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Chronic blockade of extrasynaptic NMDA receptors ameliorates synaptic dysfunction and pro-death signaling in Huntington disease transgenic mice

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ABSTRACT

In the YAC128 mouse model of Huntington disease (HD), elevated extrasynaptic NMDA receptor (Ex-NMDAR) expression contributes to the onset of striatal dysfunction and atrophy. A shift in the balance of synaptic–extrasynaptic NMDAR signaling and localization is paralleled by early stage dysregulation of intracellular calcium signaling pathways, including calpain and p38 MAPK activation, that couple to pro-death cascades. However, whether aberrant calcium signaling is a consequence of elevated Ex-NMDAR expression in HD is unknown. Here, we aimed to identify calcium-dependent pathways downstream of Ex-NMDARs in HD. Chronic (2-month) treatment of YAC128 and WT mice with memantine (1 and 10 mg/kg/day), which at a low dose selectively blocks Ex-NMDARs, reduced striatal Ex-NMDAR expression and current in 4-month old YAC128 mice without altering synaptic NMDAR levels. In contrast, calpain activity was not affected by memantine treatment, and was elevated in untreated YAC128 mice at 1.5 months but not 4 months of age. In YAC128 mice, memantine at 1 mg/kg/day rescued CREB shut-off, while both doses suppressed p38 MAPK activation to WT levels. Taken together, our results indicate that Ex-NMDAR activity perpetuates increased extrasynaptic NMDAR expression and drives dysregulated p38 MAPK and CREB signaling in YAC128 mice. Elucidation of the pathways downstream of Ex-NMDARs in HD could help provide novel therapeutic targets for this disease.

Introduction

In Huntington disease (HD), progressive neurodegeneration is attributed to a polyglutamine (polyQ) expansion near the N-terminus of the protein huntingtin (mutant huntingtin, mtHtt) (Huntington's Disease Collaborative Research Group, 1993). Whereas wildtype huntingtin is vital for normal cellular function, mtHtt interferes with essential intracellular processes, including gene expression, Ca^{2+} homeostasis and vesicular trafficking (Zuccato et al., 2010). The polyQ expansion primarily affects GABAergic medium-sized spiny projection neurons (SPNs) of the striatum, which exhibit up to 95% neuronal loss at late stages of the disease (Vonsattel et al., 1985). However, the mechanisms underlying selective vulnerability of the striatum to mtHtt-induced death remain unclear.

Increased functional expression of N-methyl-D-aspartate glutamate receptors (NMDARs) in HD could contribute to selective striatal excitotoxicity (Cepeda et al., 2001; Chen et al., 1999; Fan et al., 2007; Li et al., 2003; Milnerwood et al., 2010; Starling et al., 2005; Zeron et al., 2001, 2002; Zhang et al., 2008). Previously, we reported elevated expression of GluN2B-containing extrasynaptic NMDARs (Ex-NMDARs) in presymptomatic YAC128 mice (Milnerwood et al., 2010), in which the yeast artificial chromosome is used to express full-length human huntingtin with 128 CAG repeats (Slow et al., 2003). Synaptic NMDARs activate pro-survival pathways, while Ex-NMDARs trigger cell death (Hardingham and Bading, 2010; Hardingham et al., 2002). A shift in the balance of synaptic to Ex-NMDAR signaling contributes to HD pathology, as chronic Ex-NMDAR blockade attenuates mtHtt-induced striatal atrophy and motor learning deficits in YAC128 mice (Milnerwood et al., 2010; Okamoto et al., 2009).

