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Research Paper

Altered glutamate response and calcium dynamics in iPSC-derived striatal neurons from XDP patients

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ABSTRACT

X-linked dystonia-parkinsonism (XDP) is a neurodegenerative disorder endemic to Panay Island (Philippines). Patients present with generalizing dystonia and parkinsonism. Genetic changes surrounding the TAF1 (TATA-box binding protein associated factor 1) gene have been associated with XDP inducing a degeneration of striatal spiny projection neurons. There is little knowledge about the pathophysiology of this disorder.

Our objective was to generate and analyze an *in-vitro* model of XDP based on striatal neurons differentiated from induced pluripotent stem cells (iPSC).

We generated iPSC from patient and healthy control fibroblasts (3 affected, 3 controls), followed by directed differentiation of the cultures towards striatal neurons. Cells underwent characterization of immunophenotype as well as neuronal function, glutamate receptor properties and calcium dynamics by whole-cell patch-clamp recordings and calcium imaging. Furthermore, we evaluated expression levels of AMPA receptor subunits and voltage-gated calcium channels by quantitative real-time PCR.

We observed no differences in basic electrophysiological properties. Application of the AMPA antagonist NBQX led to a more pronounced reduction of postsynaptic currents in XDP neurons. There was a higher expression of AMPA receptor subunits in patient-derived neurons. Basal calcium levels were lower in neurons derived from XDP patients and cells with spontaneous calcium transients were more frequent. Our data suggest altered glutamate response and calcium dynamics in striatal XDP neurons.

1. Introduction

X-linked dystonia-parkinsonism (XDP, DYT/PARK-TAF1, or DYT3 in the old nomenclature) is a rare neurodegenerative disorder initially restricted to the Island of Panay (Visayas, Philippines). Extremely rare on a global scale, the prevalence in the Philippines is 0.31 per 100,000 and as high as 5.74 per 100,000 on Panay Island (Lee et al., 2011). Thus, it represents a considerable challenge to the local healthcare system in this circumscribed region. The disease is characterized by an initial dystonic stage, followed by combined dystonia-parkinsonism, and eventually, a parkinsonian phase (Rosales, 2010). Histopathological studies proposed an initial loss of striatal GABAergic spiny projection neurons (SPN) in the striosomal compartment, followed by a degeneration of SPN throughout the entire striatum (Goto et al., 2005). The reason for this focal degeneration is unclear. The disease locus has been mapped to a ~ 427 kbp region on Xq13.1 (Domingo et al., 2015) Although no protein-coding genetic variation could be identified, five disease-specific single nucleotide changes (DSC1, DSC2, DSC3, DSC10, DSC12), one 48-bp deletion, and one 2.6-kbp retrotransposon insertion segregate in affected individuals (Domingo et al., 2015; Nolte et al., 2003). All changes lie either within intronic regions, intergenic DNA segments, or in a proposed non-conventional exon of the neighboring *TAF1* gene (*TATA-box binding protein associated factor 1*). Down-regulation of *TAF1* and retention of intron 32 (Aneichyk) have been proposed as disease mechanism (Herzfeld et al., 2013; Sako et al., 2011; Makino et al., 2007). However, it is unknown how reduced expression levels of this ubiquitously expressed gene lead to a tissue-specific degeneration of SPN. Recent data strongly support the pathogenicity of the SVA retrotransposon insertion in *TAF1* (Aneichyk et al., 2018; Rakovic et al., 2018). However, the complexity of the genetic variations

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in XDP and their uncertain effects complicate the generation of appropriate animal models.

Induced pluripotent stem cells (iPSC) are reprogrammed from patient specific somatic cells (Takahashi et al., 2007). Due to their pluripotency, the directed differentiation to the desired cell types (including striatal neurons) is possible (Carri et al., 2013; Stanslowsky et al., 2016a). The patient-specific neurons harbor all genetic changes inherent to the original cell type reprogrammed. Therefore, this approach seems particularly feasible for disease modeling when the causative genetic changes are unknown or unclear.

At present, there is no study available about the mechanisms leading to the neuronal degeneration in XDP.

We sought to analyze striatal neurons derived by directed differentiation from human iPSC lines. Three XDP patients and three ethnically- and age-matched healthy volunteers served as donors. We focused on functional analyses using whole-cell patch-clamp as well as calcium imaging with a special emphasis on the glutamatergic transmission and calcium dynamics. In addition, we measured expression levels of AMPA-receptor subunits and voltage-gated calcium channels (CaV) by quantitative real-time polymerase chain reaction (qPCR).

2. Methods

2.1. Generation and cultivation of human iPSC lines

Fibroblast cultures from three genetically proven and clinically affected XDP patients in their dystonic phase and three matched healthy volunteers from the Philippines (see Table 1 for control and patient details), who gave informed written consent according to the ethical regulations of the University of Lübeck, were included in this study (AZ12–219).

