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Vitamin C Transport Deficiency Alters Striatal Dopamine Gene Expression and Metabolism in YAC128 Huntington Disease Mice

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ABSTRACT

Neurodegeneration in Huntington disease (HD) contributes to dopaminergic system dysfunction via the loss of striatal medium spiny neurons expressing dopamine receptors. Given the key role for ascorbic acid (vitamin C) in dopamine synthesis and neurotransmission, we investigated whether mild cellular ascorbate deficiency accelerates dopaminergic dysfunction in the development of HD pathology and behavioral deficits. YAC128 mice expressing mutant human huntingtin were crossed with SVCT2$^{+/-}$ mice, which carry a heterozygous knockout of the sodium-dependent vitamin C transporter, to generate mice with approximately 30% decreased neuronal vitamin C as well as progressive changes in dopamine signaling. Behavioral and neurochemical outcomes were assessed at early disease stages. At 14 and 20 weeks, YAC128 and SVCT2$^{+/-}$ YAC128 mice showed similar deficits in grip strength, locomotor activity, and rotarod performance compared to controls, suggesting modest ascorbate deficiency did not accelerate motor phenotypes. Gene expression analysis revealed six significantly upregulated genes in the striatum of SVCT2$^{+/-}$ YAC128 mice, including those involved in dopamine synthesis, packaging, and transport. Notably, striatal dopamine and serotonin and their metabolites were decreased in both single mutant mouse lines (YAC128 and SVCT2$^{+/-}$) but without a compounding effect of the double mutation (SVCT2$^{+/-}$ YAC128). These results indicate that while moderate ascorbate deficiency may not worsen early behavioral phenotypes in the YAC128 model, it does impact dopamine system regulation at the molecular level. These findings highlight the potential importance of ascorbate in modifying disease progression and suggest that humans with HD, who cannot synthesize ascorbate, may be particularly vulnerable to vitamin C deficiency effects on dopamine dynamics.

1 | Introduction

Huntington disease (HD) is an autosomal-dominant inherited, progressive neurodegenerative disease. It is rooted in dopaminergic system dysfunction which produces an array of abnormal behavioral phenotypes. The complete combination of mechanisms by which dopamine transmission is altered in HD is unclear, and a stage-dependent imbalance has been proposed, in which dopamine levels may be increased in early-stage (presymptomatic) disease and decreased in late-stage disease [1, 2]. Ascorbic acid (ascorbate, vitamin C) is a well-known antioxidant molecule that is highly concentrated in the brain and neuroendocrine tissue such as the adrenals. Humans do not synthesize ascorbate and thus must

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consume it via their diet. Ascorbate deficiency through diet is common in many populations [3, 4] and genetic variants in genes coding for transporters may also impact cellular uptake [5]. Energy-dependent mechanisms, including the sodium-dependent vitamin C transporter type 2 (SVCT2) expressed on neuronal, mitochondrial, microglial, and oligodendrocyte membranes [6–8], are required to achieve the high millimolar (2–10 mM) range concentrations of ascorbate found in neuron-rich brain areas. Under steady state conditions, ascorbate from the bloodstream enters the brain parenchyma via the SVCT2 and Glut12, both expressed in the choroid plexus. Glut12 can transport ascorbate in a sodium-independent and bidirectional manner, and both transporters are critical for maintaining adequate ascorbate concentrations in the brain [9–11]. Astrocytes play an important role in ascorbate recycling in the brain by taking up dehydroascorbate (DHA; the oxidized form of ascorbate), reducing it back to ascorbate, and releasing it into the extracellular space. Strong homeostatic mechanisms keep neuronal ascorbate concentrations within very tight limits. Urinary excretion depletes circulating and organ ascorbate, but it is difficult to deplete brain ascorbate except for in cases of prolonged deficiency or significant oxidative stress [12]. In most mammals, including rodents, that synthesize their own ascorbate in the liver, such depletion is not observed.

This sustained high concentration of neuronal ascorbate underscores its importance as a neuromodulator, particularly of the glutamate and dopamine neurotransmitter systems. Ascorbate modulates the dopaminergic neurotransmitter system directly through its action as a cofactor for dopamine beta-hydroxylase [13], thus deficiency limits its metabolism to norepinephrine. Effective antioxidant and reactive oxygen species scavenging properties also help protect localized glutathione metabolism and the preservation of dopamine from oxidation [14]. Ascorbate also protected against amphetamine-induced dopamine depletion in the striatum [15] and amphetamine may directly decrease ascorbic acid in the striatum [16]. Other studies suggest that ascorbate can potentiate dopamine-dependent behaviors such as amphetamine-induced conditioned place preference without directly changing striatal dopamine levels in rats [17]. A later study demonstrated that ascorbate can directly prevent dopamine oxidation and therefore increases extracellular dopamine availability in the rat striatum [18]. Similarly, global ascorbate deficiency decreases dopamine release in the nucleus accumbens of mice [19]. In the case of neurodegenerative diseases or neuropsychiatric disorders in which there may be a neurotransmitter imbalance or increase in oxidative stress, then the consequences of ascorbate deficiency on the dopaminergic system and redox balance are intensified [19–21].

Mutant *Huntingtin* (*Htt*), the functional allele that produces the huntingtin protein (Htt) is the driving force leading to a phenotype of aberrant dopaminergic signaling and underlying pathology. The greater the number of CAG expansion repeats in the gene sequence, the more likely symptoms will be observed, and the earlier they present, due to the production of abnormally long huntingtin protein. Nevertheless, understanding modifiable factors that may further contribute to disease phenotype expression provides the opportunity to improve disease severity or treatment efficacy in individuals [22]. Dietary and environmental exposures influencing neuronal ascorbate availability can directly impact dopaminergic signaling and are viable candidates for study in this respect.