Along with elevated Ex-NMDAR activity, intracellular Ca^{2+} signaling pathways that couple to survival or death are also dysregulated early in HD. Activity of the Ca^{2+}-dependent protease calpain is elevated in striatal tissue of post-mortem HD human brains and presymptomatic 1–2 month old YAC128 mice (Cowan et al., 2008; Gafni and Ellerby, 2002; Gladding et al., 2012). Calpain potentiates HD-associated striatal degeneration by cleaving mtHtt into toxic fragments and triggering pro-apoptotic cascades in parallel with caspases (Gafni et al., 2004; Kim et al., 2001). Calpain also contributes to Ex-NMDAR surface mislocalization in 1–2 month old YAC128 mice by cleaving the GluN2B C-terminus and thus altering NMDAR surface stability (Gladding et al., 2012). Activity of the p38 mitogen-activated protein kinase (MAPK), shown previously to be downstream of Ex-NMDARs (Xu

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et al., 2009), is also elevated in YAC128 mouse striatum at 1–2 months of age (Fan et al., 2012). The p38 MAPK mediates enhanced NMDA-induced toxicity in YAC128 cultured striatal neurons (Fan et al., 2012) and thus contributes to susceptibility of mtHtt-expressing striatal neurons to excitotoxic death. In addition, activity of the pro-survival transcription factor cAMP response element binding protein (CREB) is reduced in striatal tissue of 1 and 4 month-old YAC128 mice (Milnerwood et al., 2010). While synaptic NMDAR signaling promotes CREB activity, Ex-NMDARs trigger dephosphorylation and inactivation of CREB via dominant pathways (Hardingham et al., 2002). Additionally, CREB signaling is restored by chronic suppression of Ex-NMDAR activity in YAC128 mice (Milnerwood et al., 2010), suggesting a link between Ex-NMDARs and CREB shut-off.

Together, elevated Ex-NMDAR activity and dysregulated intracellular signaling could contribute to mtHtt-induced striatal degeneration. However, whether aberrant Ca^{2+} signaling in HD is a direct result of enhanced Ex-NMDAR activity or a consequence of other effects of mtHtt remains unclear. Here, we examined the role of Ex-NMDARs in HD-associated aberrant Ca^{2+} signaling.

Methods

Memantine treatment

WT and YAC128 (line 55) mice (Slow et al., 2003) bred on the FVB/N background were maintained at the University of British Columbia (UBC) Faculty of Medicine Animal Resource Unit, according to guidelines of the Canadian Council on Animal Care. Animals were housed in identical conditions (2–4 mice/cage) in a 12-h light/dark cycle, with full access to food and water. WT and YAC128 mice were treated with memantine as described previously (Milnerwood et al., 2010; Okamoto et al., 2009). Briefly, memantine at 1 or 10 mg/kg/day was provided to mice *ad libitum* in their drinking bottles, starting at 2 months of age (±10 days) for 2 months. Control mice received water (vehicle). Memantine solution concentrations were adjusted on a semi-monthly basis according to mouse body weight and daily solution intake to ensure consistent dosing throughout the treatment period. Cohorts alternated between male and female mice (a total of 4 female cohorts and 6 male cohorts). No differences in daily solution intake were detected between treatment groups compared to control groups for either genotype or sex (data not shown); thus, the mice had no apparent aversion to the taste of memantine.

Striatal dissection, subcellular and nuclear fractionations

Striatal tissue from memantine-treated (1 and 10 mg/kg/day) and untreated WT and YAC128 mice was collected after the treatment period and paired on the day of dissection. Mice were decapitated following halothane vapour anesthesia. Brains were rapidly removed and striatal sections were dissected and homogenized in 200 μL ice-cold sucrose buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4). Striatal cytosolic, synaptosomal, and nuclear fractions were obtained by subcellular and nuclear fractionation as described previously (Milnerwood et al., 2010). Synaptic (postsynaptic density, PSD) and extrasynaptic-enriched (non-PSD) synaptosomal fractions were isolated based on the principle that the non-PSD is Triton-X-soluble, whereas the PSD is Triton-X-insoluble. All buffers contained 'complete' protease and phosphatase inhibitor cocktails (Roche), as well as 15 μM calpeptin (Calbiochem), 1 mM EDTA, 1 mM EGTA, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 30 mM sodium fluoride. The purity of the subcellular fractionation was confirmed by enrichment of the PSD marker PSD-95 in the PSD fraction, and the presynaptic marker synaptophysin in the non-PSD (not shown). The purity of the nuclear fractionation was confirmed by enrichment of the nuclear marker histone deacetylase (HDAC) in the nuclear matrix fraction (not shown). Fractions were stored at −80 °C until use.

