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Visualizing synaptic disruptions in the release and regulation of dopamine hotspots in Huntington's Disease

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ABSTRACT

Dopamine neuromodulation is a critical process that facilitates learning, motivation, and motor control. Disruption of these processes has been implicated in several neurodegenerative disorders including Huntington's Disease (HD). While dopaminergic signaling is a therapeutic target for treating physical and psychiatric HD symptoms, the mechanism by which dopaminergic dysfunction occurs during HD is unknown. New tools for the visualization of dopamine dynamics at the spatiotemporal resolution of neuromodulator release (ms) and dopaminergic boutons (μm) provide a richer understanding of how dopamine signaling is disrupted in HD. Here we employ near-infrared fluorescent catecholamine nanosensors (nIRCats) to image dopamine release within the striatum of R6/2 Huntington's Disease model mice of either sex. We find that dorsal striatal dopamine release decreases with progressive degeneration and that these deficits are primarily driven by a decrease in the number of nIRCat imaged dopamine release sites, termed dopamine hotspots, combined with decreased release fidelity. Using nIRCat's high spatial resolution, we track individual dopamine hotspots over repeated stimulations and pharmacological applications to measure dopamine release fidelity from individual sites. Compellingly, we found that D2-receptor (D2R) antagonist sulpiride drives increased fidelity of dopamine hotspot activity in wild type striatum but not in late-disease HD striatum, suggesting that D2R regulation of dopamine release is compromised in late HD. These findings, enabled by nIRCats, provide more detailed insights into how dopamine release is disrupted and dysregulated during Huntington's Disease.

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SIGNIFICANCE STATEMENT

Huntington's Disease (HD) is a neurodegenerative disorder with no cure. Dopamine signaling is known to deteriorate in HD but has not been studied at the level of individual release sites.

Here, we image dopamine release from individual dopamine release sites in R6/2 HD mouse brain slices containing the striatum with novel dopamine nanosensors. We find that dopamine release site number and release fidelity are decreased in late HD. Furthermore, we demonstrate that D2-receptor signaling may be altered in late disease R6/2 HD mice, and that these disruptions are likely to drive decreased dopamine release fidelity over multiple stimulations. These findings suggest dopaminergic neurons projecting to the striatum as a potential therapeutic target for HD treatment to complement more commonly targeted medium spiny neurons.

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INTRODUCTION

Huntington's Disease (HD) is a neurodegenerative disorder caused by aberrant expansion of CAG repeats within the Huntingtin Gene leading to early cognitive symptoms such as difficulty multi-tasking, depression, and apathy, as well as motor dysfunction that begins with *chorea* and progresses to *bradykinesia* (Finkbeiner, 2011). Neurodegeneration in HD occurs primarily in the striatum via selective dysfunction and death of medium spiny neurons (MSN) (Tang et al., 2005; Lebouc et al., 2025; Mei et al., 2025), coincident with the formation of mutant huntingtin protein (mhtt) aggregates and disruptions in neuronal signaling within the striatum (Arrasate et al., 2004; Takahashi et al., 2008; Callahan et al., 2022). The neurodegenerative and behavioral changes observed during HD are hypothesized to arise through combined cell autonomous (mhht) and non-cell-autonomous (synaptic) effects (Cepeda and Levine, 2020; Galimberti et al., 2024; Cheng et al., 2025). In this light, study of synaptic dysfunction during HD at size-scales relevant to HD molecular events may provide a richer understanding of how HD manifests as a neurodegenerative disease.

Healthy motor function relies on dopamine release from neurons projecting from the substantia nigra pars compacta to the striatum, where dopamine differentially modulates striatal MSNs via dopamine D1 receptors (D1R) and dopamine D2 receptors (D2R) (Bariselli et al., 2019). Striatal dopamine can also shape future dopamine release through presynaptic feedback inhibition via D2-autoreceptors on dopaminergic axons or D2-heteroceptors on cholinergic interneurons (CIN) (Beaulieu and Gainetdinov, 2011; Threlfell et al., 2012). Bi-phasic changes in dopamine release during HD progression have been observed in both human patients and animal models, with elevated dopamine release coinciding with *chorea*, and decreased dopamine release coinciding with *bradykinesia* (Yamamoto et al., 2000; Ortiz et al., 2010, 2011; Callahan and Abercrombie, 2011; Choi et al., 2014; Chuhma et al., 2014). These changes in dopamine release are accompanied by early loss of D2R expressing MSNs, further disrupting dopamine signaling (Callahan et al., 2022; Lebouc et al., 2025). While overall dopamine release trends across HD disease progression are well understood, less is known about how HD dopamine signaling changes at the level of release sites. The development of tools enabling high spatio-temporal dopamine imaging has been instrumental in furthering this field of study, in particular non-genetically encoded tools such as near infrared catecholamine sensors (nIRCats) (Beyene et al., 2019).

nIRCats serve as adept dopamine sensors in the dorsal striatum capable of imaging 2 µm dopamine release sites that demonstrate strong, non-photobleaching fluorescence in response to dopamine release in ex vivo brain slices (Beyene et al., 2018, 2019). The non-genetically encoded aspect of these sensors allows them to be readily deployed in animal models of disease at a wide range of ages, and their structural dissimilarity to endogenous dopamine receptors facilitates studies incorporating dopamine receptor pharmacology. In this work we utilize nIRCat's high spatial resolution to study changes in dopamine release in ex vivo brain slices from R6/2 HD model mice over disease course, and measure changes in the fidelity

of dopamine release across repeat stimulations to examine the reliability of dopamine release at the level of single hotspots. These analyses are combined with nIRCat's pharmacological compatibility to explore how the sensitivity of dopamine hotspots to extracellular Ca^{2+} concentration, D2R signaling, and acetylcholine (ACh) release from CIN changes over disease course. We find that dopamine release in R6/2 HD mice is progressively disrupted at the level of individual release sites, with early alterations in Ca^{2+} sensitivity followed by loss of dopamine hotspots and release fidelity in late disease. Furthermore, we find D2R antagonism fails to restore hotspot recruitment or fidelity in late HD, suggesting compromised presynaptic regulation of dopamine release.

MATERIALS AND METHODS

Animals

Male B6CBA-Tg(HDexon1)62Gpb/3J mice (R6/2 mice) were purchased from Jackson Labs and bred at 6 weeks with 10-week female C57BL/6 mice. Pups were weaned and genotyped for the human HD fragment at 3 weeks. Mice were housed at three to five animals per cage with food and water available *ad libitum* and maintained in a temperature-controlled environment on a 12h dark/light cycle with light-on at 7:00 am and light-off at 7:00 pm. All animal procedures were approved by the University of California Berkeley Animal Care and Use Committee.

nIRCat Nanosensor Synthesis and Characterization

nIRCat nanosensor was synthesized and characterized as described previously in (Yang et al., 2021). A single-walled carbon nanotube (SWNT) slurry was created by combining 1050 mg of hydrated HiPco SWNTs purchased from NanoIntegris with 25 mL of molecular grade

water in a 50 mL Falcon Tube and probe sonicating the solution for 2 minutes at 10% amplitude until the slurry was visually distributed. To create nIRCat nanosensors, 100 µL of SWNT slurry was mixed with 1 mg of $(GT)_6$ oligonucleotides purchased from Integrated DNA Technologies (standard desalting) in 100 mM NaCl and bath sonicated for 10 minutes (Branson Ultrasonic 1800) followed by 5 minutes of rest at room temperature. The solution was then sonicated on ice for 10 minutes using a probe-tip sonicator (Cole-Parmer Ultrasonic Processor, 3-mm diameter tip, 5 W power) followed by 5 minutes of rest on ice. The sonicated solution was incubated at room temperature for 30 mins and centrifuged at 16,000 g (Eppendorf 5418) for 30 minutes to removed unsuspended SWNT bundles and amorphous carbon. The supernatant was removed for use and stored at 4˚C for 30 minutes before characterization. Final supernatant was stored at 4˚C until use.

nIRCat nanosensors were synthesized in 1 mL batches and combined for characterization. Nanosensor concentrations were determined using absorbance at 632 nm with an extinction coefficient of $\varepsilon = 0.036\ (mg/L)^{-1}cm^{-1}$. To characterize the visible and nIR absorption spectrum, nanosensors were diluted to a concentration of 5 mg/L in 1x PBS and measurements were taken using a UV-VIS-nIRC spectrophotometer (Shimadzu UV-3600 Plus). To test nanosensor fluorescence response to dopamine (DA) administration, each sensor batch was diluted to a working concentration of 5 mg/L in 1x PBS and 99 µL aliquots were made into a 96-well plate. Baseline fluorescence was taken using a 20x objective on an inverted Zeiss microscope (Axio Observer D1) coupled to a Princeton Instruments spectrograph (SCT 320) and a liquid nitrogen cooled Princeton Instruments InGaAs linear array detector (PyLoN-IR). Nanosensors were excited using a 721-nm laser (Opto Engine LLC). After the baseline fluorescence was taken, 1 µL of 10 mM DA in 1xPBS was added to the 99 µL of nanosensor solution to produce a final concentration of 100 µM DA in the well, and a robust fluorescence response to DA was confirmed. To characterize the full dynamic range of the nanosensor.

including the limit of detection and saturation, nIRCat fluorescence response to DA concentrations ranging from 1 nM to 10 mM DA in NaCl, ACSF, and human CSF (Lee Biosolutions 991-19-P) were tested in separate wells. Mixing was achieved through pipette-based addition followed by rapid diffusion in the well volume. To obtain the dissociation constant K_d, the concentration dependence data was fitted with the Hill equation, which is defined as normalized $\frac{dF}{F_0} = \frac{[Dopamine]^n}{[Dopamine]^n + K_d}$, where n and K_d were fitting parameters. The limit of detection was determined by extrapolating the value that is 3 times the standard deviation from the baseline in the fitted results.