Altered ascorbate transport (via SVCT2 expression) and metabolism (ascorbic acid: dehydroascorbic acid ratio) were identified in the striatum and ventral tegmental area in a mouse model lacking *Trkb* [14]. While not a direct model of HD, the brain-derived neurotrophic factor (BDNF)-TrkB signaling pathway is known to be impaired in the medium spiny neurons of the striatum [23, 24]. Importantly, these changes reported by Malik et al. coincided with area-specific changes in dopamine levels and metabolism, and mitochondrial energy metabolism deficits, but preceded motor phenotypes. The R6/2 transgenic model of HD shows diminished extracellular striatal ascorbic acid (ascorbate, vitamin C) availability as measured by in vivo voltammetry, presumably due to impaired uptake by neurons and release by both astrocytes and neurons [25, 26]. Repeated and frequent injections of ascorbate improved behavioral phenotypes even though these studies were conducted in a mouse model with severe HD phenotypes that may be more indicative of responses during late-stage disease [27]. The YAC128 mouse is a transgenic model of HD expressing the full-length mutant human *Htt* gene, displaying an initial hyperactive phenotype starting around 2 months of age, followed by hypoactivity as the disease progresses [28]. This change in behavior correlates with selective striatal neuron loss and recapitulates many of the phenotypes observed in affected human patients [29]. SVCT2 expression is decreased at synaptic terminals in YAC128 mice [30–32] which may be a result of less ascorbate release from glia into the extracellular space or an independent change. Using cell models with different CAG repeat numbers (STHdhQ7 and Q111) the authors further demonstrated that the required SVCT2 trafficking to the membrane was disrupted under active and resting cell conditions. Evidence from YAC128 and GFAP-HD160Q mouse models suggests that decreased free ascorbic acid and decreased release from astrocytes via extracellular vesicles directly impacts cellular metabolism as well as redox balance in early stages of disease, including in corticostriatal slices from 2- to 3-month-old YAC128 mice [33].

An important gap in the literature that prevents full translation of these findings to humans is that most of the work has been completed in rodents under physiologically replete ascorbate conditions. In contrast, humans without the ability to synthesize ascorbate, and who carry genetic variants in solute carrier family 23 members A1 and/or A2 (*SLC23A1* and *SLC23A2*) that can alter transport efficiency of their respective proteins SVCT1 and SVCT2, are more likely to experience deficiency. More than 40 single-nucleotide polymorphisms (SNPs) have been identified in *SLC23A2* [34–36], and while the study of these SNPs in HD patients is not known, there are documented instances of *SLC23A2* variants increasing the risk of glaucoma and other neurodegenerative conditions [36–39]. Here, we crossed YAC128 mice with SVCT2$^{+/-}$ mice to produce a novel HD mouse model line with approximately 30% decreased neuronal vitamin C to examine the interaction with vitamin C deficiency and dopaminergic dysfunction at an early disease stage. We intentionally chose a genetic manipulation of neuronal vitamin C as opposed to a global dietary deficiency to better understand the direct impact of this neuronal deficiency

without risking ill health or general weakness in the animals [20, 40, 41]. We hypothesized that a decrease in neuronal ascorbate would exacerbate the existing dopaminergic abnormalities in YAC128 mice.

2 | Methods

2.1 | Animals

Female YAC128 mice (previously backcrossed to a C57BL/6J background as in [42]) which have three copies of the Huntington gene compared to their wild-type (WT) littermates were crossbred with male SVCT2 heterozygous mice [9, 11]. SVCT2$^{+/-}$ mice lack one gene encoding the neuronal sodium-dependent vitamin C transporter, leading to an approximately 30% decrease in brain ascorbic acid. These animals were also maintained on a C57BL/6J background. Breeder sex and genotype were the same for all pairings to ensure that placental ascorbate transport via SVCT2 was not impacted in WT and YAC128 mice. These breedings produced four unique genotypes and final numbers for each group are listed in Table 1. All animals underwent behavioral testing. Tissues were acquired from the same mice for biochemical analyses. Due to tissue quantity and available resources for biochemical experiments, subsets of the animals were used for postmortem studies and group numbers are outlined in their respective methodological sections. YAC128 and SVCT2 genotype were confirmed using Transnetyx qPCR-based genotyping as previously described [42]. Male and female mice were used to test the potential for known sex differences in ascorbate and dopaminergic signaling to impact the results [43–45]. Throughout the study, mice were group housed in cages of two to five animals in a temperature and humidity-controlled housing room (22°C ± 2°C) kept on a 12-h light/dark cycle with lights on/off at 7 a.m./7 p.m. Mice were fed standard lab chow (Purina 5001) and water *ad libitum*, with cage changes occurring every 2–3 weeks but not immediately prior to behavioral testing. Vanderbilt University Institutional Animal Care and Use Committee approved all procedures using animals for this study.

2.2 | Behavioral Testing

Mice began their first behavioral battery at 14 weeks of age, which is when YAC128 mice on this background typically begin to show behavioral deficits [28], and were tested again at 20 weeks of age. All testing parameters were kept the same between the two timepoints. Locomotor activity and grip strength were assessed on the same day. Rotarod testing began the following day. For behavioral testing, experimental groups included all animals shown in Table 1.

TABLE 1 | Group numbers for each genotype used in the study, data from males and females were combined for each group.

	WT	YAC128
WT	19 (9F, 10M)	17 (10F, 7M)
SVCT2$^{+/-}$	25 (13F, 12M)	19 (10F, 9M)

Note: Mice generated for this study did not differ from the expected 1:1:1:1 ratio according to expected genetic outcomes, $\chi^2 = 1.9$, $p = 0.615$.

2.3 | Locomotor Activity

Mice were placed in MedAssociates locomotor chambers (32 × 32 cm, light level measured at 36–48 lux) for a 30-min trial to measure exploratory behavior. The chambers use infrared beams to track movement and total distance traveled throughout the trial. Each mouse was placed in the same chamber at both test ages.

2.4 | Grip Strength

Mice were placed on a grip strength meter (San Diego Instruments) in which all 4 paws were able to grasp the metal mesh grid. For five trials, their tails were gently pulled to record the maximum grip strength used to hold on to the mesh grid, and an average grip strength was calculated.

2.5 | Rotarod

Mice were given three 5-min trials over the course of three consecutive days on the Rotarod (UgoBasile). Over the course of the trial, the rotarod speed increased from 4 to 40 rpm, and the time of first full rotation and when they fell off was recorded.

2.6 | Tissue Collection and Analyses

Mice were euthanized within 72 h of the completion of the 20-week behavioral battery. Mice were first anesthetized using isoflurane before cervical dislocation and decapitation. The brain was removed from the skull, and the cortex and striatum from both hemispheres were collected rapidly by microdissection using forceps. Liver tissue was also collected by cutting a piece of tissue from the largest lobe. All tissue was immediately snap frozen on dry ice and stored at −80°C until used for biochemical analyses. Tissue was used for gene expression, protein expression, and neurochemistry as described below.