Western blotting

Protein concentration was assessed by a BCA protein assay (Pierce). Freshly-thawed samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by heating (3–5 min, 80–85 °C), in 3X protein sample buffer (PSB) (6% SDS, 0.4 mM Tris (pH 6.8), 30% glycerol, pyronin Y, 70 mg/mL DTT). Equal amounts of protein (5–15 μg for non-PSD, PSD, nuclear matrix fractions, or 20–40 μg for cytosolic fractions) were separated in 10% (w/v) SDS-polyacrylamide gels, and transferred to polyvinylidene fluoride (PVDF) membranes by semi-dry electrophoresis (BioRad). Membranes were blocked in TBS with 0.5% Tween-20 (TBST) and 3% BSA (or 5% milk for spectrin blots) (1 h, RT), incubated in primary antibodies (overnight, 4 °C), then washed and incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (2 h, RT). Blots were then washed and visualized using an enhanced chemiluminescence substrate (ECL, Amersham) and developed by exposure to film (Amersham), except for $pCREB^{Ser13}$ and CREB blots which were developed using an automated ChemiDoc XRS Molecular Imager (BioRad). Blots for which total p38 was probed were subsequently reprobed to quantify phosphorylated p38 (P-p38), using alkaline phosphatase (AP)-conjugated secondary antibodies and a Lumi-phosWB Chemiluminescent Substrate detection system (Pierce).

The following primary antibodies were used: rabbit N-terminal anti-GluN2B (AGC-003; Alomone, 1:500), rabbit anti-spectrin (cleaved) (AB38, gift from Dr. David Lynch, University of Pennsylvania, Philadelphia, PA, 1:2000), rabbit anti- $pCREB^{Ser133}$ (06-519, Millipore, 1:500), rabbit anti-CREB (9197, Cell Signaling, 1:500), rabbit anti-P-p38 MAPK (4511S, Cell Signaling, 1:200), mouse anti-p38 MAPK (sc-7972, Santa Cruz, 1:200), mouse anti-PSD-95 (MA1-045, Pierce, 1:500), goat anti-β-actin (sc-1616, Santa Cruz, 1:1500), goat anti-α-tubulin (sc-9935, Santa Cruz, 1:1500), goat anti-HDAC (sc-6268, Santa Cruz, 1:500), and mouse anti-synaptophysin (S5768, Sigma, 1:1000). All primary antibodies were diluted in TBST with 3% BSA, except for anti-spectrin, which was diluted in TBST with 5% milk. The following secondary antibodies were used: anti-mouse HRP-conjugated (NA931V, Amersham, 1:5000), anti-rabbit HRP-conjugated (NA934V, Amersham; 1:5000), anti-rabbit AP-conjugated (S372, Promega, 1:5000), and anti-goat HRP-conjugated (sc-2020; Santa Cruz, 1:5000). All secondary antibodies were diluted in TBST with 1% BSA.

Image analysis

For blots developed using film, the optical density of bands was quantified using Image J software (NIH) after background subtraction. Band intensities of GluN2B in synaptosomal fractions were normalized to β-actin (loading control), whereas bands for cytosolic spectrin were normalized to α-tubulin, which gave a clearer signal in the cytosolic fraction. P-p38 bands were normalized to p38 bands probed on the same membrane. For quantification of $pCREB^{Ser133}$/ CREB ratios, CREB and $pCREB^{Ser133}$ levels were probed on separate gels, and each was normalized to HDAC (loading control), as probing for CREB and $pCREB^{Ser133}$ on the same membrane did not provide clear results. $pCREB^{Ser133}$ and CREB blots, which were developed using the ChemiDoc XRS Molecular Imager, were quantified using Image-Lab Analysis Software (4.1, BioRad).

Brain slice preparation

Ex-NMDAR currents were recorded from coronal brain slices made from memantine-treated (1 mg/kg/day) and untreated WT and YAC128 mice. Mice were halothane-anesthetized and decapitated. Brains were immersed in ice-cold oxygenated (95% O_2, 5% CO_2)