To test nanosensor sensitivity to Ca^{2+} concentration, individual solutions of increasing Ca^{2+} concentration were prepared in 10 mM NaCl, where molarity of NaCl was held constant and molarity of $CaCl_2$ was varied to reflect final Ca^{2+} concentrations of 0 mM (control), 1 mM, 2 mM, and 4 mM. Next, nanosensor was diluted to a concentration of 1 mg/L in each Ca^{2+} (0 mM - 4 mM) solution and 99 µL aliquots were made into separate wells of a 96-well plate. Baseline fluorescence was taken using a 20x objective on an inverted Zeiss microscope (Axio Observer D1) coupled to a Princeton Instruments spectrograph (SCT 320) and a liquid nitrogen cooled Princeton Instruments InGaAs linear array detector (PyLoN-IR). Nanosensors were excited using a 721-nm laser (Opto Engine LLC). After the baseline fluorescence was taken, 1 µL of 10 mM DA in water was added to achieve a well concentration of 100 µM DA and nIRCat fluorescence response to DA was measured. Mixing was achieved through pipette-based addition followed by rapid diffusion in the well volume. For no-DA controls, one set of three wells with 0 mM Ca^{2+} in 10 mM NaCl received 1 µL 10 mM NaCl instead of 10 mM DA.

Phenotypic Motor Coordination Assessment

The accelerating rotarod test was used to evaluate changes in motor coordination in R6/2 HD and WT mice. For accelerating rotarod tests, mice were placed on a Ugo Basile

rotarod for 1 min a 5 rpm to adjust to the apparatus. At the end of the 1 min adjustment period, the speed of the rotarod was increased at a constant rate to a final speed of 40 rpm over 350 s. The trial was terminated after mice either fall off the rod, tumbled on the rod for two consecutive rotations, or “maxed out” the rod speed at 360 s. Starting at four weeks, mice were introduced to the rotarod and completed the test for 3 consecutive days before their rotarod times plateaued and performance was recorded on the fourth day. For subsequent weeks, mice completed the rotarod only once a week.

nIRCat dopamine Imaging

Acute ex vivo brain slices were prepared using protocols previously described (Yang et al., 2021). Briefly, mice are deeply anesthetized via intraperitoneal ketamine/xylazine cocktail and perfused transcardially using cold cutting buffer (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4,3.5 mM $MgCl_2$, 10 mM glucose, and 0 mM $CaCl_2$). The brain was then rapidly dissected, mounted on a vibratome stage (Leica VT1200 S) using super glue, and cut into 300 µm thick slices containing the dorsal striatum. Slices were then collected and incubated at 37˚C for 30 minutes in carbogen (95% O_2/5% CO_2) saturated ACSF (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose, and 2 mM $CaCl_2$) followed by 30-minute incubation at room temperature in carbogen saturated ACSF. All slices were maintained at room temperature in carbogen saturated ACSF until imaging and used within 6 hours of preparation.

Slices were labeled through passive incubation in 5 mL of room temperature carbogen saturated ACSF containing nIRCat nanosensor at a concentration of 2 mg/L for 15 minutes. After incubation, the slices were transferred through 3 wells of a 24-well plate containing ACSF to rinse off non-localized nIRCat sensor and then left to rest at room temperature ACSF for 15 minutes before transfer to the 32˚C recording chamber. The slices were maintained at 32˚C in

the recording chamber for the duration of all dopamine imaging. Once placed in the recording chamber, slices equilibrated for 15 minutes during which a tungsten bipolar stimulation electrode was positioned at a field of view in the dorsal-lateral striatum using a 4x objective (Olympus XLFluor 4/ 340). Under a 60x objective the electrode was moved 200 µm away from the selected field of view and brought into contact with the surface of the brain slice. In all experiments, 600 total images were acquired into an image-stack at a rate of 9 frames per second. A single stimulation of 0.3 mA was applied after 200 frames of baseline were collected. Videos of stimulation were collected in 3 replicates or in 10 replicates as indicated. All slices were given 5 minutes between each stimulation with the excitation laser path shuttered. Prior to stimulation, the laser was un-shuttered for 1 minute.

nIRCat Imaging Calcium (Ca^{2+}) Concentration and Sulpiride Application

To image nIRCat-labeled acute brains slices at multiple extracellular Ca^{2+} concentrations, buffers were prepared at three Ca^{2+} concentrations: 1 mM Low Ca^{2+} Buffer (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose, and 1 mM $CaCl_2$), 2 mM Normal Ca^{2+} Buffer (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose, and 2 mM $CaCl_2$), 4 mM High Ca^{2+} Buffer (119 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10 mM glucose, and 4 mM $CaCl_2$). Following stimulation in 2 mM Normal Ca^{2+} Buffer, 4 mM High Ca^{2+} Buffer was flowed into the imaging chamber for 15 minutes (full bath turnover in ~3 minutes). After buffer transfer, the slice was stimulated at 0.3 mA in triplicate as described for 2 mM Normal Ca^{2+} Buffer. The Buffer in the imaging chamber was then exchanged to 1 mM Low Ca^{2+} Buffer by flowing 1 mM Low Ca^{2+} Buffer into the imaging chamber for 15-minutes. The slice was stimulated at 0.3 mA in triplicate.

To nIRCat image acute brain slices in the presence of the D2R-antagonist sulpiride, sulpiride was dissolved in sterile DMSO and frozen in 100 µL aliquots at -20˚C. Prior to use, single aliquots were thawed and added to 100 mL of ACSF to produce a 10 µM sulpiride solution. Acute brain slices were stimulated at 0.3 mA in triplicate in sulpiride-free ACSF. ACSF containing 10 µM sulpiride was then flowed into the imaging chamber for 15 minutes before stimulating the slice at 0.3 mA in triplicate.

To nIRCat image acute brain slices in the presence of the acetylcholine receptor antagonist Dihydro-β-erythroidine hydrobromide (DHβE), DHβE was dissolved in sterile DMSO and frozen in 100 µL aliquots at -20˚C. Prior to use, single aliquots were thawed and added to 100 mL of ACSF to produce a 1 µM DHβE solution. Acute brain slices were stimulated at 0.3 mA in triplicate in DHβE-free ACSF. DHβE-containing ACSF was flowed into the imaging chamber for 15 minutes before stimulating the slice at 0.3 mA in triplicate.

Image Stack Processing and Data Analysis of nIRCat Data

Raw image stack files were processed using a custom-built, publicly available MATLAB program (https://github.com/jtdbod/Nanosensor-Imaging-App). Image processing procedures are described in depth in Yang, del Bonis O'Donnel et al. and briefly summarized here (Yang et al., 2021). Regions of dopamine release were identified by large changes in nIRCat dF/F response. To minimize bias and improve stack processing time, regions of high dF/F response (dopamine hotspots) were identified by defining a grid of 2 µm squares across the field of view. For each grid square, dF/F was calculated using the formula $(F- F_0) /F_0$, where F_0 is defined by the average fluorescence of the grid square over the first 30 frames of the image stack and F is the fluorescence intensity of the grid square as it changes over the 600 collected frames. Grid squares were identified as regions of interest if they exhibit behavior that is 3 standard deviations above the baseline F_0 activity around time of stimulation (200 frames).

Dopamine hotspots were identified for each stimulation replicate image stack taken at a given field of view on a brain slice. The peak dF/F of each dopamine hotspot in the image stack was averaged to give the average image stack peak dF/F. The average image stack peak dF/Fs from the three stimulation replicates were then averaged to give the slice average peak dF/F. Similarly, the number of dopamine hotspots identified from each stimulation replicate image stack was averaged to give the slice average hotspot number. Mean dopamine release and reuptake traces were produced by averaging the average traces from each slice (3 stimulations per slice, 1 slice per animal). For Ca^{2+} concentration experiments, normalized mean peak dF/F was calculated as (mean peak dF/F) / (mean peak dF/F at 2 mM Ca^{2+}) and normalized mean hotspot number was calculated as (mean hotspot number) / (mean peak hotspot number at 2 mM Ca^{2+}).

To track hotspot fidelity, each initially defined grid square was assigned a unique position number, allowing the position of each identified dopamine hotspot within an image stack to be recorded. For a set of triplicate image stacks, an array of all unique hotspots active across the stimulation replicates was generated. Python code was then used to analyze whether each unique hotspot was active in each stimulation replicate. The number of stimulations a unique hotspot was active in was summed across the three replicates and assigned as the dopamine release fidelity (e.g. hotspot “82” is active in 2 out of 3 stimulations and is assigned release fidelity = 0.66). The same procedure was used to identify the dopamine release fidelity of hotspots active after drug application. Hotspots were then separated into three groups for mean peak dF/F analysis: hotspots that are active and above nIRCat’s detection threshold (post-threshold) both before and after drug application (shared hotspots), hotspots that become active and post-threshold after drug application (added hotspots), and hotspots that are only active before drug application. Mean hotspot fidelity for an ex vivo brain slice was calculated by first averaging the fidelity of all hotspots active in a stimulation and then averaged for all stimulation replicates. Hotspot fidelity was analyzed for Ca^{2+} concentration and sulpiride

application data taken with 3 stimulation replicates per slice. Additional sulpiride application data was taken with 10 stimulation replicates per slice.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

All nIRCat Imaging data was processed using a custom-built, publicly available MATLAB program (https://github.com/jtdbod/Nanosensor-Imaging-App). Statistical analyses were conducted using Graphpad Prism. All bar graphs show the mean with error bars denoting standard deviation (SD). All single data points correspond to a single slice taken from an animal. Data comparing two variables was analyzed using a two-way repeated measures with bath condition as the within-subject factor (e.g., sulpiride, ACSF, Ca^{2+} concentration) and disease state as the between-subject factor (e.g., HD, WT). Sidak's multiple-comparisons test was used as a post-hoc test if two-way ANOVA analyses indicated significant differences. Data comparing multiple values of one variable were analyzed using an one-way ANOVA with a Dunnet multiple-comparisons post-hoc test. Group sizes were determined based on previous literature (Adil et al., 2018).

CODE ACCESSIBILITY

All analyses were performed using in-house developed code using either MATLAB or Python. Code to process nIRCat image stacks is available on GitHub: https://github.com/jtdbod/Nanosensor-Imaging-App.

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RESULTS

Imaging high spatio-temporal dopamine release from hotspots in ex vivo slices

HD related changes in dopamine signaling are well documented in both human HD patients and murine animal models (Suzuki et al., 2001; Jahanshahi et al., 2010; Bédard et al., 2011; Cepeda et al., 2014; Cepeda and Levine, 2020). Foundational work investigating dopamine release in R6/2 HD mice has employed Fast Scan Cyclic Voltammetry (FSCV) and in vivo microdialysis to show that dopamine tone and evoked dopamine release decreases over the course of degeneration in HD models (Johnson et al., 2006, 2007; Ortiz et al., 2010; Callahan and Abercrombie, 2011). Both FSCV and in vivo microdialysis provide measurements of dopamine release by locally averaging dopamine from multiple dopaminergic release sites along the length of the probe. We build upon this work by imaging dopamine release at higher spatiotemporal resolution through the use of nIRCat nanosensors capable of visualizing dopamine release from individual dopamine release sites termed dopamine hotspots. The insights gleaned from the ability to study dopamine release at the level of individual hotspots enable a quantitative understanding of how dopamine release sites and their release characteristics are altered during the course of degeneration in HD models.