2.7 | Gene Expression

Frozen striatal tissue from one hemisphere was thawed on ice and RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN, cat # 74136). RNA was reverse transcribed into cDNA using the RT^2 First Strand kit (QIAGEN, cat # 330404). Gene expression was measured by quantitative PCR (qPCR) by loading the resulting cDNA and RT^2 SYBR Green ROX FAST Mastermix (QIAGEN, cat # 330620) on the Mouse Dopamine and Serotonin Pathway RT^2 Profiler PCR Array 96-well plate (QIAGEN, cat # 330231), which profiles the expression of 84 genes associated with the dopaminergic and serotonergic neurotransmitter systems. All kits were used according to the manufacturer's instructions. For gene expression analysis, experimental groups comprised three male and three female mice per genotype.

2.8 | Protein Expression

To prepare protein lysates, frozen striatal tissue from one hemisphere was thawed on ice and homogenized by hand with a plastic pestle in 200 μL of Pierce RIPA lysis buffer (Thermo Scientific, cat # 89900) containing protein and phosphatase inhibitors: cOmplete EDTA-free protease inhibitor cocktail (Roche, cat # 04693132001), 1:100 phosphatase inhibitor cocktail 3 (MilliporeSigma, cat # P0044), and 1 mM sodium orthovanadate per 10 mL of RIPA buffer (Thermo Scientific, cat # 89900). Samples were centrifuged at 12,000*g* for 5 min and the supernatant was collected as the protein lysate. Total protein concentration was determined using standard bicinchoninic acid (BCA) assay protocol (Pierce BCA Protein Assay Kit, Thermo Scientific). Samples were denatured by adding NuPage LDS sample buffer (Bolt cat # B0007) and reducing agent (Bolt cat # B0009), followed by boiling at 95°C for 3 min before loading 20 μg of protein per well onto Bolt 4%–12% Bis-Tris Plus gels (Thermo Scientific, cat # NW04120BOX). Protein was transferred to nitrocellulose membranes using the iBlot2 system (Thermo Scientific, cat # IB23001). After transfer, membranes were blocked with 5% nonfat milk in tris-buffered saline with 0.1% Tween-20 (TBST) for 30 min, followed by blocking in 5% bovine serum albumin (BSA) in TBST for 30 min. Blots were incubated with primary antibody (pTH Ser31, anti-rabbit, Cell Signaling Technologies, cat # 3370, 1:1000 in 5% BSA in TBST) overnight at 4°C, then with the appropriate secondary antibody (1:5000 in 5% nonfat milk) for 2 h. Protein bands were detected via chemiluminescence (Western Lightning Plus ECL, Perkin Elmer, cat # 103E001EA). Blots were also probed for TH (anti-rabbit, Cell Signaling Technologies, cat # 2792, 1:1000 in 5% BSA in TBST), DAT (anti-rat, Millipore Sigma, cat # MAB369, 1:1000 in 5% BSA in TBST), and β-actin (anti-mouse, *Santa Cruz Biotechnology*, cat # 4778, 1:1000 in 5% BSA in TBST). Between sequential probes, blots were stripped for 20 min (Restore Stripping Buffer, Thermo Fisher Scientific, cat # 21059), followed by 3 × 10 min washes in TBST, then reblocked prior to primary antibody incubation. Protein bands were quantified using the NIH ImageJ gel analysis tool. Each sample was normalized first to its own β-actin and then to the average WT control value for that blot. Both sexes were run on each blot and data were combined for analysis after confirming no sex differences. For Western blotting, experimental groups were as follows: WT = 13 (7F, 6 M), SVCT2$^{+/-}$ = 14 (7F, 7 M), YAC128 = 11 (6F, 5 M), and SVCT2$^{+/-}$ YAC128 = 14 (7F, 7 M).

2.9 | Ascorbic Acid

Ascorbic acid concentrations were measured by using ion pair HPLC on cortical tissue and liver. Tissue was homogenized with a plastic pestle after adding 10 μL per mg of tissue using a 7:2 ratio of 25% w/v metaphosphoric acid: 100 mM sodium phosphate. After being homogenized, samples were centrifuged at 15,000 rpm for 5 min, and the supernatant was collected to determine ascorbic acid concentration at a 1:1000 dilution. For ascorbic acid measurements, experimental groups were as follows: WT = 16 (7F, 9 M), SVCT2$^{+/-}$ = 19 (9F, 10 M), YAC128 = 14 (8F, 6 M), and SVCT2$^{+/-}$ YAC128 = 15 (7F, 8 M).

2.10 | Biogenic Amines

Concentrations of biogenic amines and major metabolites were measured from striatal tissue from one hemisphere by mass spectrometry by the Vanderbilt University Neurochemistry Core in two separate cohorts, and by HPLC in a third cohort. All sexes and genotypes were represented in each cohort, and to allow comparison among them, the data were converted to *Z*-scores using the formula $z = (x - \mu)/\sigma$. For neurotransmitter measurements, experimental groups were as follows: WT = 11 (6F, 5 M), SVCT2$^{+/-}$ = 15 (7F, 8 M), YAC128 = 12 (7F, 5 M), and SVCT2$^{+/-}$ YAC128 = 12 (7F, 5 M).

2.11 | Statistical Procedures

All statistical analyses, unless otherwise noted, were conducted using IBM SPSS 28.0 or GraphPad Prism version 10.4 or higher. For ANOVA analyses, the model used did not assume sphericity (equal variability of differences) and a Geisser–Greenhouse correction was used. For body mass, males and females were analyzed separately due to known differences in body weight, and a repeated-measures (RM) factorial ANOVA was conducted with SVCT2 genotype (WT SVCT2$^{+/+}$ or SVCT2$^{+/-}$), YAC128 genotype (WT or YAC128), and age (5, 10, 14, and 20 weeks) as fixed variables. Sex was first included as a fixed variable in all behavioral analyses and biochemical analyses, but due to the lack of significant effects, data were collapsed across sex and run together for all subsequent analyses. Two-factor ANOVA (two levels of SVCT2 genotype, two levels of YAC128 genotype) was conducted for behavioral tests with a single dependent variable. Significant interaction effects were followed with post hoc comparisons using Tukey's multiple comparisons test. For rotarod, which includes multiple trials, an RM-ANOVA with the same between-subjects factors as above was conducted with test day (3) as the repeated measure. For protein expression, ascorbate concentrations, and biogenic amine concentrations, two-factor ANOVA (2 SVCT2 genotype × 2 YAC128 genotype) was conducted. Post hoc comparisons were made following a significant interaction effect. Data are reported as mean ± SEM. unless otherwise reported, and an alpha of 0.05 was considered statistical significance for all analyses.

