Neurobiology of Disease 87 (2016) 80–90

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

# Impaired development of cortico-striatal synaptic connectivity in a cell culture model of Huntington's disease

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ARTICLE INFO

*Article history:*
Received 18 August 2015
Revised 13 December 2015
Accepted 18 December 2015
Available online 19 December 2015

*Keywords:*
Huntington's disease
Corticostriatal co-culture
Dendritic arborization
Excitatory synapse
Glutamate vesicle pool replenishment
Inhibitory synapse
Screening tool

ABSTRACT

Huntington's disease (HD) is a genetically inherited neurodegenerative disease caused by a mutation in the gene encoding the huntingtin protein. This mutation results in progressive cell death that is particularly striking in the striatum. Recent evidence indicates that early HD is initially a disease of the synapse, in which subtle alterations in synaptic neurotransmission, particularly at the cortico-striatal (C-S) synapse, can be detected well in advance of cell death. Here, we used a cell culture model in which striatal neurons are co-cultured with cortical neurons, and monitored the development of C-S connectivity up to 21 days *in vitro* (DIV) in cells cultured from either the YAC128 mouse model of HD or the background strain, FVB/N (wild-type; WT) mice. Our data demonstrate that while C-S connectivity in WT co-cultures develops rapidly and continuously from DIV 7 to 21, YAC128 C-S connectivity shows no significant growth from DIV 14 onward. Morphological and electrophysiological data suggest that a combination of pre- and postsynaptic mechanisms contribute to this effect, including a reduction in both the postsynaptic dendritic arborization and the size and replenishment rate of the presynaptic readily releasable pool of excitatory vesicles. Moreover, a chimeric culture strategy confirmed that the most robust impairment in C-S connectivity was only observed when mutant huntingtin was expressed both pre- and postsynaptically. In all, our data demonstrate a progressive HD synaptic phenotype in this co-culture system that may be exploited as a platform for identifying promising therapeutic strategies to prevent early HD-associated synaptopathy.


## 1. Introduction

Neurodegenerative disorders are characterized by progressive neuronal death, however substantial variability exists in the rate, magnitude and neuronal selectivity of cell death that occurs among different central nervous system conditions. Despite this variability, a recent surge in evidence indicates that early synaptic dysfunction can be detected well in advance of cell death in neurodegenerative disease (Brose et al., 2010; Selkoe, 2002; Marttinen et al., 2015). The emerging view that such diseases are initially disorders of the synapse, or "synaptopathies," is accompanied by the hypothesis that early interventions aimed to restore synaptic function are likely to provide greater therapeutic value than attempts to prevent cell death once neurons are severely compromised (Li et al., 2003). This highlights the importance of understanding the specific mechanisms of early synaptic dysfunction associated with neurodegenerative disease.

Huntington's disease (HD) is a neurodegenerative disorder that has strong evidence for synaptic dysfunction preceding cell death (Raymond et al., 2011; Li et al., 2003). HD is caused by a mutation in the huntingtin protein, which results in a toxic gain of function of the mutant protein and can also interfere with the normal physiological function of huntingtin (Cattaneo et al., 2005). Huntingtin is known to interact with a wide variety of intracellular proteins, many of which are responsible for synaptic neurotransmission (Shirasaki et al., 2012), and the mutation or altered expression of wild-type huntingtin can influence its interaction with, and the localization of, synaptic proteins (Fan et al., 2009; Parsons et al., 2014; Milnerwood et al., 2010; Smith et al., 2005a). The effects of mutant huntingtin are particularly striking in the striatum, and therefore, most studies have focused on the synaptic dysfunction that occurs at a major excitatory projection to the striatum; the cortico-striatal (C-S) synapse.