We have previously shown that nIRCat nanosensors robustly image dopamine release at the level of hotspots in ex vivo brain slices and effectively capture release site level variability in dopamine release intensity, and sensitivity to pharmacological perturbation (Beyene et al., 2019; Yang et al., 2021). These nIRCat nanosensors utilize single-walled carbon nanotubes as the source of their fluorescence in the near-infrared, allowing for sustained detection of dopamine release that is not subject to photobleaching and is insensitive to competing neurotransmitters including γ-aminobutyric acid, glutamate, and acetylcholine (ACh) (Beyene et al., 2017, 2019; Yang et al., 2021) (Fig 1a). Previous *in vitro* studies have shown that nIRCat produces detectable fluorescence changes for dopamine concentrations within a range of 10 nM to 100 μM when suspended in 100 mM NaCl (Beyene et al., 2019). As the performance of nIRCat sensors has been reported to change with the presence of different salts and proteins, we first sought to

determine the detection range of nIRCat *in vitro* in the presence of biologically relevant salts and protein concentrations. Our findings indicate that nIRCat suspended in both ACSF and human cerebral spinal fluid show detectable fluorescence changes for dopamine concentrations within a range of 1 μM to 100 μM (Fig. S1a). Because evoked dopamine concentration in brain tissue can exceed 1 μM (Beyene et al., 2017), these results indicate that nIRCat imaging in ex vivo brain slices is capable of detecting changes in dopamine concentration within a range physiologically relevant to striatal dopamine release. Furthermore, we evaluated the effect of Ca^{2+} concentrations within a range of 0 mM to 4 mM on the *in vitro* fluorescence response of nIRCat to dopamine. Although omnibus ANOVA indicated variation between groups, Dunnett's post-hoc test showed no significant differences between nIRCat fluorescence response to 100 μM dopamine at 0 mM Ca^{2+} and all other tested Ca^{2+} concentrations (Fig. S1b, One-way ANOVA with Dunnett's post-hoc: 0 mM Ca^{2+} vs 1 mM Ca^{2+} $p = 0.7399$, 0 mM Ca^{2+} vs 2 mM Ca^{2+} $p = 0.6737$, 0 mM Ca^{2+} vs 4 mM Ca^{2+} $p = 0.0736$).

Dopamine release deficits in late HD are driven by decreased number of dopamine hotspots

We next sought to perform nIRCat dopamine imaging in ex vivo brain slices prepared from WT and R6/2 HD mice at three time points: immediately at the onset of motor degeneration (p32-35, 4 weeks), mid-degeneration (p64-66, 9 weeks), and late in disease (p87-93, 12 weeks) (Fig. 1b). WT and HD animals were aged without need for sensor injection or expression, and perfused at designated timepoints to produce acute ex vivo brain slices. The slices were then passively incubated with nIRCat following dissection to allow nanosensor intercalation into the extracellular space of each slice, after which they were transferred to a near-infrared capable microscope rig for imaging (Fig. 1b). Building upon foundational FSCV studies, we adapted an electrical stimulation paradigm previously utilized to measure changes in striatal dopamine release in HD model mice for use in our nIRCat imaging experiments (Ortiz et al., 2010; Johnson et al., 2007;

Johnson et al., 2006). A single-pulse 0.3 mA electrical stimulation was used to elicit dopamine release from nIRCat labeled brain slices and the microscope objective was positioned over the dorsal lateral striatum (DLS), 200 μm away from the stimulating electrode, to capture changes in fluorescence corresponding to dopamine release.

We have previously shown in C57BL/6 mice that nIRCat dopamine imaging in the dorsal striatum reveals approximately 2μm-wide regions of fluorescence change – termed dopamine hotspots – that emerge in response to electrical or optogenetic stimulation of dopamine release (Beyene et al., 2019). As such, we sought to develop a framework by which to investigate changes in dopamine release in HD through examination of the number and behavior of active dopamine hotspots (Fig. 1c). We recorded the average number of dopamine hotspots active in response to electrically stimulated dopamine release in both R6/2 HD and WT slices. We also utilized the high spatial resolution of dopamine release afforded by nIRCat imaging to record the dopamine release profile of each dopamine hotspot, which were averaged to produce the metric mean peak dF/F, a measure that encompasses the average dopamine release intensity across hotspots (Fig. 1c). Together, these two metrics provide a means of understanding changes in dopamine release across the space of the DLS. For example, decreases in dopamine release measured via tools that detect dopamine by spatially averaging across multiple hotspots may be driven by decreases in dopamine hotspot number, decreases in mean peak dF/F, or both.

We next examined how the number of dopamine hotspots active in response to a single 0.3 mA electrical pulse changed with the age of R6/2 HD and WT mice. WT brain slices showed no significant change in the number of dopamine hotspots across 4, 9, and 12 weeks. In contrast, R6/2 HD mice showed a significant decrease in dopamine hotspot number between 9 weeks and 12 weeks (mean difference = 159.7 hotspots, *p=0.0454 Sidak's post-hoc test) (Fig.1d). In addition, the number of dopamine hotspots present in R6/2 HD slices at 12 weeks is significantly lower than the number of dopamine hotspots in WT slices at 12 weeks (mean difference = 231.1

hotspots, ***p=0.0007 Sidak's post-hoc test) (Fig. 1d). Neither WT nor R6/2 HD brain slices showed a significant pairwise difference in dopamine hotspot mean peak dF/F over 4, 9, and 12 weeks despite a significant main effect (two-way ANOVA: age $F(2,32)=3.534$, *p = 0.0410; disease state $F(1,32)=0.9824$, p=0.329, interaction $F(2,32)=0.266$, p=0.7681. Sidak's post-hoc test shows no significant difference between 4 wks vs 9 wks for WT (p = 0.9636) or HD (p =0.9975) slices. No significant difference between 9 wks vs 12 wks for WT (p = 0.5218) or HD (p =0.1488)) (Fig. 1e). Moreover, the mean peak dF/F of dopamine hotspots at 12 weeks did not differ significantly between WT and R6/2 HD slices (p=0.5604 Sidak's post-hoc test) (Fig. 1e).

The magnitude of this observed decrease in dopamine release from 12-week R6/2 HD slices using nIRCat imaging is consistent with the magnitude of decreased dopamine release previously measured via spatially averaged methods such as FSCV and microdialysis (Fig. S1c) (Johnson et al., 2006, 2007; Ortiz et al., 2010; Callahan and Abercrombie, 2011). However, in contrast to previous studies, we do not find a difference in either hotspot number or mean peak dF/F between R6/2 HD and WT slices at 9 weeks despite R6/2 HD animals showing decreased latency to fall on the rotarod at this timepoint (Fig. 1f). These findings suggest that impaired dopamine release at this mid-disease timepoint may not be captured by dopamine hotspot number and mean peak dF/F, or that current nIRCat nanosensors may not be sensitive enough to capture changes in dopamine release at 9 weeks. As such, we examined dopamine hotspot changes between early and late disease brain slices (4 weeks and 12 weeks) in subsequent experiments.

HD dopamine hotspot mean peak dF/F release intensity shows increased sensitivity to extracellular Ca^{2+} concentration in early disease

Striatal dopamine release occurs in a Ca^{2+}-dependent manner, with the entry of Ca^{2+} ions into the dopamine varicosity triggering the fusion of dopamine-containing synaptic vesicles (Leviel et al., 1994). Increased extracellular Ca^{2+} concentration has been shown to modulate

neurotransmitter release by increasing the probability of vesicle release, increasing the effective size of readily-releasable pool vesicles, and recruiting boutons with low release probability to more active states (Leitz and Kavalali, 2011; Thanawala and Regehr, 2013). Changes in neuronal Ca^{2+} handling have been reported in HD, though these studies have largely focused on aberrant N-methyl-D-aspartate receptor signaling or mitochondrial Ca^{2+} uptake in MSNs (Tang et al., 2005 p.200; Mackay et al., 2018). Given observed differences in dopamine hotspot number between WT and R6/2 HD mice at 12 weeks, we sought to understand how extracellular Ca^{2+} concentration would affect the behavior of dopamine hotspots in R6/2 HD and WT slices early and late in disease.

Previous studies have demonstrated that nIRCat imaged dopamine hotspots from healthy C57BL/6 mice showed increased mean peak mean dF/F at high extracellular Ca^{2+} concentrations that were abolished when slices are bathed in 0 mM Ca^{2+} ACSF (Beyene et al., 2019). We adapted these studies for imaging in slices taken from R6/2 HD and WT mice, including tracking how the number of active dopamine hotspots changes with increasing extracellular Ca^{2+} concentration. Electrically-evoked dopamine release was measured from 4-week R6/2 HD and WT slices that were sequentially imaged in standard ACSF (2 mM Ca^{2+} ACSF), high Ca^{2+} concentration ACSF (4 mM Ca^{2+} ACSF), and low Ca^{2+} concentration ACSF (1 mM Ca^{2+} ACSF). As expected, increasing extracellular Ca^{2+} concentration from 2 mM to 4 mM Ca^{2+} increased the number of imaged dopamine hotspots in both R6/2 HD and WT slices, whereas a corresponding decrease to 1 mM Ca^{2+} resulted in fewer imaged dopamine hotspots in both R6/2 HD and WT slices (Fig. 2a). No significant difference in the number of dopamine hotspots in 4-week R6/2 HD and 4-week WT slices was found at any extracellular Ca^{2+} concentration (Fig. 2a). The mean peak dF/F of dopamine hotspots was also found to increase with extracellular Ca^{2+} concentration in both WT and R6/2 HD mice (Fig. 2b, Fig. S2a-d). Interestingly, dopamine hotspots in 4-week R6/2 HD slices were found to have a higher mean

peak dF/F at 4 mM Ca^{2+} compared to their 4-week WT counterparts (mean difference = 0.0145, *p=0.0253 Sidak's post-hoc test) (Fig. 2b). These results suggest that dopamine hotspots in R6/2 HD slices at 4 weeks – a timepoint preceding observed changes in motor ability in R6/2 mice – release relatively more dopamine than their 4-week WT counterparts at high Ca^{2+} conditions where the probability of neurotransmitter release is globally increased.