Gene expression was analyzed using QIAGEN GeneGlobe RT^2 Profiler PCR Data Analysis Tool (available from: https://geneglobe.qiagen.com/us/analyze). The fold change ($2^{(-\Delta\Delta CT)}$) method, normalizing to the group average of the arithmetic mean of the housekeeping genes *Actb* and *Gapdh*, was used to generate volcano plots and bar chart figures. The associated *p*-value for each fold change was calculated by the software based on a Student's *t* test of the replicate $2^{(-\Delta CT)}$ values for each gene in the WT control compared to the experimental test group (either SVCT2$^{+/-}$, YAC128, or SVCT2$^{+/-}$ YAC128).

2.12 | Excluded Data

Data were excluded from two female SVCT2$^{+/-}$ mice on locomotor activity at 20 weeks. Distance travelled was more than twofold the average distance travelled for the group and likely reflects

measurement error in the chambers rather than real hyperactivity. Exclusion of these data did not alter the main effects observed and reported below. For qPCR data, one SVCT2$^{+/-}$ sample was omitted from further analysis due to genomic DNA contamination as identified by the data quality control tool within GeneGlobe and low CT values. One SVCT2$^{+/-}$ YAC128 sample was removed due to being an outlier for housekeeping gene expression reflecting compromised sample integrity. Data from neurochemistry analyses were only excluded for three cases in which high-value outliers for one or two amino acids per mouse indicated technical issues (one YAC128 mouse for DA and DOPAC, and one WT mouse for 5-HIAA). Three samples for 3-MT registered <1 or below detection level and were also removed due to likely technical error.

3 | Results

3.1 | Behavior

Both male and female mice showed the expected effect of increased body mass with age, and YAC128 mice were heavier compared to mice that did not express the transgene. In females, YAC128 mice were heavier than littermates (age: $F_{3, 114}=81.89$, $p<0.001$; YAC128 genotype: $F_{1, 38}=11.03$, $p=0.002$, Figure 1A). SVCT2$^{+/-}$ mice were also heavier overall than WT mice (SVCT2 genotype: $F_{1, 38}=4.39$, $p=0.04$). YAC128 mice were also heavier overall in male mice (age: $F_{3, 102}=206.4$, $p<0.001$; YAC128 genotype: $F_{1, 34}=4.92$, $p=0.033$, Figure 1B) although this effect was modified by SVCT2 genotype (YAC128×SVCT2 genotype: $F_{1, 34}=6.53$, $p=0.015$).

No significant differences were observed between male and female mice among groups on other behavioral measures, so data were combined across sex for further analysis. Grip strength was weaker overall in YAC128 transgenic animals compared to nontransgenic animals, regardless of SVCT2 genotype, at both 14 weeks (YAC128 genotype: $F_{1, 76}=8.29$, $p=0.005$; SVCT2 genotype: $F_{1, 76}=1.69$, $p=0.20$, Figure 1C) and 20 weeks of age (YAC128 genotype: $F_{1, 76}=5.23$, $p=0.025$; SVCT2 genotype: $F_{1, 76}=0.60$, $p=0.44$, Figure 1F). Locomotor exploration during a 30-min trial was similar across genotypes at 14 weeks of age (Fs<1.88, ps>0.17, Figure 1D). By 20 weeks of age, decreased locomotor activity was observed in YAC128 mice, regardless of SVCT2 genotype (YAC128 genotype: $F_{1, 72}=14.33$, $p=0.0003$; SVCT2 genotype: $F_{1, 72}=1.052$, $p=0.31$, Figure 1G). Mice were first trained on the accelerating rotarod at 14 weeks of age, and all groups showed the expected improvement in latency to fall across days (day: $F_{2,52}=15.86$, $p<0.001$). Mice that carried the YAC128 transgene performed more poorly than their littermates (YAC128 genotype: $F_{1, 76}=13.64$, $p=0.0004$; Figure 1E) with no other main effects or interactions (Fs<2.0, ps>0.16). At 20 weeks, the deficit in rotarod performance in the YAC128 mice persisted ($F_{1, 76}=19.43$, $p<0.0001$; Figure 1H) and there were no further effects of learning and no difference according to SVCT2 genotype (Fs<2.24, ps>0.11).

3.2 | Gene Expression

A volcano plot displaying fold change ($2^{(-\Delta\Delta CT)}$) for all 84 genes in the array against the −log10(p-value) for each of the three experimental groups compared to WT controls was generated (Figure 2A–C). Gene expression was considered significantly and substantially altered if $p<0.05$ *and* the fold change was either greater than 2 (indicating two-fold upregulation compared to WT control) or less than 0.5 (indicating two-fold downregulation compared to WT control). None of the 84 genes were significantly *and* substantially downregulated compared to WT control. No genes were significantly and substantially upregulated in SVCT2$^{+/-}$ striatal tissue compared to WT, although *dopamine receptor D2 (Drd2)* was upregulated 2.05-fold with a p value approaching significance ($p=0.06$) (Figure 2A and Table 2). In YAC128 striatal tissue, *dopamine receptor D3 (Drd3)* was upregulated 2.15-fold ($p=0.017$) (Figure 2B and Table 2). Six genes were significantly and substantially upregulated in the striatum of SVCT2$^{+/-}$ YAC128 mice, including *Drd3, solute carrier family 18 member A1* (*Slc18a1*) coding for vesicular monoamine transporter 1 (VMAT1), *solute carrier family 6 member A3* (*Slc6a3*) coding for the dopamine transporter (DAT), *tyrosine hydroxylase* (*Th*) coding for the TH protein, *tryptophan 2,3-dioxygenase* (*Tdo2*) coding for the TDO2 protein, and *solute carrier family 6 member A4* (*Slc6a4*) coding for the sodium-dependent serotonin reuptake transporter (SERT) (Figure 2C and Table 2). An additional 17 genes presented with a statistically significant change in expression level in SVCT2$^{+/-}$ YAC128 mice, but these did not meet the threshold of substantial change (<0.5- or >2.0-fold change) (Table 2). Five of the genes upregulated in SVCT2$^{+/-}$ YAC128 mice (*Slc18a1, Slc6a3, Slc6a4, Tdo2*, and *Th*) showed a stepwise increase in expression, such that SVCT2$^{+/-}$ YAC128 mice show the greatest fold-change, followed by YAC128 mice and then SVCT2$^{+/-}$ mice compared to control (Figure 2D). Genes significantly upregulated in SVCT2$^{+/-}$ YAC128 are involved in neurotransmitter synthesis (*Th*, dopamine synthesis; *Tdo2*, tryptophan metabolism), monoamine packaging (*Slc18a1*; VMAT1), and monoamine transport (*Slc6a3*, DAT; *Slc6a4*, SERT serotonin transporter).