Cortical projections to striatal spiny projection neurons (SPNs), the most vulnerable cell type in HD, represent the predominant excitatory input to this region, and the majority of excitatory synapses in the striatum are comprised of vesicular glutamate transporter 1-expressing cortical terminals that synapse onto SPN dendritic spines (Deng et al., 2013). Our lab and others have studied this synapse in relative isolation

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**Available online on ScienceDirect (www.sciencedirect.com).**

http://dx.doi.org/10.1016/j.nbd.2015.12.009
0969-9961/

through the use of a cell co-culture model in which embryonic or early postnatal cortical neurons are simultaneously grown on coverslips with striatal neurons. Under such conditions, robust excitatory connectivity develops between the cortical and striatal neurons (Kaufman et al., 2012; Segal et al., 2003). As cell cultures are highly amenable to genetic manipulation and therapeutic screening, the C-S co-culture system may provide a useful platform for identifying both the mechanisms underlying HD-associated synaptic dysfunction as well as potential treatments that may prevent or restore these synaptic alterations. Indeed, in the YAC128 mouse model of HD, we have previously shown that the early increase in cell death-associated extrasynaptic N-methyl-D-aspartate (NMDA) receptors detected in brain slice is also recapitulated in this co-culture model, suggesting that at least some of the synaptic phenotype observed in animal models can be modeled in this isolated system.

From animal models, it has been reported that the HD mutation can also affect release probability at C-S synapses, promote the loss of C-S connectivity and SPNs dendritic complexity, and reduce cell survival signaling, in a disease stage-dependent manner (Lerner et al., 2012; Joshi et al., 2009; Klapstein et al., 2001; Steffan et al., 2000). Here, we used C-S co-cultures from wild-type (WT; FVB/N) and YAC128 mice to study C-S synaptic connectivity, SPN morphology and intracellular signaling as the co-cultures developed up to three weeks *in vitro*. Our results demonstrate that a number of HD-associated synaptopathic features are recapitulated in co-cultures within this three week period. We discuss the potential value of this system to unlocking the mechanisms and treatment of synaptic dysfunction in HD.

## 2. Materials and methods

### 2.1. Culture preparation

All procedures were approved and guided by the University of British Columbia Committee on Animal Care and Canadian Council on Animal Care regulations. Cultures were prepared on embryonic day 17–18 from wild-type (WT) FVB/N mice and/or transgenic YAC128 (line 55; FVB/N background) mice expressing full-length human huntingtin with 128 CAG repeats (Slow et al., 2003). Co-cultures were prepared and maintained, and striatal neurons were identified by transfecting a yellow fluorescent protein (YFP) marker at day of plating, as previously described (Milnerwood et al., 2012; Parsons et al., 2014). To assess the effects of mutant huntingtin expression when isolated to one neuronal-type, a chimeric co-culture strategy was employed, in which WT striatal neurons were co-plated with YAC128 cortical neurons, or vice versa.

### 2.2. Electrophysiology

Whole-cell patch-clamp recordings were performed with an Axopatch 200B amplifier and pClamp 10.2 software (Molecular Devices, Palo Alto, CA). YFP-expressing striatal SPNs were targeted for recording at various stages of co-culture development, which are described in the text. Cells were clamped at −70 mV and intrinsic membrane properties were determined by the current response to a 10 mV hyperpolarizing step applied immediately after achieving whole-cell access. For all experiments, a series resistance of up to 25 MΩ was tolerated, with the large majority of recordings under 20 MΩ. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in artificial cerebrospinal fluid (ACSF) containing (mM): 167 NaCl, 2.4 KCl, 10 Glucose, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, 0.05 picrotoxin (PTX; to block $GABA_A$ receptors), 0.0003 tetrodotoxin (TTX; to block action potentials), pH 7.3 with NaOH, 310–320 mOsm. The recording electrode (3–6 MΩ) was filled with internal solution containing (mM): 145 K-Gluconate, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 MgATP, 0.5 $Na_2GTP$, pH 7.3 with KOH, 280–290 mOsm. For miniature inhibitory postsynaptic currents (mIPSCs), cells were also clamped at −70 mV, but with a high chloride internal solution containing (mM): 145 CsCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 2 MgATP, 0.5 $Na_2GTP$, pH 7.3 with CsOH, 280–290 mOsm. The ACSF used for mIPSCs was as above, except with 10 μM DNQX (to block AMPA-type glutamate receptors) instead of PTX. Both mEPSCs and mIPSCs were recorded for approximately 2 min; typically 100–1000 consecutive events were analyzed per cell. To assess the size of the readily-releasable pool of excitatory vesicles (Rosenmund and Stevens, 1996), ACSF containing 500 mM sucrose was applied for 4 s through a theta tube placed in close proximity to the recorded cell. Recording conditions were as above for mEPSCs. The rate of replenishment of the readily-releasable pool was assessed by a second application of sucrose 3 s after the termination of the first application. All electrophysiological data were analyzed in Clampfit 10.2 (Molecular Devices, Inc.)