High extracellular Ca^{2+} concentration increases dopamine hotspot number and mean peak dF/F release intensity in late disease

We next investigated how dopamine hotspot number and mean peak dF/F change with extracellular Ca^{2+} concentration after significant striatal degeneration has occurred in 12-week R6/2 HD mice. As observed in preceding sections, nIRCat imaging in standard 2mM Ca^{2+} ACSF shows significantly fewer active dopamine hotspots in R6/2 HD slices compared to their WT counterparts (Fig. 1d). We sought to investigate whether this loss was primarily driven by the physical degeneration of dopaminergic axons in late disease or by decreased dopamine release from hotspots that could be increased by raising Ca^{2+} concentration. Transition to 4 mM Ca^{2+} ACSF after nIRCat imaging in 2 mM Ca^{2+} ACSF resulted in an increase in the number of dopamine hotspots in both 12-week R6/2 HD and WT slices, suggesting that observed dopamine hotspot loss in 12-week R6/2 HD slices is not solely the result of physical degeneration of dopamine release sites (Fig. 2c). Instead, diminished dopamine release from hotspots – which can be partially rescued by increasing extracellular Ca^{2+} concentration – may contribute to the observed decrease in dopamine hotspot number in 12-week R6/2 HD slices at physiological conditions. Notably, the increase in dopamine hotspot number in R6/2 HD slices at 4 mM Ca^{2+} is not large enough to rescue hotspot number to WT levels (Fig. 2c), suggesting that physical degeneration of dopamine axons may still contribute to diminished dopamine release in 12-week R6/2 HD slices, or that dopamine hotspots may experience additional non-Ca^{2+}-dependent impairments of dopamine release.

Mean peak dF/F increases as Ca^{2+} concentration increases, with no difference in the mean peak dF/F of WT and HD hotspots at 1 mM Ca^{2+} or 2 mM Ca^{2+} (Fig. 2d). Slices taken from 12-week R6/2 HD mice showed decreased mean peak dF/F values in 4 mM Ca^{2+} ACSF compared to their 12-week WT counterparts, suggesting that the overall dopamine release capacity of R6/2 HD hotspots late in disease is decreased (mean difference = 0.0162, *p=0.0180 Sidak's post-hoc test) (Fig. 2d–h). Given that increasing Ca^{2+} concentration results in an increase in both dopamine hotspot number and mean peak dF/F, it is possible that the observed increase in hotspot number is driven in part by low peak dF/F hotspots below nIRCat's threshold of detection ("pre-threshold hotspots") entering into nIRCat's detection range at high Ca^{2+} concentrations ("post-threshold hotspots"). In previous sections, we report that nIRCat's dynamic range of detection extends up to 100 µM dopamine (Fig. S1a). As such, dF/F signal saturation as a result of striatal dopamine release is unlikely at physiological conditions. To more deeply examine the process of hotspots crossing into nIRCat's detection threshold with increasing Ca^{2+} concentration, we utilized nIRCat's ability to track individual dopamine hotspots across ACSF conditions to identify, within a brain slice, the subset of "shared" dopamine hotspots that are post-threshold in both 2 mM Ca^{2+} and 4 mM Ca^{2+}ACSF, as well as the subset of "added" dopamine hotspots that are pre-threshold at 2 mM Ca^{2+}ACSF but post-threshold at 4 mM Ca^{2+} ACSF. We compared the distribution of peak dF/Fs of shared dopamine hotspots at 4 mM Ca^{2+} ACSF to that of added dopamine hotspots at 4 mM Ca^{2+} ACSF and found that increasing Ca^{2+} concentration shifts both populations towards higher peak dF/F values. Furthermore, the distribution of peak dF/F values from added hotspots mirrored the peak dF/F distribution of shared and added hotspots combined at 4 mM Ca^{2+} (all post-threshold hotspots at 4 mM Ca^{2+}) (Fig. S5a-d). There was no observed skew of the added hotspots towards lower peak dF/F values, suggesting that hotspots that are newly post-threshold at 4 mM Ca^{2+} exhibit similar dopamine release characteristics at 4 mM Ca^{2+} to hotspots that are post-threshold at both 2 mM Ca^{2+} and 4 mM Ca^{2+} (Fig. S5a-d). Altogether, these results suggest that threshold

effects alone do not fully account for the increase in dopamine hotspot number observed at 4 mM Ca^{2+}.

During spatially-averaged measurements such as FSCV, measured dopamine release is a function of both dopamine hotspot number and dopamine hotspot release intensity as measured by peak dF/F. As a result, HD-driven changes in dopamine hotspot number could be potentially masked by the lack of change in mean peak dF/F. The spatial resolution achieved by nIRCat imaging allows us to separate dopamine hotspot number and dopamine hotspot release intensity and examine each feature individually to reveal changes in Ca^{2+}-dependent dopamine release within 12-week R6/2 HD slices. In this vein, we compared our findings to previous studies employing FSCV that report no significant change in the Ca^{2+} dependence of normalized stimulated dopamine release between 12-week R6/2 HD mice and their WT counterparts by normalizing mean peak dF/F and mean hotspots number values to their measurements at 2 mM Ca^{2+} (Johnson et al., 2007). In contrast to these previous studies, we found that while there is no significant difference in mean peak dF/F values at 1 mM Ca^{2+} or 4 mM Ca^{2+}, there is a significant increase in the normalized mean hotspot number in 12-week R6/2 HD slices compared to their WT counterparts (Fig. S2e-f). Thus, though 12-week R6/2 slices showed an overall diminished number of hotspots compared to WT at 4 mM Ca^{2+}, 12-week R6/2 slices also showed a larger proportional increase in hotspot number between 2 mM Ca^{2+} and 4 mM Ca^{2+}. These findings underscore the utility of nIRCat's improved spatial resolution to bring new insights to our understanding of dopamine transmission. In the context of HD-driven neurodegeneration, these findings suggest that dopamine hotspot loss in 12-week R6/2 HD mice is driven by both a physical loss of dopamine hotspots and a diminished dopamine release from hotspots. This may manifest in the form of dopamine axon degeneration, molecular changes in dopamine release machinery, a compensatory decrease in dopamine tone to mitigate the behavioral hyperactivity resulting from the imbalance between direct and indirect

pathway MSNs, or a combination of multiple mechanisms (Callahan et al., 2022). Altogether, our findings indicate that increased dopamine can be elicited from dopamine hotspots in late-disease R6/2 HD slices by changing the conditions for neuromodulator release through non-physiological methods such as raising extracellular Ca^{2+} concentration.

Sulpiride antagonism of D2Rs increases mean peak dF/F release intensity but not hotspot number in late disease

Axonal dopamine release in the striatum is regulated at multiple stages such that Ca^{2+} influx into the dopamine release site alone does not determine whether dopamine release occurs (Liu and Kaeser, 2019). As such, we next sought to drive increased dopamine release through the physiologically relevant mechanism of D2R antagonism. R6/2 HD model mice show broad decreases in D2R transcription and expression, including early degeneration of D2R expressing MSNs and decreased ACh release from D2R expressing CINs (Callahan et al., 2022; Pancani et al., 2023). Less is known about how D2-autoreceptors are affected during disease progression (Vashishtha et al., 2013; Achour et al., 2015). To this end, we utilized nIRCat's compatibility with dopamine receptor pharmacology to examine the effect of the selective D2R antagonist sulpiride. Sulpiride antagonism of D2Rs drives increased dopamine release through antagonism of D2-autoreceptor action on K_v1 channels, disinhibition of dopamine synthesis via tyrosine hydroxylase, and antagonism of D2Rs on CINs to increase ACh release onto dopaminergic axons (Trabucchi et al., 1975; O'Connor and Brown, 1982; Beaulieu and Gainetdinov, 2011; Chuhma et al., 2014). For all mechanisms, sulpiride application should result in an increase in dopamine hotspot number and mean peak dF/F.

We first examined the effect of sulpiride antagonism of D2Rs on 4-week R6/2 HD and WT brain slices by imaging dopamine release elicited from a single electrical pulse. Sulpiride antagonism is often examined using a set of 2 or more electrical pulses in succession to establish initial dopamine tone within the ex vivo brain slice (Phillips et al., 2002). In this work,

533 we elected to use a single-pulse stimulation paradigm to assess the impact of sulpiride
534 antagonism. This mirrors the stimulation paradigm we employed in preceding experiments,
535 allowing for comparison of how sulpiride antagonism and Ca^{2+} concentration impact dopamine
536 hotspot number and release intensity.

537 With a fixed field of view, slices were first imaged in standard ACSF and then imaged in
538 ACSF containing 10 μM sulpiride. Interestingly, we find that at this early 4-week time point
539 sulpiride application drives an increase in dopamine release in response to a single electrical
540 pulse by increasing the number of dopamine hotspots in both R6/2 HD and WT brain slices (Fig.
541 3a). In contrast, sulpiride application increased mean peak dF/F in R6/2 HD brain slices only
542 (Fig. 3b, Fig. S3a-d). No difference was observed between R6/2 HD and WT brain slice hotspot
543 number or mean peak dF/F at 4 weeks (Fig. 3a-b). The observed increase in dopamine hotspot
544 number from WT slices after sulpiride application stands in contrast to previous studies utilizing
545 FSCV, which have reported no sulpiride-driven increase in measured dopamine release after
546 single-pulse electrical stimulation (Phillips et al., 2002). This unique finding underscores the
547 utility of nIRCat imaging's improved spatial resolution over spatially averaged dopamine
548 detection methods in visualizing how sulpiride may heterogeneously affect dopamine hotspots
549 across a given brain slice. We again isolated the distribution of peak dF/F values of sulpiride
550 added post-threshold dopamine hotspots after application of 10 μM sulpiride and found that
551 their distribution mirrors the peak dF/F distribution of all post-threshold dopamine hotspots
552 observed after 10 μM sulpiride application (Fig S5e-f).

553 We next assessed the sulpiride response of R6/2 HD and WT slices after advanced
554 neurodegeneration at 12 weeks. Sulpiride application increased dopamine hotspot number and
555 mean peak dF/F in 12-week WT brain slices. However, in 12-week R6/2 HD slices, sulpiride
556 increased dopamine mean peak dF/F but not dopamine hotspot number (Fig. 3c-d, Fig. 3g-h). In
557 addition, despite sulpiride increasing the mean peak dF/F of both R6/2 HD and WT hotspots,

the overall mean peak dF/F of R6/2 HD hotspots remained significantly lower than that of WT hotspots after sulpiride application (Fig. 3d, Fig. 3e-f). Taken together, these results suggest that D2R signaling is disrupted in late HD such that sulpiride antagonism of D2R pathways that increase dopamine hotspot release intensity are still functional, but D2R pathways that recruit hotspots to be newly post-threshold following sulpiride application are impaired.