3.3 | Protein Expression

Given the changes in genes coding for key dopaminergic proteins, we followed up these findings with western blots in the same brain area, striatum. Relative densities were calculated relative to WTs within each blot of up to 14 samples per blot. Each blot was run with samples of one sex only and included at least two of each of the four genotypes for a total of 11–14 samples per group between the blots and up to four blots run per antibody tested. No significant changes in protein expression were identified for DAT, TH, pTH, or the ratio of TH:pTH (Fs<4.12, ps>0.15, *data not shown*).

3.4 | Neurochemistry

We first confirmed that brain ascorbate levels were decreased in mice with 50% lower expression of SVCT2 (SVCT2 genotype: $F_{1, 60}=33.40$, $p<0.0001$, Figure 3A). As expected [9], there were no changes in liver ascorbate levels since all mice were able to synthesize ascorbate de novo in the liver (SVCT2 genotype: $F_{1, 46}=0.13$, $p=0.72$, Figure 3B). YAC128 genotype did not impact either brain or liver ascorbate levels (Fs<1.48, ps>0.23).

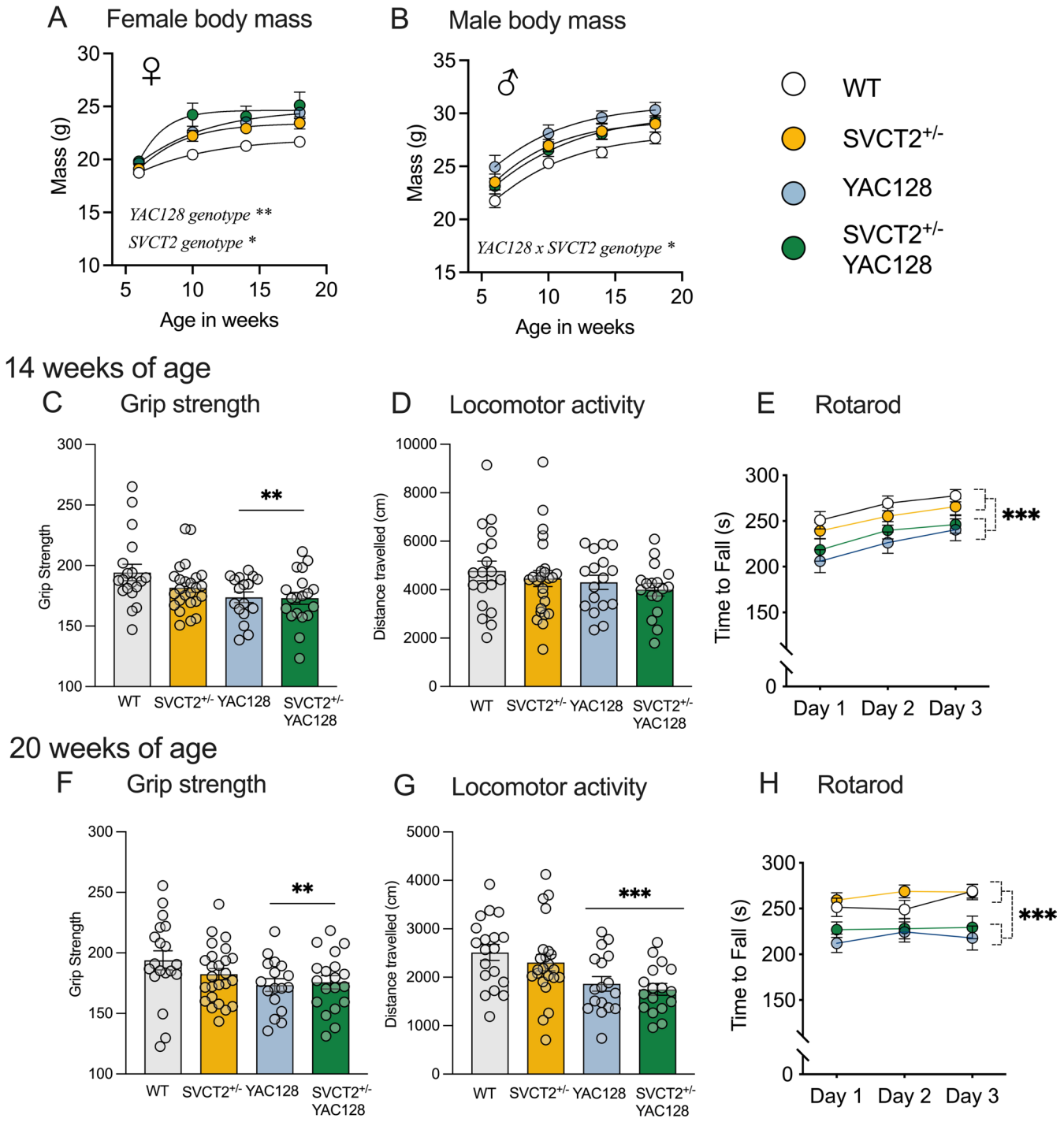

FIGURE 1 | Increased body mass and decreased grip strength, locomotor activity and motor ability in YAC128 mice. Changes in body weight over time are shown for (A) female and (B) male mice at 5, 10, 15, and 20 weeks of age. Data analyzed by repeated measures ANOVA. Weight data are shown as group mean and standard error of the mean, with non-linear line of best fit. (C, F) Average four-paw grip strength across five trials is shown for mice tested at 14 weeks (C) and 20 weeks of age (F). (D, G) Distance travelled in locomotor activity chambers over 30 mins. are shown in mice tested at 14 weeks (D) and when tested again in the same chambers at 20 weeks of age (G). (E, H) Three days of rotarod training were given at 14 weeks (E) and again using the same training parameters at 20 weeks of age (H). (C–H) Data shown are mean with standard error of the mean and individual data points where possible. Significant main effects of YAC128 genotype following two-way ANOVA are marked $^{**}p < 0.01$, $^{***}p < 0.001$.

