scan cyclic voltammetry at carbon-fiber microelectrodes, microdialysis sampling, and fluorescence microscopy. We currently use these methods to measure dopamine release and uptake in rodents that model HD, including R6/2 mice and HDtg rats. We obtained breeding pairs of these rats through a collaboration with Prof. Stephan von Hörsten, Erlangen University, Germany, and Prof. Olaf Riess, Tubingen University, Germany. Another important aspect of this project is the measurement of behavior. For these studies we are collaborating with Prof. Stephen C. Fowler, Department of Pharmacology and Toxicology, University of Kansas, in order to measure behaviors at millisecond timescales, and also to correlate these behaviors with millisecond timescale voltammetric measurements. Finally, we have entered into a collaboration with Dr. Dave Johnson and Donna Johnson, Pinnacle Technology Inc., Lawrence, KS, to develop a wireless fast-scan cyclic voltammetry system. This technology will enable us to obtain voltammetric measurements in the context of behavioral paradigms of increasing complexity. All of these collaborations have been invaluable in strengthening our experimental approaches by expanding our repertoire of capabilities. For example, we have been able to directly correlate neurochemical signaling events with behaviors in rats by obtaining our fast-scan cyclic voltammetry measurements simultaneously with behavioral measurements collected using a force-plate actometer, developed by S.C. Fowler.

Reserve pool measurements in R6/2 mice

Recent evidence, much of it collected by our laboratory, show that vesicular dopamine release is impaired in the striata of multiple types of HD model rodents. Previous results have indicated that dopamine release is impaired in R6/2 mice compared to WT control mice. Moreover, our data suggest that reserve pool dopamine, which is available for periods of extended synaptic activity, is depleted in R6/2 mice. Therefore, we sought to assess how well dopamine reserve pool vesicles are mobilized. It has been shown recently that cocaine, a powerful psychostimulant that

impairs dopamine uptake by competitively inhibiting the dopamine transporter, also increases dopamine release in mice by mobilizing a synapsin-dependent dopamine reserve pool (Venton et al., 2006). Therefore, we used cocaine as a tool to mobilize dopamine reserve pools in striatal brain slices from R6/2 and WT mice (Fig. 1). This experiment was carried out by treating brain slices with αMPT, which blocks dopamine synthesis, while providing a single electrical stimulus pulse every 5 minutes to deplete releasable vesicles of dopamine. Dopamine release and uptake were measured using FSCV. After dopamine release disappeared during treatment with αMPT, cocaine was applied to the brain slice, in addition to both αMPT treatment and the ongoing application of the stimulus pulses. In both R6/2 and WT brain slices, dopamine release almost immediately reappeared and increased to about 20% of pre-drug release, presumably due to the mobilization of vesicular reserve pools. However, dopamine release from R6/2 brain slices disappeared more quickly than WT (~35 min versus ~105 min). Therefore, these data support a scenario in which vesicles from both R6/2 and WT slices are mobilized effectively; however, it appears that there are less reserve pool vesicles available for mobilization in R6/2 slices.

Behavioral and neurochemical measurements in HDtg rats

A synchronized behavioral /neurochemical approach was employed in which microdialysis sampling was used to measure trends in extracellular dopamine levels while behavior was simultaneously measured at 100 samples/s using the force plate actometer (Fig. 2). Male hmHDtg rats and male WT control rats, 9 months old, were injected with AMPH (5.0 mg/kg, i.p.) and behavior was measured in the actometer for 240 minutes (Fig. 2A). During this time, microdialysis samples were collected from the dorsal lateral striatum every 15 minutes. Dialysates were subsequently analyzed for dopamine concentration using high performance liquid chromatography with electrochemical detection. Our results show that, after injection with AMPH, extracellular dopamine levels increased dramatically and then decreased gradually in both hmHDtg and WT rats (Fig. 2B). During this increase in extracellular dopamine levels, the space used in the actometer, which serves as a measure of focused stereotypy spatial confinement, also was initially elevated. WT rats used significantly less space (more stereotypy) compared to hmHDtg rats 30 to 135 minutes after AMPH injection despite the fact that there was not a difference in extracellular dopamine levels during this time or throughout the entire 240-minute measurement period. Therefore, hmHDtg rats appear to be resistant to AMPH-induced spatial confinement and focused stereotypy, while the WT control rats are not. Additionally, there was no difference in pre-injection dopamine levels or in dopamine levels after injection with saline vehicle (data not shown). Consequently, this experiment is important because it suggests that differences in psychostimulant-induced behavior between HDtg and WT rats do not result from differences in basal extracellular dopamine levels aggregated across 15 min. Thus, we propose an alternate mechanism: behavioral differences between genotypes arise from differences in the characteristics, such as frequency of occurrence, of dopamine transients, which cannot be detected by microdialysis. Our experimental approach, therefore, was to use FSCV to measure striatal dopamine release transients