### 2.3. pCREB and synaptic protein immunofluorescence

To quantify nuclear localization of phosphorylated cAMP response element-binding protein (pCREB), cells were fixed at DIV14 or DIV21, as indicated, in 4% paraformaldehyde (PFA) + 4% sucrose for 15–20 min, washed with phosphate buffered saline (PBS), permeabilized with PBST (1% triton-X in PBS) and blocked for 45 min with normal goat serum (NGS; 10% in PBST). Cultures were then incubated with a mouse monoclonal anti-pCREB antibody (1:500, Millipore, 05-667) together with a chicken anti-GFP antibody (1:1000–1:2000, AbCam, ab13970) in 2% NGS-containing PBST at room temperature (RT) for 3 h under 40 rpm agitation. After thorough washing with PBST, cultures were incubated with goat anti-mouse Alexa 568 (1:1000, Invitrogen, A-11031) and goat anti-chicken (1:1000, Invitrogen, A11039) secondary antibodies in 2% NGS-containing PBST for 1.5 h at RT with 40 rpm agitation. Following another extensive wash with PBST, coverslips were subject to 10 min of Hoechst 33342 staining (5 μM; H21492, Invitrogen), followed by a final wash prior to mounting on glass slides (Corning, 2948-75 × 25) with Fluoromount-G (SouthernBiotech, 0100-01). Images were acquired with a Zeiss Axiovert 200 M fluorescence microscope (63× magnification, 1.4 NA) running ZEN 2012 software. Using a z-step of 0.23 μm, 10–15 images were taken per cell and the best 3–5 sections, including the focal planes of the cell body, were flattened using the extended focus function within the ZEN 2012 program. Flattened images were saved as TIFF files and imported into ImageJ, where they were analyzed for pCREB fluorescence intensity in the nucleus (defined by Hoechst staining) and in the cytosol (defined by the average of three regions of interest drawn within the YFP-filled cell body but outside of the Hoechst-stained nucleus; these were drawn to optimize coverage of the maximum cytoplasmic area). The nuclear-to-cytoplasmic (n/c) ratio was calculated by dividing the nuclear pCREB staining intensity by the average of the 3 regions of cytoplasmic pCREB staining intensity.

For synaptic protein staining, cells were live-stained for AMPA receptor subunit GluA2 using a previously published protocol (Wierenga et al., 2005). Live cells were incubated at 37 °C and 5% $CO_2$ with mouse anti-GluA2 antibodies (1:200; Millipore) diluted in plating medium for 1 h, followed by 1.8 mL of plating medium for another 1 h. Coverslips were then fixed with 4% PFA and 4% sucrose for 20 min and washed 3 times with PBS. Coverslips were blocked in PBS with 10% normal goat serum (30 min at RT) and incubated with secondary antibodies for 1.5 h at RT. The secondary antibody mixture consisted of Alexa Fluor 568-conjugated donkey anti-mouse (1:500; Invitrogen) diluted in PBS with 2% normal goat serum. Cells were washed 3 times with PBS and then permeabilized with methanol (5 min at −20 °C), washed 3 more times with PBS, and incubated in PBS with 0.03% Triton X-100. Coverslips were blocked in PBS with 10% normal goat serum (30 min at RT) and incubated with primary antibodies for 1 h at room temperature and then overnight at 4 °C. The primary antibody mixture consisted of guinea pig anti-VGLUT1 (1:1000; Millipore) and chicken anti-GFP (1:2000; Abcam) diluted in PBS with 2% normal goat serum. Coverslips were washed 3 times with PBS and then incubated with secondary antibodies for 1.5 h at room temperature. The secondary antibody mixture