Monoamine neurotransmitters and metabolites, GABA and glutamate, were measured by mass spectrometry and HPLC. Neurotransmitter analysis was run in three independent cohorts, with mice from each sex and genotype represented in each cohort. Differences in standards and running conditions can limit direct comparison between data sets, so we calculated Z-scores ($z = (x - \mu)/\sigma$) to account for differences in means and variability between the groups. The data showed that both SVCT2 and YAC128 genotype decreased dopamine and serotonin and their metabolites compared to WT controls. Interestingly, there was not a compounding effect of the double genotype. Significant SVCT2 × YAC128 genotype interactions

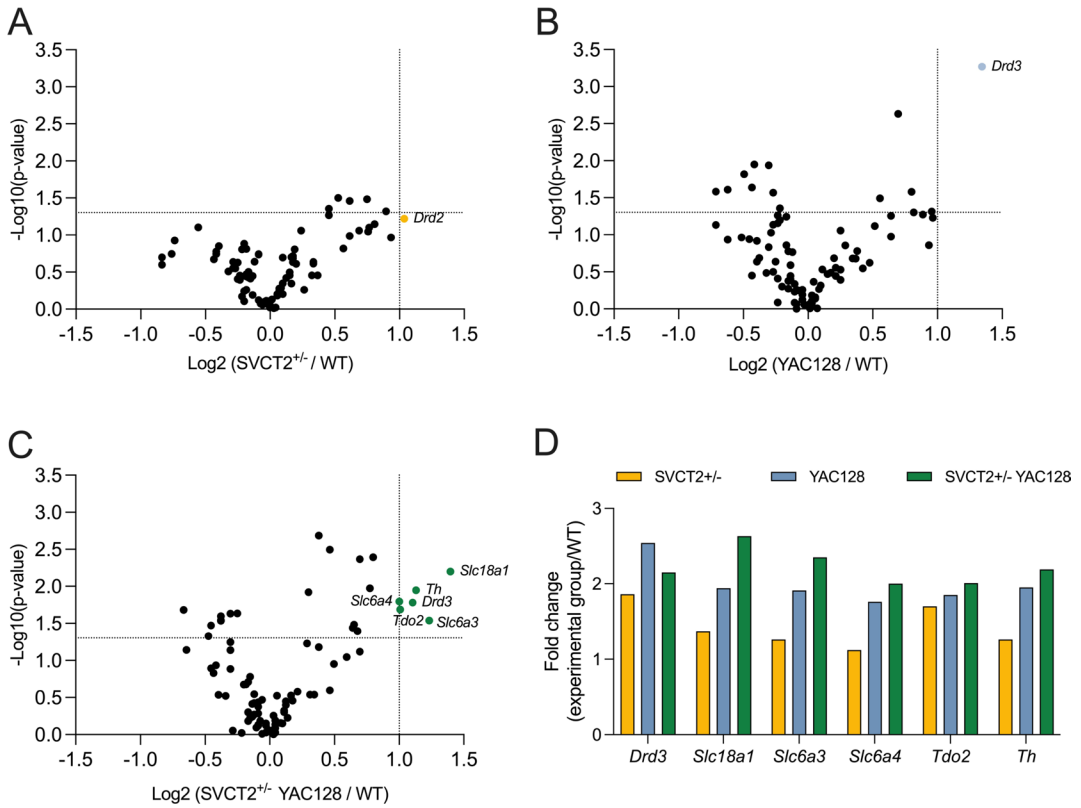

FIGURE 2 | Gene expression changes in the striatum of SVCT2$^{+/-}$ and YAC128 mice. A vertical line denotes the minimum threshold for substantial upregulation (twofold) on each volcano plot (A–C) and a horizontal line indicates the threshold for significance ($p < 0.05$) on each volcano plot. (A) No genes were significantly upregulated greater than twofold in SVCT2$^{+/-}$ mice compared to WT control. (B) Drd3, dopamine receptor subtype 3, was significantly upregulated in YAC128 compared to WT control. (C) Six genes, Slc18a1, Slc6a3, Th, Drd3, Tdo2, and Slc6a4, were significantly upregulated at least twofold in SVCT2$^{+/-}$ YAC128 mice compared to WT. (D) A stepwise increase in gene expression for five genes in which SVCT2$^{+/-}$ YAC128 mice show the greatest fold-change increase, followed by YAC128 mice and lastly SVCT2$^{+/-}$ mice. Drd3 was significantly upregulated in all genotypes compared to WT, but only considered substantial for both YAC128 (2.54-fold) and SVCT2$^{+/-}$ YAC128 (2.15-fold) mice.

were observed for dopamine ($F_{1, 46} = 9.22$, $p = 0.004$), DOPAC ($F_{1, 46} = 10.43$, $p = 0.002$), HVA ($F_{1, 46} = 6.51$, $p = 0.014$), 3-MT ($F_{1, 45} = 12.05$, $p = 0.001$), serotonin ($F_{1, 46} = 5.72$, $p = 0.021$), and 5-HIAA ($F_{1, 46} = 6.48$, $p = 0.014$; Figure 3C–H). Tukey's multiple comparisons were conducted following the significant interactions, which revealed that differences between WT and SVCT2$^{+/-}$YAC128 were not significant for dopamine and serotonin, 3-MT, and 5-HIAA. We did not observe any change in levels of GABA or glutamate in the striatum ($Fs_{1,33} < 2.76$, $ps > 0.11$, *data not shown*).

4 | Discussion

The present study demonstrated that SVCT2$^{+/-}$ YAC128 mice exhibit subtle changes in the dopaminergic neurotransmitter system revealed through differences in gene expression compared to single mutant littermates. We confirmed a 35% decrease in brain ascorbate levels in mice heterozygous for SVCT2, and this was not further decreased in SVCT2$^{+/-}$ YAC128 mice. The data suggest that the observed subtle changes are not simply due to a more extreme decrease in ascorbate availability, but rather the compounded effect of ascorbate deficiency in an already sensitive dopaminergic system due to the HD mutation. Decreased availability of monoamine neurotransmitters was observed in both SVCT2$^{+/-}$ and YAC128 mice, and there was not a compounding impact of the double mutation.

We initially hypothesized that SVCT2$^{+/-}$ YAC128 mice would display more severe behavioral phenotypes, or faster onset of deficits, compared to YAC128 with sufficient ascorbate. The experiments in the current study do not support this aspect of the hypothesis, as SVCT2$^{+/-}$ YAC128 performed similarly to YAC128 at both 14 and 20 weeks of age when examining overall locomotor activity levels and rotarod performance. Early work with YAC128 mice on an FVB/N background reported *hyper*activity as early as 3 months of age [29] in shorter (10 min) trials, during the dark cycle. We did not observe the same phenotype in the YAC128 mice used here on the C57Bl/6J background when tested during daylight hours. It is possible that the initial slight hyperactive phenotype [29] (typically associated with increased dopamine release and concentrations) in these mice occurred before 14 weeks of age, or that there is an interaction with circadian rhythms, but these possibilities were not directly addressed in the current study. Hypoactivity was observed in both YAC128 and SVCT2$^{+/-}$ YAC128 mice at 20 weeks of age. Although it is possible that differences in onset time may have been detected in the double mutant animals given more time points or continuous measuring, the magnitude of the change in both groups was

TABLE 2 | Fold change gene expression in SVCT2$^{+/-}$ and YAC128 mice.