in ambulatory HDtg rats and WT control rats.

Dopamine transient frequency of occurrence in HDtg rats

We quantified the frequency of occurrence of dopamine release transients in hmHDtg rats and WT control rats (n = 3 hmHDtg and 3 WT; age = 12 months). Shown in Fig. 3 are representative data taken from a 12-month old hmHDtg rat and a 12-month old WT littermate control rat. The release plots (center trace of each picture) for the WT and hmHDtg rats are expressed here in terms of current and show the occurrence of dopamine transients measured at a sampling rate of 10 cycles/s. A CV, corresponding to the peak marked with an asterisk, is shown above the trace and confirms the presence of dopamine. Color plots, constructed by unhinging sequential CVs, stacking them from left to right, and expressing current as color, are also shown. For a given rat, files collected 15 to 21 minutes after attaching the rat to the voltammetry system were analyzed (six 1-minute files per rat). Peaks that represent dopamine transients were identified by inspection of the respective CVs. The frequency of transients was calculated by averaging the number of transients identified by the number of minutes of measurement. The analysis of the data yielded an average of 7.2 ± 2.3 transients/min occurring in the hmHDtg rats and an average of 2.6 ± 0.6 transients/min occurring in the WT rats ($p = 0.084$, t-test). For these analyses, each group consisted of two male rats and one female rat. It is interesting to note that the female rats of both genotypes had a dopamine transient frequency roughly half that of the respective male rats. The recent paper by Bode *et al*. (2008) revealed sex differences present in the HD rats, and the differences seen between male and female rats in our studies may be reflective of these findings. Overall, these data are significant because they suggest that HDtg rats may release dopamine transients at a greater frequency than WT control rats. These increased dopamine signaling events may, therefore, impact MSN neuron firing properties, discussed in the following sub-section.

Simultaneous collection of behavioral and neurochemical data in rats

Collecting neurochemical and behavioral measurements separately allows for behavioral and neurochemical comparisons to be made between age groups and drug treatments. Nevertheless, simultaneous data collection will, due to the close temporal association of the data, allow for even more direct comparisons between behavior and neurochemistry. Additionally, this method also maximizes the use of each rat. To demonstrate feasibility, voltammetry data were collected from a normal male Sprague-Dawley rat behaving on a force plate actometer (Fig. 4). FSCV and actometer measurements were electronically synchronized. The rat received an ip (5 mg/kg) injection of AMPH 60 minutes after the start of data collection. As can be seen from these data, during the last ~24 min, the rat developed focused stereotypy, indicated by the presence of a 10 Hz Z-axis force peak ("Power Spectra"; behavioral data at top of Fig. 4) and by the lack of X-Y movement on the force plate ("Track"). Moreover, naturally occurring dopamine release transients, measured soon after injection, and a longer series of transients, measured 16 minutes after injection, are shown. These data demonstrate the feasibility of simultaneously measuring naturally-occurring dopamine transients and behavioral alterations at near-millisecond temporal resolutions.

Concluding remarks

The data presented here are made available not only by the hard work of personnel in the Johnson laboratory, but also are the product of important collaborations that have been established. These collaborations were necessary to obtain the transgenic HDtg rats (S. von Hörsten and O. Riess, Germany) and also to obtain behavioral measurements (S.C. Fowler). These types of complimentary efforts are expected to become increasingly important for neuroscience research as newer, more specialized techniques are developed.