Individual dopamine hotspot tracking reveals hotspot release fidelity is decreased in late disease

The high spatial-temporal resolution of nIRCat imaging allows the visualization of dopamine release across not only multiple dopamine hotspots, but also over the course of multiple stimulations. With nIRCat, we sought to track whether individual dopamine hotspots within a slice reliably release dopamine over multiple events of electrically stimulated dopamine release. Here we term “hotspot release fidelity” as the ability of the same dopamine hotspot to release dopamine upon repeated electrical stimulations separated by 5 min intervals of rest (Fig. 4a). Notably, these protocols can be combined with nIRCat’s compatibility with dopamine receptor pharmacology to investigate how dopamine hotspot release fidelity is affected by dopamine receptor agonism or antagonism.

We first sought to understand how dopamine hotspot fidelity changed in the presence of altered extracellular Ca^{2+} concentration (Leitz and Kavalali, 2011; Thanawala and Regehr, 2013). We re-analyzed the Ca^{2+} nIRCat imaging data presented in Figure 2, tracking individual dopamine hotspots across the three stimulations and recording the number of stimulations out of three that each hotspot was active (Fig. 4a). Hotspots that responded in three of three total stimulations were assigned a hotspot release fidelity of 1.0. We found that increasing extracellular Ca^{2+} concentration from 2 mM Ca^{2+} to 4 mM Ca^{2+} increased mean dopamine hotspot fidelity (Fig. 4b). Similarly, decreasing extracellular Ca^{2+} concentration to 1 mM Ca^{2+} decreased mean dopamine hotspot fidelity (Fig. 4b). We also examined how hotspot fidelity is

583 altered over the course of HD progression and found that R6/2 HD and WT hotspots showed
584 similar fidelity at 4 weeks, but R6/2 HD hotspots showed decreased fidelity compared to their
585 WT counterparts at 12 weeks (Fig. 4c, Fig. 4b). Increasing extracellular Ca^{2+} concentration to 4
586 mM Ca^{2+} increased the fidelity of dopamine hotspots in 12-week R6/2 HD slices, but was not
587 sufficient to restore fidelity to the level of WT hotspots at 4 mM Ca^{2+} (Fig. 4b). These findings
588 underscore the utility of fidelity as a measure for characterizing dopamine release and disease
589 progression.

590 **Sulpiride D2R antagonism is unable to shift low-fidelity hotspots in late-disease HD slice**
591 **to high-fidelity release states**

592 We next examined the effect of sulpiride antagonism on dopamine hotspot fidelity.
593 Sulpiride antagonism through either D2-autoreceptors or D2-heteroreceptors on CINs is
594 expected to increase the probability of dopamine release. Early disease 4-week R6/2 HD slices
595 showed similar fidelity to WT hotspots both in ACSF and after sulpiride application. Interestingly,
596 sulpiride application did not increase the fidelity of dopamine hotspots in either R6/2 HD or WT
597 slices (Fig. 4d). In contrast, late disease 12-weeks WT dopamine hotspots showed increased
598 fidelity in response to sulpiride, while R6/2 HD hotspots showed no change in fidelity after
599 sulpiride application (Fig. 4e). These findings suggest that the decreased fidelity observed in 12-
600 week R6/2 HD hotspots may be driven by alterations in D2R signaling.

601 To better characterize the effect of sulpiride antagonism on late-stage HD mice, we
602 conducted a second set of nIRCat imaging experiments that increased the total number of
603 single-pulse stimulations from 3 replicates to 10 replicates separated by 5 min intervals of rest.
604 The increased granularity afforded by higher stimulation number allowed for greater
605 visualization of the distribution of hotspots fidelities (Fig. S4a-b). In ACSF, the majority of
606 dopamine hotspots identified over multiple slices were low fidelity hotspots that were only active
607 in response to 1- 2 stimulations over a total of 10 stimulation events (Fig. 4h-i). This resulted in

a distribution of dopamine hotspots that was heavily skewed towards low fidelities. Application of sulpiride increased the mean fidelity of hotspots by making lower fidelity hotspots release dopamine in response to more stimulations, resulting in a rightwards shift of the histogram towards higher fidelity states (Fig. 4h-i). This shift towards higher fidelity states in response to sulpiride was not observed in 12-week R6/2 HD hotspots (Fig. 4i). In these 10-replicate stimulation experiments, 12-week R6/2 HD hotspots showed decreased mean fidelity in comparison to 12-week WT hotspots after sulpiride application, but not in ACSF only (Fig. 4f). This is likely due to the heavy skew of dopamine hotspots towards low fidelities in ACSF (Fig. 4h-i). However, 12-week WT slices did show an increased number of "high fidelity hotspots" – defined as hotspots with fidelity of 0.33 or greater—in comparison to 12-week R6/2 HD slices (Fig. 4g).

The majority nIRCat imaged dopamine hotspots are pre-threshold after DHβE application

Dopamine release in the dorsal striatum can be triggered by the release of ACh from CINs onto nicotinic acetylcholine receptors (nAChR) on dopaminergic terminals. This ACh-dependent dopamine release plays a critical role in influencing dopamine release imaged with genetically encoded sensors in ex vivo brain slices within 500 μm of stimulating electrode (Matityahu et al., 2023). As such, we sought to understand what role ACh signaling plays in nIRCat imaged dopamine release at our experimental conditions that measure 200 μm away from the location of stimulation. We conducted nIRCat imaging in ex vivo brain slices superfused with the nicotinic acetylcholine receptor antagonist Dihydro-β-erythroidine hydrobromide (DHβE) and found that application of DHβE results in a sharp decrease in the number of observed post-threshold dopamine hotspots in 12-week WT mice, suggesting that the majority of nIRCat hotspots imaged 200 μm from the stimulator required CINs to induce striatal dopamine release (Fig. 5a). Application of DHβE resulted in no observed high-fidelity hotspots (fidelity = 1) in both R6/2 and WT slices, consistent with findings that ACh signaling plays a critical role in coordinated dopamine

release in the striatum (Fig. 5d-g) (Threlfell et al., 2012; Matityahu et al., 2023). A small number of active dopamine hotspots are still observed in WT slices after DHβE application, demonstrating that some population of imaged hotspots still release dopamine without ACh signaling. However, these DHβE insensitive dopamine hotspots show lower mean peak dF/F and mean fidelity (Fig. 5b-c). In contrast, dopamine hotspots in 12-week HD mice do not show a significant decrease in dopamine hotspot number, mean peak dF/F, or mean fidelity after DHβE (Fig. 5a-c). These results may be due to the low number of post-threshold dopamine hotspots present in 12-week R6/2 HD slices prior to DHβE application, providing limited range for showing further decrease of hotspot number.

DISCUSSION

In this work we investigated spatial changes in dopamine release over the course of disease in R6/2 HD mice using high spatio-temporal resolution nIRCat nanosensors to examine the behavior of R6/2 HD dopamine hotspots in the presence of extracellular Ca^{2+} concentration, D2R antagonism, and nAChR antagonism. We also developed novel analyses to study hotspot release fidelity and examined how this facet of dopamine release contributes to altered dopamine transmission across disease progression in the DLS of R6/2 HD mice.

Spatially-dependent dysregulation of dopamine dynamics in Huntington's Disease

Decreased dopamine release and basal tone over HD progression has been well documented in R6/2 HD mice using bulk dopamine measurement tools such as FSCV and microdialysis (Johnson et al., 2006, 2007; Ortiz et al., 2010; Callahan and Abercrombie, 2011; Ortiz et al., 2011). Our findings show that at 4 weeks, R6/2 HD dopamine hotspots at 4 mM Ca^{2+} show increased mean peak dF/F dopamine release intensity compared to WT hotspots. Late in disease at 12 weeks, R6/2 HD slices show decreased dopamine hotspot number and decreased hotspot fidelity over multiple stimulations. In contrast, mean peak dF/F is not

diminished in 12-week R6/2 HD slices compared to WT counterparts in standard ACSF, but is diminished after application of high extracellular Ca^{2+} concentrations or sulpiride, suggesting that maximum dopamine release capacity of R6/2 HD hotspots is diminished late in disease. Combining the metrics of hotspot number and mean peak dF/F into a single measure shows a 77% decrease in dopamine levels in 12-week R6/2 HD slices compared to WT, a value aligned with levels previously reported in literature utilizing parallel dopamine measurements methods (Fig. S1c) (Johnson et al., 2006, 2007; Ortiz et al., 2010; Callahan and Abercrombie, 2011; Ortiz et al., 2011). These findings point towards a neurodegenerative process where disruption of Ca^{2+}-related dopamine hotspot activity early in HD may lead to overall degeneration of dopamine release late in disease via decreased dopamine hotspot number and release ability.

Dopamine hotspot loss in late HD appears to be the combined result of physical degeneration of dopamine axons and striatal changes that disrupt release from dopamine hotspots at physiological conditions, though further characterization is needed. Previous studies utilizing dopamine fluorescent false neurotransmitters (dFFNs) to image the DLS of GCaMP3 expressing mice have reported dopaminergic boutons showing Ca^{2+} activity in response to electrical stimulation but no accompanying dopamine release (Pereira et al., 2016). These "silent" boutons could be "unsilenced" to release dFFNs at non-physiological conditions such as high K^+ concentration (Pereira et al., 2016). We find that moderate increases in dopamine release can be achieved in late-disease R6/2 HD and WT slices through increasing extracellular Ca^{2+} concentration or D2R antagonism with sulpiride. In both conditions, application of high Ca^{2+} ACSF or sulpiride results in the appearance of new post-threshold hotspots in late-disease brain slices that were not previously observed in standard ACSF.