	SVCT2$^{+/-}$		YAC128		SVCT2$^{+/-}$ YAC128	
Gene	**Fold change (rel. to WT)**	***p***	**Fold change (rel. to WT)**	***p***	**Fold change (rel. to WT)**	***p***
Adcy1	0.60	0.119	0.61	**0.026**	0.63	**0.020**.
Adrb1	0.75	0.180	0.71	**0.015**	0.77	**0.029**
Akt1	0.86	0.156	0.81	**0.012**	0.77	**0.028**
Akt2	0.90	0.387	0.74	**0.023**	0.72	**0.047**
Alox12	1.44	**0.032**	1.43	0.076	1.62	**0.004**
Arrb1	0.88	0.361	0.85	0.070	0.77	**0.025**
Arrb2	1.04	0.658	1.19	0.298	1.38	**0.003**
Cdk5	1.05	0.528	1.07	0.483	1.23	**0.012**
Dbh	1.06	0.515	1.39	0.239	1.60	**0.040**
Drd2	**2.05**	0.060	1.08	0.293	1.08	0.477
Drd3	1.86	**0.048**	**2.54**	**<0.001**	**2.15**	**0.017**
Grk4	1.37	**0.044**	1.62	**0.002**	1.56	**0.036**
Gsk3a	0.91	0.356	0.75	**0.011**	0.81	0.056
Htr2a	0.91	0.643	1.06	0.542	1.30	**0.002**
Htr2c	1.53	**0.035**	1.74	**0.026**	1.62	0.076
Htr5a	0.68	0.079	0.65	**0.025**	0.73	0.128
Itpr1	1.04	0.636	0.86	**0.044**	0.81	**0.023**
Maoa	0.94	0.183	0.83	**0.027**	0.84	**0.023**
Pde4a	0.75	0.180	0.82	0.093	0.73	**0.034**
Pde4c	1.13	0.233	1.56	0.056	1.71	**0.011**
Slc18a1	1.37	0.054	1.94	**0.049**	**2.63**	**0.006**
Slc6a3	1.26	0.243	1.91	0.139	**2.35**	**0.029**
Slc6a4	1.12	0.452	1.76	0.050	**2.00**	**0.016**
Sncaip	1.68	**0.033**	1.47	**0.032**	1.57	**0.033**
Tdo2	1.70	0.079	1.85	0.053	**2.01**	**0.020**
Th	1.26	0.231	1.95	0.059	**2.19**	**0.011**
B2m	1.07	0.447	1.30	0.166	1.74	**0.004**

Note: Changes in gene expression that were significant ($p < 0.05$) or substantial (fold change > 2.0 or < 0.50) for at least one of the experimental groups are shown in bolded text. No genes were both significantly and substantially altered in SVCT2$^{+/-}$ mice, one gene met that requirement in YAC128 mice, and six genes met that requirement in n SVCT2$^{+/-}$ YAC128 mice.

similar, and so any additional impact of decreased neuronal ascorbate would likely have been slight. Similarly, the age at which a rotarod deficit first emerges in YAC128 mice depends on the mouse line background [28]. Here, we report a main effect of YAC128 genotype on rotarod performance beginning at 14 weeks of age and persisting through 20 weeks of age, with a minimal decline from 14 to 20 weeks of age. As with activity and grip strength, rotarod performance deficits were not exacerbated in SVCT2$^{+/-}$ YAC128 mice compared to YAC128 mice. Specific compensatory mechanisms were not evaluated, but it is likely that some resistance to further deficits associated with the YAC128 genotype was conferred by the C57Bl/6J genetic background.

Circulating ascorbate is normal in SVCT2$^{+/-}$ mice due to in situ synthesis, as indicated by the lack of change in liver ascorbate levels. Any deficiency is therefore at the cellular level driven by decreased expression of the transporter. In the typical human condition where dietary ascorbate intake determines availability, tissues achieve much lower concentrations and more extreme deficiency in both central nervous system and peripheral organs. Although this study was designed to avoid the potential for systemic depletion to contribute to overall weakness, where there is general depletion in humans there is the potential for behavioral phenotypes to be further impacted. To the best of our knowledge, the current literature has not investigated specific ascorbate deficiencies in patients with HD. However, one study