Acknowledgements

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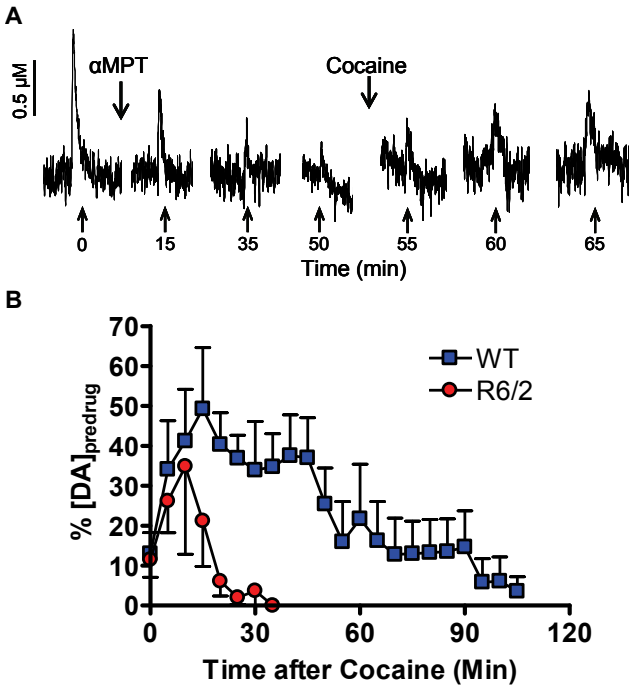

Figure 1. Cocaine-mobilized reserve pools are diminished in R6/2 mice compared to WT mice. Brain slices from 12-week old R6/2 mice and age-matched WT control mice were treated with α-methyl-p-tyrosine (50 μM). When stimulated dopamine release disappeared, cocaine (20 μM) was also added to the perfusion solution. The peak cocaine-induced increase in stimulated dopamine release in R6/2 slices was not significantly less than that observed in WT slices, but was substantially shorter in duration (n = 5 WT and 5 R6/2 mice).