All parts of this project were approved by the ethical committee of the University of Lübeck and were conducted in full accordance to their regulations. Genotyping of patients, collection of skin biopsies and generation of fibroblast cultures were performed in a former study from our group (Domingo et al., 2016). Cells were kept in basic fibroblast medium (see supplementary Table 1 for an overview of media composition) in T-300 flasks (Cornig) and passaged by TrypLE (Thermo Fisher Scientific). Fibroblasts were reprogrammed integration-free by commercially available Sendai virus vectors according to the manufacturer's guidelines (CytoTune®-iPS Reprogramming Kit, Invitrogen). We picked emerging iPSC colonies manually and cultivated them on irradiated mouse embryonic fibroblasts (MTI-GlobalStem) in iPSC medium with 10 ng/µl of freshly added fibroblast growth factor 2 (FGF2, Millipore). Established iPSC lines were characterized by immunofluorescent stainings for pluripotent markers (for an overview of employed antibodies see supplementary Table 2). RNA was extracted from all iPSC clones as well as spontaneously differentiated embryoid bodies (EBs), transcribed into cDNA and analyzed by quantitative real-time PCR (please see below). Expression levels of iPSC lines were compared to fibroblasts for the pluripotency markers *NANOG*, *GDF3*, *OCT4*, and *SOX2* and relative values calculated. EBs, generated as published (Xu et al., 2001), were analyzed for the expression of germ layer markers, *i.e.* endoderm (*AFP*, *GATA4*, *SOX17*), mesoderm (*RUNX1*, *MSX1*, *MYH6*), and ectoderm (*NCAM*, *PAX6*). Relative expression levels of these values were calculated by comparing them to the levels of the initial iPSC lines. Evaluation of genomic stability of each iPSC line in comparison to the parental fibroblast line was accomplished by SNP analysis with the Infinium OmniExpress-24 beadchip (Illumina) according to the manufacturer's protocol. DNA was extracted from all iPSC clones and compared to the DNA of the respective fibroblast line. Retrieved data was analyzed using the Karyo Studio software (Illumina).

Table 1
Characteristics of control subjects and XDP patients as skin fibroblast donors in this study.

Controls:		
ID code	Sex	Age at biopsy
L8357	m	37
L8360	m	35
L8361	m	35

XDP patients:					
ID code	Sex	Age at biopsy	Age at onset	Disease duration (y)	Disease stage
L-5748	m	44	38	6	parkinsonian > dystonic
L-7994	m	37	33	4	parkinsonian > dystonic
L-7149	m	51	48	3	dystonic > parkinsonian

2.2. Directed differentiation of iPSC lines to striatal neurons

For the striatal differentiation of iPSC, we adapted an already established protocol recently published elsewhere (Stanslowsky et al., 2016a): After manual removal of areas suspicious of spontaneous differentiation, iPSC colonies were incubated with collagenase IV (Gibco) for 10 min at 37 °C. Colonies were removed from the culture dish, triturated carefully to smaller pieces and plated on ultra-low attachment culture plates (Cornig) for the formation of free-floating aggregates known as EB. EBs were kept for two days in knockout serum replacement (KSR) medium with the addition of the following small molecules: The effectivity of neural induction was enhanced by 100 nM LDN-193189 (Stemgent) and 10 µM SB-431542 (Tocris) from day 0–6 (Chambers et al., 2009). The Rho kinase inhibitor Y-27632 (Stemcell Technologies) was added at a concentration of 10 µM for the enhancement of cell survival during the first two days (Ungrin et al., 2008). Rostral patterning was enhanced by the addition of 1 µM of the antagonist at the WNT-pathway IWP-2 (Stemcell Technologies) from day 0–10, while 0.2 µM of the smoothened agonist Purmorphamine from day 4–10 enhanced ventral patterning (Li et al., 2009). Medium was changed to KSR/N2 medium 1:1 on day two. From day 4 onwards, EBs were kept in N2-medium until day 12. EBs were reduced in size by careful trituration and plated on 2.5% Matrigel (MG, Cornig) coated 12-well plates in N2B27 medium with BDNF 20 ng/ml (PeproTech), GDNF 10 ng/ml (PeproTech) and db-cAMP 50 µM (Enzo Life Sciences). Each time when reaching full confluency (~ day 20 and day 40), cells were removed mechanically from the culture dish, triturated to smaller clumps and replated on matrigel (MG) coated culture ware. For terminal differentiation, aggregates of neural cells were plated on poly-D-lysine (Sigma Aldrich) and laminin (Roche) coated coverslips (Hecht). For enhanced survival, at each passaging step, 10 µM of Y-27632 were added to the medium. Medium was changed every other day. Cells were analyzed after 90 days. For an overview of the protocol, see Fig. 2a. Detailed media composition is listed in supplementary Table 1. We performed a total of two differentiations of each iPSC line (3 cell lines each for XDP and controls).

2.3. Immunofluorescent staining and microscopy

Immunofluorescent stainings were performed at three time points: 1. After the first replating step at day 20 in order to evaluate early transcription factors associated with ventral forebrain identity. 2. After day 90 to quantify the amount of terminally differentiated SPN. 3. After whole-cell patch-clamp recordings to quantify the amount of correctly identified SPN (see below).

Cells on coverslips were fixed for 15 min in 4.5% freshly prepared paraformaldehyde (PFA, Sigma-Aldrich). After washing cells 3× for 15 min with phosphate-buffered saline (PBS, Gibco), unspecific