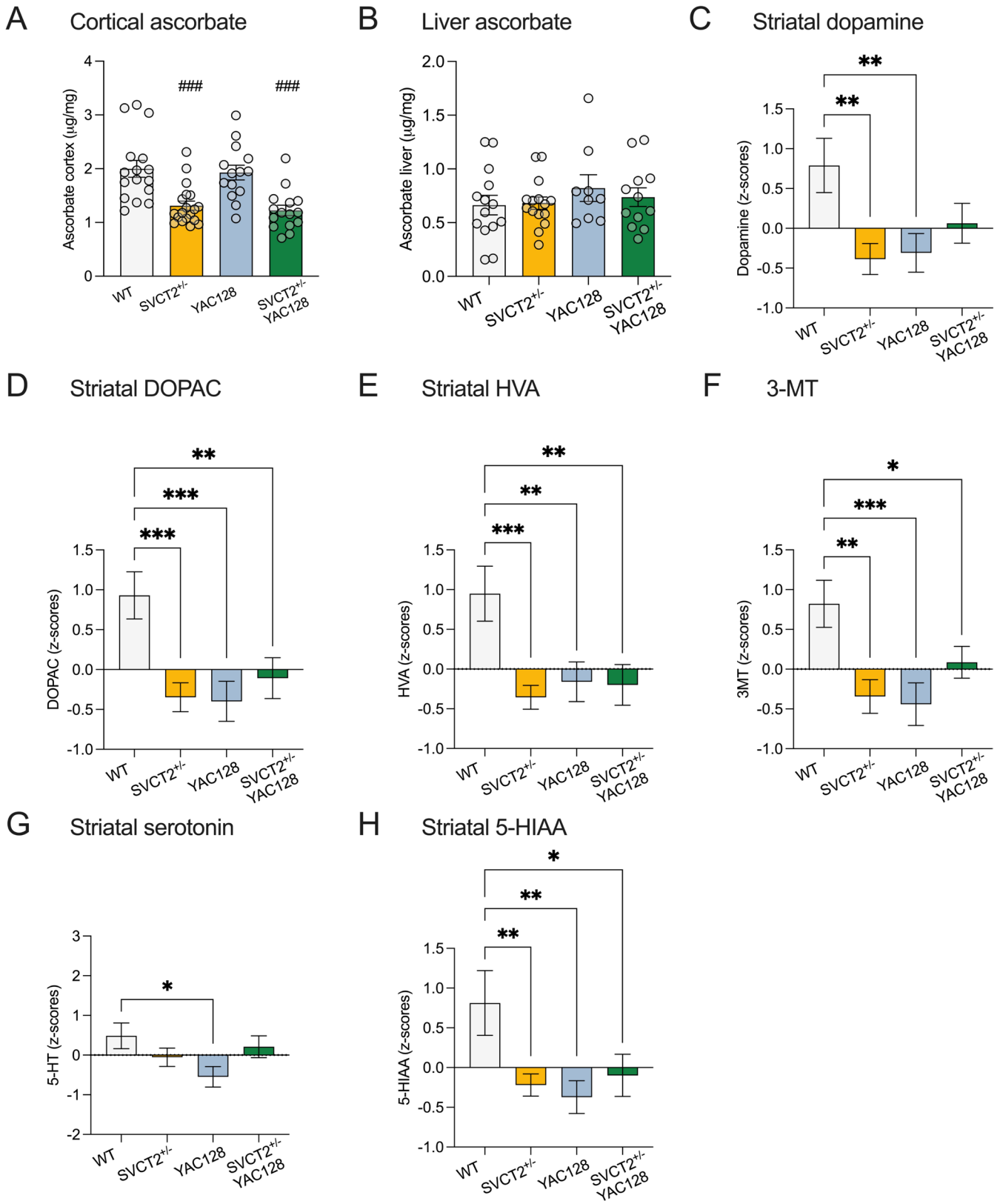

FIGURE 3 | Dysregulated dopamine regulation in SVCT2$^{+/-}$ and YAC128 mice. SVCT2 and YAC128 genotype were confirmed by qPCR. (A) Ascorbate was measured in cortex as a representative brain level area to confirm approximately 30%–40% decrease in SVCT2$^{+/-}$ mice. (B) Liver ascorbate was similar in all mice. (C) Dopamine, DA, (D) DOPAC, (E) HVA, (F) 3-MT, (G) serotonin, 5-HT, and (H) 5-HIAA, are reported as *Z*-scores to compare among analysis batches. Main effect of SVCT2 genotype denoted as $^{###}p < 0.001$. Significant SVCT2 × YAC128 genotype interactions were followed by Tukey's post hoc comparisons.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ differences as marked. Data shown are mean ± SEM.

documenting self-reported adherence to the Mediterranean diet (which included higher vitamin C consumption) had higher quality of life and lower motor impairment scores [46]. More broadly, the overall nutritional status of these patients is a well-known concern as motor impairments complicate feeding and can lead to lower-than-average body weight and general malnutrition that is observed in HD patients [46, 47].

Subtle changes in gene expression and metabolite levels in SVCT2$^{+/-}$ or YAC128 only mice were augmented in SVCT2$^{+/-}$ YAC128 double mutants. When evaluating the gene expression profile of dopamine and serotonin pathways, the greatest number of genes were upregulated in SVCT2$^{+/-}$ YAC128 compared to WT mice. Six out of 84 genes tested were upregulated significantly and substantially in these mice, and four of these were directly related to the dopamine neurotransmitter system: *Drd3, Slc18a1, Slc6a3*, and *Th*. When assessing striatal dopamine and serotonin and metabolites, we observed statistically significant decreases in both and in their metabolites in SVCT2$^{+/-}$ mice and YAC128 mice compared to WT. Not only did SVCT2$^{+/-}$ YAC128 mice not exhibit the same statistically significant decrease, in some cases the decrease relative to WT littermates was not statistically significant. This pattern of results may indicate a compensatory mechanism including upregulation of *Th* gene expression. Further studies that examine the dynamics of dopamine release and uptake (e.g., fast-scan cyclic voltammetry) could clarify these results of static metabolite measurements [19, 45]. The breeding schedule used in this study results in SVCT2$^{+/-}$ mice having lower ascorbate throughout development including in utero [9]. The potential for developmental differences in nutrient availability to impact neurodegenerative disease is an important concept that should also be further studied with relation to HD. SVCT2 is also expressed on mitochondrial membranes, and we previously showed that SVCT2$^{+/-}$ genotype and APP/PSEN1 genotype (a mouse model for amyloidogenesis and Alzheimer's disease pathology) had opposing abnormal effects in cellular respiration leading to diminished differences from control mice compared to either genotype alone [48]. Ascorbate deficiency exacerbated the mitochondrial oxidative stress in the APP/PSEN1 mouse model. Mitochondrial function is also impaired in HD [49, 50] and oxidative stress markers are elevated in HD [51]. Given that SVCT2 is expressed on mitochondrial membranes, this could be one possible mechanism that contributes to or exacerbates HD pathology. This was not evaluated in the present study and would be an interesting area for future research.

Gene expression differences were not reflected in modified protein levels. It is likely that decreased ascorbate directly impacted enzymatic function as a direct cofactor (dopamine beta hydroxylase), or recycling a cofactor as is the case for tetrahydrobiopterin (BH_4) and tyrosine hydroxylase. It is also possible that if protein translation was altered, it was in a cell-specific or region-specific manner that was masked in the tissue sample we tested. In the case of early disease stage, western blotting may also simply not be sensitive enough to detect small, biologically relevant changes in certain proteins, whereas more sensitive techniques like qPCR are able to indicate subtle transcriptional changes.

A role for ascorbate on dopamine levels and those of its precursors and metabolites is easily explained by its known cofactor roles as well as potential for oxidation of dopamine. Other roles for the effect of SVCT2 and ascorbate on gene regulation were not directly tested in this study but may be related to epigenetic changes [52, 53]. It also remains to be tested whether direct cellular uptake and release or overall global deficiency are more important in modifying disease progress. The minor changes observed in this model of mild ascorbate depletion and minimal pathological challenge suggest that there may be a role of ascorbate deficiency in the progression of HD pathology and that small changes that can further dysregulate dopamine dynamics in HD models, and thus presumably in humans, may further impinge on motor and behavioral outcomes. Clarification of these results will require support from models of more severe disease pathology and nutrient depletion such as R6/1 [54] R6/2 [55–57] Q175 [58] HD rats [59]. It is also important to gather targeted data from human patient samples to understand whether these mechanisms may have an impact on patient trajectories.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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