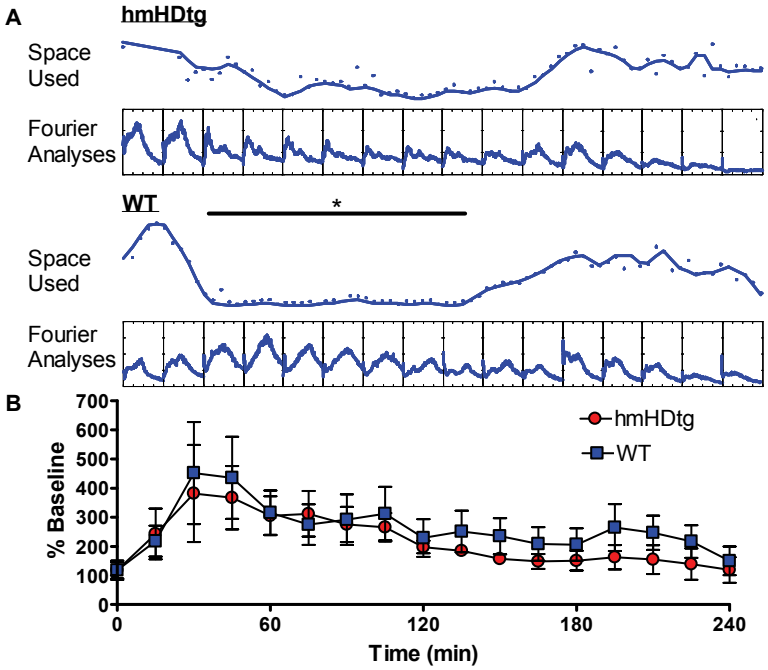

Fig. 2. Transgenic HD rats are less spatially confined than WT rats after AMPH injection even though extracellular dopamine levels are the same. Rats were injected i.p. (5.0 mg/kg) with AMPH at t = 0 min and synchronized behavioral and neurochemical measurements were collected. ***A***, Space used (y-axis) was measured using the force plate actometer. The asterisked bar denotes 15-min time blocks in which the space used was significantly different between WT and hmHDtg rats ($p < 0.01$). Force spectra (arbitrary units), derived from Fourier analyses, are shown below respective plots of Space Used. WT rats develop classic 8 to 10 Hz focused stereotypy, while hmHDtg rats express an altered force response at lower frequencies. Force is normalized to body weight for all force spectra. ***B***, Plot of average (± SEM) extracellular dopamine levels obtained by microdialysis sampling conducted simultaneously with the force plate actometer measurements. Values are normalized against the same rats injected with saline three days prior. Samples were collected from 4 HDtg rats and 5 WT rats while behavior was simultaneously measured using the force-plate actometer. Therefore, the behavioral data directly correspond to the force spectra plots collected within 15 minute time periods between adjacent pairs of data points.

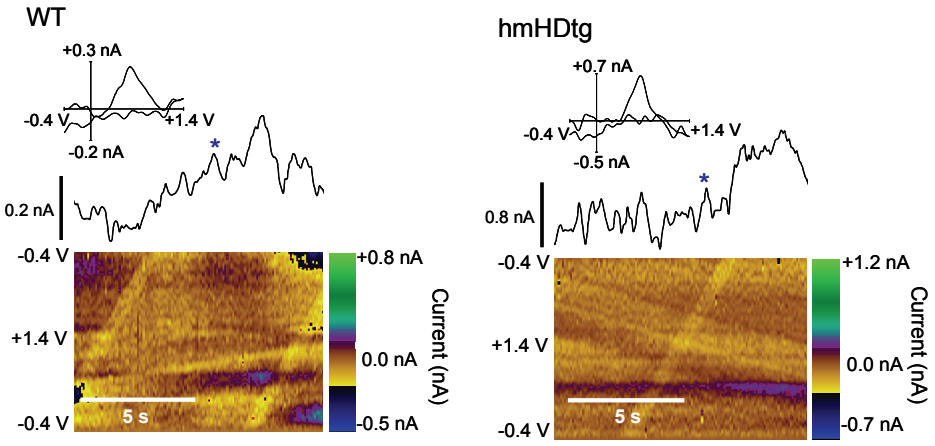

Figure 3. Alterations in dopamine release transients measured from HDtg rats. FSCV was used to measure transient spikes in extracellular dopamine concentration in the dorsolateral caudate of male WT and hmHDtg rats. The release plots of dopamine transients are shown above the color plots. Current was sampled at about +0.6 V for each successive CV. A sample CV corresponding to one of the peaks, denoted by an asterisk, confirms the presence of dopamine. Corresponding color plots of successive CVs, unhinged and stacked, are shown below the release plots. Different currents are expressed as different colors (scale shown on right side of plot). The time scale bar on the color plot also applies to the release plot. Scan rate: 400 V/s, CV update rate: 10 CVs/s.