Dopamine release in R6/2 HD mice shows increased sensitivity to extracellular Ca^{2+} concentration in early and late disease

Fast-degenerating mouse models with long CAG repeats such as R6/2 do not typically undergo biphasic motor changes. However, striatal TH activity in R6/2 mice has been shown to be biphasic, with elevated activity at 4 weeks and diminished activity at 12 weeks (Cepeda and Levine, 2020). We examined dopamine hotspot activity in 4-week R6/2 HD mice, a previously uninvestigated pre-symptomatic timepoint in this mouse model, and found that R6/2 HD hotspots showed elevated dopamine release intensity compared to WT hotspots at 4 mM Ca^{2+}. This increased sensitivity to Ca^{2+} concentration and elevated dopamine-release profile may be particularly notable during the early critical period between birth and P28 when dopamine is known to shape MSN excitability (Lieberman et al., 2018). Furthermore, this early disruption in dopamine release may play a role in altered motor function early in disease or subsequent dopamine dysregulation at later HD timepoints (Lieberman et al., 2018). The non-genetically encoded nature of nIRCat may be particularly advantageous for further exploration of early-disease dopamine release in tissues taken from human HD Patients (Lee et al., 2025).

Our findings also reveal new insights in dopamine release in late disease. Previous work using FSCV reported that 12-week R6/2 HD and WT mice showed comparable shifts in dopamine release in response to increasing extracellular Ca^{2+} concentration (Johnson et al., 2007). In contrast, our nIRCat imaging shows that though dopamine hotspots in 12-week R6/2 HD and WT slices exhibit similar shifts in mean peak dF/F with increasing extracellular Ca^{2+} concentration, 12-week R6/2 HD slices gain proportionally more dopamine hotspots than WT slices after transition to 4 mM Ca^{2+} ACSF. Our findings suggest that early targeting of Ca^{2+} channels or further investigation into their ability to modulate nIRCat imaged dopamine release in late disease may present exciting opportunities to understand the role of Ca^{2+} in altered dopamine release dynamics in HD.

Dopamine hotspot fidelity, a new facet to characterize neurodegeneration

The timing and fidelity of dopamine release in the DLS is critical to functions ranging from action selection to sensorimotor learning (Burton et al., 2015; Bariselli et al., 2019). Here we provide a powerful new method of studying dopamine hotspot release fidelity using nIRCat imaging that is capable of directly visualizing the dopamine release profile of hundreds of individual dopamine hotspots at once in both healthy and diseased tissue. In concordance with single vesicle tracking experiments done with pHluorin sensors, our nIRCat imaging shows that dopamine hotspots increase in number, dopamine release intensity, and release fidelity at high extracellular Ca^{2+} concentrations (Leitz and Kavalali, 2011; Thanawala and Regehr, 2013). This effect is observed in both R6/2 HD and WT slices, with an overall decrease in mean dopamine hotspot fidelity persisting in 12-week R6/2 HD slices at high extracellular Ca^{2+} concentration.

Studying dopamine hotspot fidelity is particularly powerful in combination with nIRCat's dopamine receptor pharmacological compatibility, allowing for examination of changes in the effect of D2R antagonists such as sulpiride. Decreased overall expression and transcription of striatal D2Rs is well established in both human HD patients and R6/2 mice (Ariano et al., 2002; Vashishtha et al., 2013). This includes D2-autoreceptors expressed on dopaminergic boutons and D2-heteroreceptors expressed on MSNs and CINs. Use of sulpiride has been explored for treatment for HD associated dyskinesia— however, the precise mechanism of action is unknown (Quinn and Marsden, 1984). We observe an increase in nIRCat imaged evoked dopamine release from a single electrical pulse after sulpiride application. This stands in contrast to previous FSCV measurements in Wistar rats that report no effect of the D2R antagonist metoclopramide on dopamine release in response to single electrical pulses (Phillips et al., 2002). The increased spatial resolution of nIRCat nanosensors may allow for the imaging of hotspots that are close together and able to quickly establish a local basal dopamine tone, as well as lone hotspots that experience no basal tone between stimuli. This heterogeneity is likely

lost during FSCV measurements, which measures dopamine that has diffused to the electrode from multiple release sites.

Sulpiride antagonism of late-disease R6/2 HD dopamine hotspots shows altered behavior in contrast to their WT counterparts, with sulpiride application in late R6/2 HD slices driving increases in hotspot mean peak dF/F but not an increase in hotspot number or hotspots fidelity. These results suggest that the ability of D2Rs to regulate dopamine release intensity is still intact in late disease, but the ability of D2Rs to increase dopamine hotspot number and increase dopamine release fidelity is compromised. Notably, we find late disease R6/2 HD dopamine hotspot fidelity remains sensitive to extracellular Ca^{2+}concentration, suggesting that decreases in hotspot fidelity are driven by changes in D2R signaling rather than an overall inability of late disease HD hotspots to modulate fidelity. The partial preservation of sulpiride's ability to modulate mean peak dF/F may suggest that D2-autoreceptor signaling is differentially affected from the degeneration of other dopamine release related D2R signaling pathways such as those involving CIN D2-heteroreceptors.

The role of acetylcholine signaling in dopamine hotspot release behavior

Release of ACh from CIN in the dorsal striatum plays a critical role in shaping reliability of dopamine release (Lim and Surmeier, 2021; Pancani et al., 2023). Consistent with findings from genetically encoded dopamine sensors, we find that the majority of dopamine hotspots imaged by nIRCat in WT slices 200 mm away from electrical stimulation are DHbE sensitive, suggesting a critical role for ACh signaling (Matityahu et al., 2023). Application of DHbE dramatically decreased the number of dopamine hotspots in WT slices and decreased hotspot mean peak dF/F and fidelity. In contrast, application of DHbE in late disease R6/2 HD slices showed a trending decrease in dopamine hotspot number and mean peak dF/F. Signaling from CINs appears to play a powerful role in shaping hotspot fidelity under these imaging conditions. The contributions of DHbE signaling to nIRCat imaging of dopamine hotspots in healthy slices at

different stimulation distances and via different methods presents an exciting area of future research beyond the scope of this work (Lim and Surmeier, 2021; Pancani et al., 2023).

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Figure Captions

Figure 1. R6/2 HD slices show progressive decrease in number of dopamine hotspots over disease progression but not a change in individual dopamine dF/F hotspot release intensity a. Graphical overview of the nIRCat synthesis process. Single walled carbon nanotubes (SWNT) are sonicated with single-stranded $(GT)_6$ DNA oligos to form the SWNT-DNA complex named nIRCat. NIRCat nanosensors increase fluorescence in the presence of catecholamines such as dopamine. **b.** Graphical overview of the experimental protocol used to image ex vivo brain slices using nIRCat nanosensors. R6/2 HD and WT mice are perfused at 4 weeks, 9 weeks, and 12 weeks of age to produce acute coronal brain slices. The slices are passively labeled with nIRCat nanosensors via 15-minute incubation with nIRCat nanosensors suspended in ACSF and then imaged in the perfusion chamber of a near-infrared imaging microscope **c.** Graphical overview of the data analysis process used to examine the number of dopamine hotspots active after electrical stimulation, and the dopamine release profile of each hotspot. The term mean peak dF/F is used to refer to the average peak fluorescence value measured from all the dopamine hotspots active in a brain slice after a stimulation event. **d.** The mean number of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 4-week, 9-week, and 12-week mice. Brain slices were taken from 4-week WT (N = 8), 4-week HD (N = 5), 9-week WT (N = 6) , 9-week HD (N = 6), 12-week WT (N = 8), and 12-week HD (N = 8) animals. (two-way ANOVA: age $F(2,32)= 2.064$, $p = 0.1435$; disease state $F(1,32)=6.882$, *$p=0.0132$, interaction $F(2,32)=5.396$, **$p=0.0096$. Sidak's multiple-comparisons test showed no significant difference for 4 wks vs 9

wks in WT slices (p = 0.5898) or HD slices (p =0.9349). No significant difference was shown for 9 wks vs 12 wks in WT slices (p = 0.9999), but a significant difference was shown for 9 wks vs 12 wks in HD slices (*p = 0.0454). No significant difference was shown for HD vs WT at 4 wks (p = 0.9362) or 9 wks (p = 0.6343), but a significant difference was shown for HD vs WT at 12wks (***p = 0.0007). **e.** The mean peak dF/F of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 4-week, 9-week, and 12-week mice (two-way ANOVA: age F(2,32)=3.534, *p = 0.0410; disease state F(1,32)=0.9824, p=0.329, interaction F(2,32)=0.266, p=0.7681. Sidak's multiple-comparisons test showed no significant difference for 4 wks vs 9 wks in WT slices (p = 0.9636) or HD slices (p =0.9975). No significant difference was shown for 9 wks vs 12 wks in WT slices (p = 0.5218) or HD slices (p =0.1488). No significant difference was shown for HD vs WT at 4 weeks (p = 0.9570), 9 weeks (p = 0.9990), or 12 weeks (p = 0.5604)). **f**. Performance on accelerated rotarod motor test of R6/2 HD and WT animals imaged at ages 4, 9, and 12 weeks. R6/2 HD animals show significantly shorter latency to fall compared to WT animals at 9 weeks and 12 weeks (two-way ANOVA: age F(2,32)= 4.579, p = 0.0178; disease state F(1,32)=72.22, p=<0.0001, interaction F(2,32)=8.152, p=0.0014. Sidak's multiple-comparisons test showed no significant difference between HD vs WT at 4 weeks (p = 0.1781), but significant differences were shown for HD vs WT at 9 weeks (****p < 0.0001) and 12 weeks (****p < 0.0001)). For all figures, brackets indicate significant Sidak-adjusted pairwise comparisons following two-way ANOVA. Non-significant comparisons are omitted for clarity. All data are presented as mean ± standard deviation (SD).