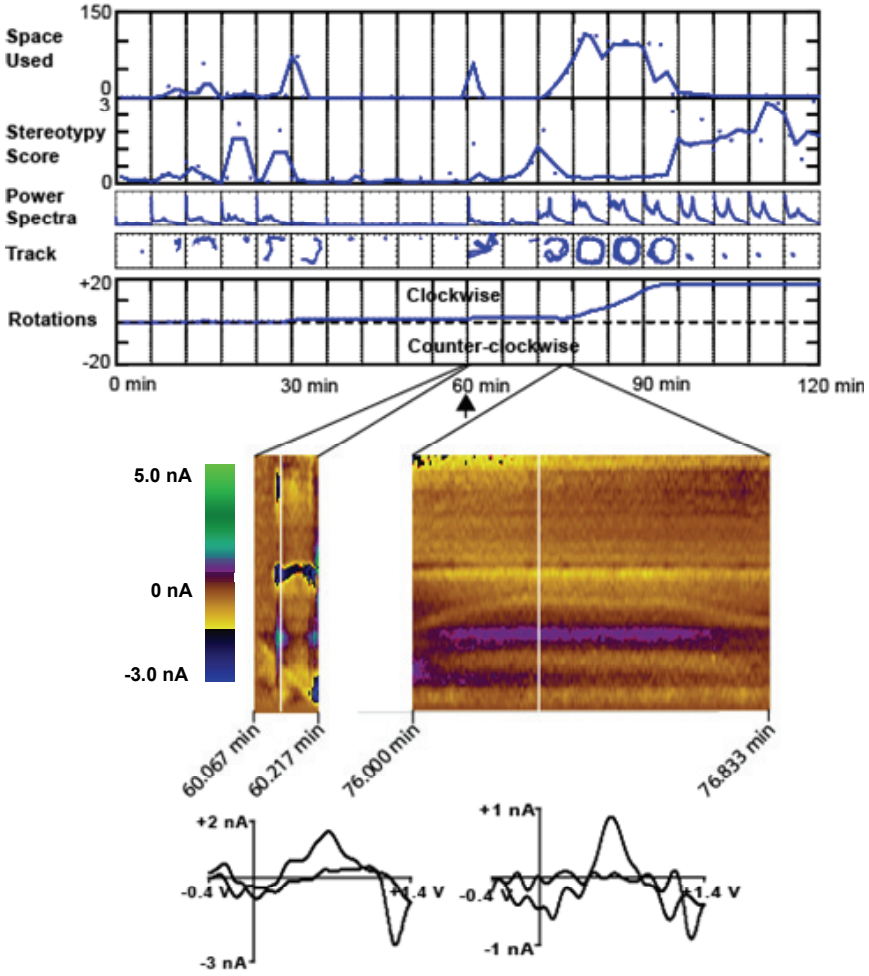

Fig. 4. Simultaneous, near-millisecond measurements of dopamine release transients and behavior. A male Sprague-Dawley rat (weight 500 g) was injected i.p. (5 mg/kg) with AMPH (indicated by arrow) 60 min after initiation of behavioral and voltammetric measurements. The collection of actometer data, including space used, stereotypy score, power spectra, X-Y track, and number of rotations (top panel) was synchronized with the collection of voltammetry files (bottom color plots and CVs). Behavioral data, collected at 100 samples/s, was synchronized to the nearest ms with the voltammetry data, collected at 10 CVs/s. The CVs were sampled from the color plots at the white vertical lines. Voltammetry data sampling ranges are shown beneath the respective color plots.

The Nebraska Center for Molecular Biology of Neurosensory Systems: A Collaborative COBRE Project

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The National Center for Research Resources (NCRR) at the National Institutes of Health has developed the Institutional Development Award (IDeA) program to enhance biomedical research where NIH funding has been in the lower tier. Twenty-three states and Puerto Rico qualify for IDeA programs, including Nebraska, Kansas, and Oklahoma in our region. The Centers for Biomedical Research Excellence (COBRE) program is one of these mechanisms. COBREs are each organized around a central scientific theme, and the growth of research in that area is facilitated in three ways: support of core facilities to serve as resources, support of individual research projects primarily for junior faculty, and development of a mentoring program to ensure that the research projects result in independent NIH funding. In addition to building a network of successful researchers within a COBRE, regional interaction of COBREs and other IDeA programs is encouraged.

In building the research infrastructure of a state, a COBRE program has a particularly unique aspect: the research projects associated with the center are generally junior investigators who have not had previous renewable NIH funding, and their projects are designed to be the basis of grant applications that are funded after 2 -3 years of Center support. When external funding starts, the projects rotate off Center funding and new projects take their place. The mentoring program provides advice to the investigators to help ensure that he projects are successful, and the core facilities provide technical support for the investigators as well as for other researchers in the participating institutions. Thus, the Center can aid junior faculty in establishing an independent research program, and can also help make advanced technologies available to researchers beyond the Center.

The Nebraska Center for the Molecular Biology of Neurosensory Systems is built around the characterization of the molecular mechanisms controlling the development and maintenance of neurosensory functions, particularly vision and hearing. Neurosensory cells of the inner ear and retina are not replaced after damage or degeneration, so understanding of the regulation of