Figure 2. R6/2 HD slices show increased dopamine hotspot sensitivity to extracellular Ca^{2+} at 4 weeks and retain dopamine hotspot sensitivity to high extracellular Ca^{2+} at 12 weeks a. The mean number of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 4-week mice (checker pattern bars). All slices are imaged in 1 mM Ca^{2+} ACSF, 2 mM Ca^{2+} ACSF, and 4 mM Ca^{2+} ACSF. Data taken from the same slice are joined by dotted lines. Slices used to generate data at 4 weeks were taken from WT (N = 7) and HD (N = 6) animals. (two-way repeated measures ANOVA: Ca^{2+} concentration F(2, 22) = 112.8, ****p < 0.0001; disease state F(1, 11) = 2.275, p = 0.1596; interaction F(2, 22) = 1.603, p = 0.2240. Sidak's multiple-comparisons test showed a significant increase for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (***p = 0.0009) and 2 mM Ca^{2+} vs 4 mM Ca^{2+} (****p < 0.0001) in WT slices. A significant increase was also shown for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (****p < 0.0001) and 2 mM Ca^{2+} vs 4 mM Ca^{2+} (***p = 0.0001) in HD slices. No significant difference between WT and HD was shown at any Ca^{2+} concentration) **b.** The mean peak dF/F of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 4-week mice. All slices are imaged in 1 mM Ca^{2+} ACSF, 2 mM Ca^{2+} ACSF, and 4 mM Ca^{2+} ACSF. (two-way repeated measures ANOVA: Ca^{2+} concentration F(2, 22) = 57.12, ****p < 0.0001; disease state F(1, 11) = 3.247, p = 0.0990; interaction F(2, 22) = 5.750, **p = 0.0098. Sidak's multiple-comparisons test showed no significant increase for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (p = 0.2731) in WT slices, but a significant increase for 2 mM Ca^{2+} vs 4 mM Ca^{2+} (**p = 0.0043) in WT slices. A significant increase was shown for both 1 mM Ca^{2+} vs 2 mM Ca^{2+} (***p = 0.0007) and 2 mM Ca^{2+} vs 4 mM Ca^{2+} (***p = 0.0001) in HD slices. A significant increase was shown for HD vs WT at 4 mM Ca^{2+} (*p =

0.0253)) **c.** The mean number of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 12-week mice (light solid fill bars). All slices were imaged in 1 mM Ca^{2+} ACSF, 2 mM Ca^{2+} ACSF, and 4 mM Ca^{2+} ACSF. Slices used to generate data at 12 weeks were taken from WT (N = 6) and HD (N = 6) animals (two-way repeated measures ANOVA: Ca^{2+} concentration F(2, 20) = 67.73, ****p < 0.0001; disease state F(1, 10) = 19.51, **p = 0.0013; interaction F(2, 20) = 13.84, ***p = 0.0002. Sidak's multiple-comparisons test showed a significant increase for both 1 mM Ca^{2+} vs 2 mM Ca^{2+} (****p < 0.0001) and 2 mM Ca^{2+} vs 4 mM Ca^{2+} (****p < 0.0001) in WT slices. No significant increase for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (p = 0.8431) was shown for HD slices, but a significant increase was shown for 2 mM Ca^{2+} vs 4 mM Ca^{2+} (**p = 0.0032). A significant decrease was shown for HD vs WT at 2 mM Ca^{2+} (***p = 0.0004) and 4 mM Ca^{2+} (****p < 0.0001)) **d.** The mean peak dF/F of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from WT and R6/2 HD ex vivo brain slices taken from 12-week mice. All slices were imaged in 1 mM Ca^{2+} ACSF, 2 mM Ca^{2+} ACSF, and 4 mM Ca^{2+} ACSF. (two-way repeated measures ANOVA: Ca^{2+} concentration F(2, 20) = 35.43, ****p < 0.0001; disease state F(1, 10) = 3.089, p = 0.1093; interaction F(2, 20) = 4.475, *p = 0.0248. Sidak's multiple-comparisons test showed no significant increase for 1 mM Ca^{2+} vs 2 mM Ca^{2+} in either WT slices (p = 0.3151) or HD slices (p = 0.9986). A significant increase was shown for 2 mM Ca^{2+} vs 4 mM Ca^{2+} in both WT slices (****p < 0.0001) and HD slices (*p = 0.0104). A significant decrease was shown for HD vs WT at 4 mM Ca^{2+} (*p = 0.0180)) For all figures, brackets indicate significant Sidak-adjusted pairwise comparisons following two-way repeated measures ANOVA. Non-significant comparisons are omitted for clarity. All data are presented as mean ± SD **e.** Representative dopamine release and reuptake traces from dopamine hotspots observed in brain slices from 12-week HD mice. Solid lines denote the mean dopamine release and reuptake trace of the hotspots taken from three stimulations and light shaded bands represent one standard deviation from mean behavior. A 0.3 mA stimulation is delivered at time = 0 s. Traces are shown for the same hotspots at 1 mM Ca^{2+}, 2 mM $Ca^{2+,}$ and 4 mM Ca^{2+}. **f.** Representative dopamine release and reuptake traces from dopamine hotspots observed in brain slices from 12-week WT mice. Solid lines denote the mean dopamine release and reuptake trace of the hotspots taken from three stimulations and light shaded bands represent one standard deviation from mean behavior. A 0.3 mA stimulation is delivered at time = 0 s. Traces are shown for the same hotspots at 1 mM Ca^{2+}, 2 mM $Ca^{2+,}$ and 4 mM Ca^{2+}. **g.** Representative images of dopamine hotspots imaged in a 12-week HD slice before, during, and after stimulated dopamine release in 1 mM Ca^{2+}, 2 mM Ca^{2+} , and 4 mM Ca^{2+}ACSF. Images are shown for the same slice and field of view. **h.** Representative images of dopamine release imaged in a 12-week WT slice before, during, and after stimulated dopamine release in 1 mM Ca^{2+}, 2 mM Ca^{2+}, and 4 mM Ca^{2+} ACSF. Images are shown for the same slice and field of view.

Figure 3. R6/2 HD hotspots show altered response to D2R antagonist sulpiride at 4 weeks and 12 weeks a. The mean number of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 4-week mice (checker pattern bars). All slices are imaged in ACSF and 10 μM sulpiride in ACSF. Data taken from the same slice is joined by dotted lines. Slices used to generate data at 4 weeks were taken from WT (N = 6) and HD (N = 6) animals. (two-way repeated measures ANOVA: sulpiride condition F(1, 10) = 21.75, ***p = 0.0009; disease state F(1, 10) = 0.7703, p=0.4007; interaction F(1, 10) = 0.7724, p = 0.4001. Sidak's multiple-comparisons test showed a significant increase for ACSF vs.

sulpiride in both WT slices (**p = 0.0057) and HD slices (*p = 0.0459). No significant difference was shown for WT vs HD in sulpiride (p = 0.7321) or ACSF (p = 0.5412)) **b.** The mean peak dF/F of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 4-week mice. All slices are imaged in ACSF and 10 µM sulpiride in ACSF. two-way repeated measures ANOVA: sulpiride condition F(1, 10) = 20.40, **p = 0.0011; disease state F(1, 10) = 0.5347, p = 0.4814; interaction F(1,10) = 1.855, p = 0.2031. Sidak's multiple-comparisons test showed no significant increase for ACSF vs Sulpiride in WT slices (p = 0.0972), but showed a significant increase for ACSF vs sulpiride in HD slices (**p = 0.0039). No significant difference was shown for WT vs HD in sulpiride (p = 0.5543) or ACSF (p = 0.8884)) **c.** The mean number of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 12-week mice (light solid fill bars). All slices are imaged in ACSF and 10 µM sulpiride in ACSF. Slices used to generate data at 12 weeks were taken from WT (N = 6) and HD (N = 6) animals. (two-way repeated measures ANOVA: sulpiride condition F(1, 10) = 58.38, ****p < 0.0001; disease state F(1, 10) = 19.34, **p = 0.0013; interaction F(1, 10) = 18.47, **p = 0.0016. Sidak's multiple-comparisons test showed a significant increase for ACSF vs sulpiride in WT slices (****p < 0.0001), but showed no significant increase of ACSF vs sulpiride in HD slices (p = 0.0777). A significant difference was shown for HD vs WT in both ACSF (**p = 0.0044) and sulpiride (***p = 0.0001)) **d.** The mean peak dF/F of dopamine hotspots active in response to a single 0.3 mA electrical stimulation from R6/2 HD and WT ex vivo brain slices taken from 12-week mice. All slices are imaged in ACSF and 10 µM sulpiride in ACSF. (two-way repeated measures ANOVA: sulpiride condition F(1, 10) = 42.71, ****p < 0.0001; disease state F(1, 10) = 5.971, *p = 0.0346; interaction F(1, 10) = 1.186, p = 0.3016. Sidak's multiple-comparisons test showed a significant increase for ACSF vs sulpiride in WT slices (***p = 0.0006) and HD slices (**p = 0.0064). No significant difference was shown for HD vs WT in ACSF (p = 0.0699), but a significant difference was shown for HD vs WT in sulpiride (*p = 0.0355)). For all figures, brackets indicate significant Sidak-adjusted pairwise comparisons following two-way repeated measures ANOVA. Non-significant comparisons are omitted for clarity. All data are presented as mean ± SD. **e.** Representative dopamine release and reuptake traces from nIRCat imaged dopamine hotspots observed in brain slices from 12-week HD mice. Solid lines denote the mean dopamine release and reuptake trace of the hotspots taken from three stimulations and light shaded bands represent one standard deviation from mean behavior. A 0.3 mA stimulation is delivered at time = 0 s. Traces are shown for the same hotspots in ACSF and 10 µM sulpiride. **f.** Representative dopamine release and reuptake traces from nIRCat imaged dopamine hotspots observed in brain slices from 12-week WT mice. Solid lines denote the mean dopamine release and reuptake trace of the hotspots taken from three stimulations and light shaded bands represent one standard deviation from mean behavior. A 0.3 mA stimulation is delivered at time = 0 s. Traces are shown for the same hotspots in ACSF and 10 µM sulpiride. **g.** Representative images of dopamine release imaged in a 12-week WT slice before, during, and after stimulated dopamine release in ACSF and 10 µM sulpiride. Images are shown for the same slice and field of view. **h.** Representative images of dopamine release imaged in a 12-week HD slice before, during, and after stimulated dopamine release in ACSF and 10 µM sulpiride. Images are shown for the same slice and field of view.

Figure 4. Dopamine hotspot tracking over multiple stimulations shows altered dopamine hotspot release fidelity in 12-week R6/2 HD slices a. Graphical overview of hotspot tracking to measure dopamine release fidelity. A field of view is imaged over the course of multiple stimulations, each separated by a 5-minute interval. Hotspots that are observed in 3 out of 3 stimulations have a fidelity of 1, hotspots that are observed in 1 out of 3 stimulations have a fidelity of 0.33. **b.** The impact of extracellular Ca^{2+} concentration on the mean hotspot fidelity of dopamine hotspots from 12-week R6/2 HD and WT ex vivo brain slices (light solid fill bars) imaged over 3 total stimulations. All slices underwent 3 electrical stimulations in each of the following conditions: 1 mM Ca^{2+} ACSF, 2 mM Ca^{2+} ACSF, and 4 mM Ca^{2+} ACSF. Data taken from the same slice is joined by dotted lines. Data is taken from WT (N = 6) and HD (N = 6) animals. (two-way repeated measures ANOVA: Ca^{2+} concentration $F(2, 20) = 89.02$, ****$p < 0.0001$; disease state $F(1, 10) = 15.91$; **$p=0.0026$; interaction $F(2, 20) = 10.55$, ***$p=0.0007$. Sidak's multiple-comparisons test showed a significant increase for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (****$p < 0.0001$) and 2 mM Ca^{2+} vs 4 mM Ca^{2+} (****$p < 0.0001$) in WT slices. No significant increase was shown for 1 mM Ca^{2+} vs 2 mM Ca^{2+} ($p = 0.2172$) in HD slices, but a significant increase was shown for 2 mM Ca^{2+} vs 4 mM Ca^{2+} (***$p = 0.0007$) in HD slices. There was no significant decrease shown for HD vs WT at 1 mM Ca^{2+} ($p = 0.8667$), but a significant decrease shown for HD vs WT at 2 mM Ca^{2+} (**$p = 0.0010$) and for HD vs WT at 4 mM Ca^{2+} (****$p < 0.0001$)) **c.** The impact of extracellular Ca^{2+} concentration on the mean hotspot fidelity of dopamine hotspots from 4-week R6/2 HD and WT ex vivo brain slices (checker pattern bars) imaged over 3 total stimulations. All slices undergo 3 electrical stimulations in each of the following conditions: 1 mM Ca^{2+} ACSF, 2 mM Ca^{2+} ACSF, and 4 mM Ca^{2+} ACSF. Data taken from the same slice is joined by dotted lines. Data is taken from WT (N = 7) and HD (N = 6) animals. (two-way repeated measures ANOVA: Ca^{2+} concentration $F(2, 22) = 61.69$ ****$p < 0.0001$; disease state $F(1, 11) = 0.0264$, $p = 0.8738$; interaction $F(2, 22) = 0.7065$, $p = 0.5042$. Sidak's multiple-comparisons test showed a significant increase for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (**$p =0.0065$) and for 2 mM Ca^{2+} vs 4 mM Ca^{2+} (***$p = 0.0002$) in WT slices. A significant increase was shown for 1 mM Ca^{2+} vs 2 mM Ca^{2+} (**$p = 0.0010$) and for 2 mM Ca^{2+} vs 4 mM Ca^{2+}(*$p = 0.0177$) for HD slices. There was no significant decrease shown between HD vs WT slices at 1 mM Ca^{2+} ($p > 0.9999$), 2 mM Ca^{2+} ($p = 0.8312$), or 4 mM Ca^{2+} ($p = 0.9711$)) **d.** The impact of sulpiride application on the mean hotspot fidelity of dopamine hotspots from 4-week R6/2 HD and WT ex vivo brain slices (checker pattern bars) imaged over 3 total stimulations. All slices undergo 3 electrical stimulations in ACSF and 10 µM sulpiride ACSF. Data is taken from WT (N = 6) and HD (N = 6) animals (two-way repeated measures ANOVA: sulpiride condition $F(1, 10) = 9.976$, *$p = 0.0102$; disease state $F(1, 10) = 0.2489$, $p = 0.6287$; interaction $F(1, 10) = 0.2681$, $p = 0.6159$. Sidak's multiple-comparisons test showed no significant difference for ACSF vs 10 µM sulpiride in WT slices ($p = 0.1745$) or HD slices ($p = 0.0523$). No significant difference was shown for HD vs WT in ACSF ($p = 0.7869$) or in 10 µM sulpiride ($p = 0.9349$)) **e.** The impact of sulpiride application on the mean hotspot fidelity of dopamine hotspots from 12-week R6/2 HD and WT ex vivo brain slices (light solid fill bars) imaged over 3 total stimulations. All slices underwent 3 electrical stimulations in ACSF and 10 µM sulpiride ACSF. Data is taken from WT (N = 6) and HD (N = 6) animals (two-way repeated measures ANOVA: sulpiride condition $F(1, 10) = 20.25$, **$p = 0.0011$; disease state $F(1, 10) = 17.34$, **$p = 0.0019$; interaction $F(1, 10) = 7.211$, *$p = 0.0229$. Sidak's multiple-comparisons test showed a significant increase for ACSF vs sulpiride (***$p = 0.0010$) in WT slices, but no significant increase for ACSF vs sulpiride ($p = 0.4044$) in HD slices. A significant decrease was shown for WT vs. HD in both ACSF (**$p = 0.0092$) and sulpiride (***$p = 0.0002$)). **f.** The impact of sulpiride application on mean hotspot fidelity of dopamine hotspots from 12-week R6/2 HD and WT ex vivo brain slices imaged over a higher

number of total stimulations (diagonal stripe bars). All slices underwent 10 electrical stimulations in ACSF and 10 µM sulpiride ACSF. Data is taken from WT (N = 5) and HD (N = 5) animals (two-way repeated measures ANOVA: sulpiride condition F(1, 8) = 8.721, *p = 0.0183; disease state F(1, 8) = 6.381, *p = 0.0355; interaction F(1, 8) = 1.012, p = 0.3438. Sidak's multiple-comparisons test showed a significant increase for ACSF vs sulpiride (*p = 0.0459) in WT slices, but no significant difference for ACSF vs sulpiride (p = 0.3694) in HD slices. No significant difference was shown for WT vs HD in ACSF (p = 0.1506), but a significant difference was shown for WT vs HD in sulpiride (*p = 0.0303). **g.** The impact of sulpiride application on the number of high-fidelity hotspots that are active in 3 out of 10 total stimulations or more (fidelity ≥ 0.33) in 12-week R6/2 HD and WT ex vivo brain slices imaged over 10 stimulations (diagonal stripe bars). All slices underwent 10 electrical stimulations in ACSF and 10 µM sulpiride ACSF. (two-way repeated measures ANOVA: sulpiride condition F(1, 8) = 7.399, *p = 0.0262; disease state F(1, 8) = 11.05, *p = 0.0105, interaction F(1, 8) = 1.422, p = 0.2673. Sidak's multiple-comparisons test showed a significant increase for ACSF vs sulpiride in WT slices (*p = 0.0483), but no significant increase for ACSF vs sulpiride in HD slices (p = 0.5260). A significant difference was shown for HD vs WT in ACSF (*p = 0.0464) and after sulpiride application (**p = 0.0057). All figure brackets indicate significant Sidak-adjusted pairwise comparisons following two-way ANOVA with repeated measures. Non-significant comparisons are omitted for clarity. All data are presented as mean ± SD. **h.** Histogram showing distribution of hotspots pooled from 12-week WT slices before and after sulpiride application. All slices underwent 10 electrical stimulations in ACSF and 10 µM sulpiride ACSF. WT (N = 5). **i.** Histogram showing distribution of hotspots pooled from 12-week HD slices before and after sulpiride application All slices underwent 10 electrical stimulations in ACSF and 10 µM sulpiride ACSF. HD (N = 5).

Figure 5. Application of nAChR antagonist DHβE sharply decreases the activity of nIRCat imaged dopamine hotspots in WT slices a. The mean number of dopamine hotspots active in R6/2 HD and WT ex vivo brain slices taken from 12-week mice (light solid fill bars). All slices were imaged in ACSF and 1 µM DHβE. Data taken from the same slice is joined by dotted lines. Data is taken from WT (N = 7) and HD (N = 4) animals. (two-way repeated measures ANOVA: DHβE condition F(1, 9) =15.94, **p = 0.0031; disease state F(1, 9) = 7.128, *p = 0.0256; interaction F(1, 9) = 5.046, p = 0.0513. Sidak's multiple-comparisons test showed a significant decrease for ACSF vs DHβE application in WT slices (**p = 0.0012), but no significant decrease was shown for ACSF vs DHβE application in HD slices (p = 0.5130). A significant difference was shown for HD vs WT slices in ACSF (**p = 0.0055) but not in DHβE (p = 0.9775). **b.** The mean peak dF/F of dopamine hotspots active in R6/2 HD and WT ex vivo brain slices taken from 12-week mice (light solid fill bars). All slices were imaged in ACSF and 1 µM DHβE. (two-way repeated measures ANOVA: DHβE condition F(1, 9) = 8.165, *p = 0.0189; disease state F(1, 9) = 1.408, p = 0.2658; interaction F(1, 9) = 1.893, p = 0.2022. Sidak's multiple-comparisons test showed a significant decrease for ACSF vs DHβE in WT slices (*p = 0.0132), but no significant decrease was found for ACSF vs DHβE in HD slices (p = 0.6123). No significant difference was shown for HD vs WT slices in ACSF (p = 0.1680) or DHβE (p = 0.9979). **c.** The impact of DHβE application on the mean hotspot fidelity of dopamine hotspots from 12-week R6/2 HD and WT ex vivo brain slices (light solid fill bars) imaged over 3 total stimulations. All slices underwent 3 electrical stimulations in ACSF and 1 µM DHβE. (two-way repeated measures ANOVA: DHβE condition F(1, 9) = 13.26, **p = 0.0054;

disease state F(1, 9) = 5.013, p = 0.0519; interaction F(1, 9) = 5.159, *p = 0.0493. Sidak's multiple-comparisons test showed a significant decrease for ACSF vs DHβE in WT slices (**p = 0.0017), but no significant decrease was shown for ACSF vs DHβE in HD slices (p = 0.6549). A significant difference was shown for HD vs WT in ACSF (*p = 0.0102) but not in DHβE (p = 0.9981)) All figure brackets indicate significant Sidak-adjusted pairwise comparisons following two-way ANOVA with repeated measures. Non-significant comparisons are omitted for clarity. All data are presented as mean ± SD. **d.** Representative images of dopamine release imaged in a 12-week WT slice before, during, and after stimulated dopamine release and 1 μM DHβE application. Images are shown for the same slice and field of view **e.** Representative images of dopamine release imaged in a 12-week HD slice before, during, and after stimulated dopamine release and 1 μM DHβE application Images are shown for the same slice and field of view. **f.** Histogram showing distribution of hotspots pooled from 12-week WT slices before and after 1 μM DHβE application. All slices underwent 3 electrical stimulations in ACSF and 1 μM DHβE ACSF. WT (N = 7). **g.** Histogram showing distribution of hotspots pooled from 12-week HD slices before and after 1 μM DHβE application All slices underwent 3 electrical stimulations in ACSF and 1 μM DHβE ACSF. HD (N = 4).