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HUMAN t-DARPP IS INDUCED DURING STRIATAL DEVELOPMENT

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Abstract—Human Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32, also known as *PPP1R1B*) gene codes for different transcripts that are mainly translated into two DARPP-32 protein isoforms, full length (fl)-DARPP-32 and truncated (t)-DARPP. The t-DARPP lacks the first 36 residues at the N-terminal, which alters its function. In the central nervous system, fl-DARPP-32 is highly expressed in GABAergic striatal medium spiny neurons (MSNs), where it integrates dopaminergic and glutamatergic input signaling. However, no information about human DARPP-32 isoform expression during MSNs maturation is available. In this study, our aim is to determine the expression of the two DARPP-32 isoforms in human fetal and adult striatal samples. We show that DARPP-32 isoform expression is differentially regulated during human striatal development, with the t-DARPP isoform being virtually absent from whole ganglionic eminence (WGE) and highly induced in the adult striatum (in both caudate and putamen). We next compared the four most common anti-DARPP-32 antibodies used in human specimens, to study their recognition of the two isoforms in fetal and adult human striatal samples by western blot and immunohistochemistry. The four antibodies specifically identify the fl-DARPP-32 in both fetal and adult samples, while t-DARPP form was only detected in adult striatal samples. In addition, the lack of t-DARPP recognition in human adult striatum by the antibody generated against the full-length domain produces in turn different efficacy by immunohistochemical analysis. In conclusion, our results show that expression of human DARPP-32 protein isoforms depends on the striatal neurodevelopmental stage with t-DARPP being specific for the human adult striatum.

Key words: DARPP-32, striatum, medium spiny neurons, human development, Huntington's disease, neurodevelopment.

INTRODUCTION

Human Dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (*DARPP-32*), also known as Protein phosphatase 1 (PP1) regulatory subunit 1B (*PPP1R1B*), is a phosphorylation-dependent inhibitor regulated by cyclic nucleotide dependent protein kinases (Yger and Girault, 2011). Its gene codes for alternative splice transcripts that are translated in two main protein isoforms, the full length DARPP-32 (204 a.a.) and the truncated (t)-DARPP (168 a.a.) (El-Rifai et al., 2002). The t-DARPP protein isoform lacks the aminoacidic sequence 1-36 at the N-terminus (El-Rifai et al., 2002), determining the loss of its function as a PP1 or PKA inhibitor, although the consequences of this for cell signaling are unclear. Interestingly t-DARPP is associated with tumorigenesis, inducing cell proliferation and resistance to apoptosis both *in vitro* and *in vivo* (El-Rifai et al., 2002; Vangamudi et al., 2010; Christenson and Kane, 2014; Denny and Kane, 2015), whereas in the central nervous system, t-DARPP increase in dorso-lateral prefrontal neocortex and hippocampus has been implicated in the pathogenesis of schizophrenia and cognitive disorders (Svenningsson et al., 2004; Kunii et al., 2011).

Within the brain, DARPP-32 is mainly expressed by regions receiving dopaminergic projection, such as *caudate*, *putamen*, *nucleus accumbens*, cerebral and cerebellar cortex (Ouimet et al., 1984; Berger et al., 1990; Brené et al., 1994, 1995). Neuronal DARPP-32 protein works as functional integrator of glutamatergic and dopaminergic inputs (Svenningsson et al., 2004). In fact, dopamine or glutamate stimulation induces differential signaling cascades that trigger alternative phosphorylation pattern of Thr 34 and Thr 75 residues of DARPP-32 protein, regulating the ability of DARPP-32 to interact with multifunctional serine/threonine phosphatases (Bibb et al., 1999; Svenningsson et al., 2004; Fernandez et al., 2005; Yger and Girault, 2011).

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Abbreviations: aa, aminoacids; HD, Huntington's disease; hPSC, human pluripotent stem cell; MSNs, medium spiny neurons; p.c.w., post conception weeks; pcd, post-conception days; pmd, post-mortem delay; WGE, whole ganglionic eminence.

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Table 1. Human tissue information. PMD = post mortem delay; pcd = post conceptional days; y.o. = years old; n.a. = not available

Gender	Age	PMD	Tissue
n.a.	49 pcd	n.a.	WGE, Cortex
n.a.	51 pcd	n.a.	WGE, Cortex
n.a.	53 pcd	n.a.	WGE, Cortex
n.a.	53 pcd	n.a.	WGE
n.a.	53 pcd	n.a.	WGE, Cortex
n.a.	55 pcd	n.a.	WGE
n.a.	57 pcd	n.a.	WGE
n.a.	59 pcd	n.a.	WGE, Cortex
n.a.	61 pcd	n.a.	WGE, Cortex
n.a.	63 pcd	n.a.	WGE, Cortex
Male	31 y.o.	17 h 30 min	Caudate, Putamen, Motor cortex, Hippocampus, Cerebellum
Male	39 y.o.	3 h 30 min	Caudate, Putamen, Motor cortex, Hippocampus
Female	60 y.o.	15 h 30 min	Caudate, Putamen, Motor cortex
Male	64 y.o.	3 h 30 min	Caudate, Putamen, Motor cortex, Hippocampus
Female	68 y.o.	13 h	Caudate, Putamen, Motor cortex, Cerebellum
Female	71 y.o.	8 h 30 min	Caudate, Motor cortex, Hippocampus,
Female	81 y.o.	23 h 30 min	Caudate, Putamen, Motor cortex, Cerebellum
Female	86 y.o.	12 h 20 min	Caudate, Putamen

Human neostriatum is anatomically composed of the caudate and putamen nuclei and is mainly populated by GABAergic medium-sized spiny neurons (MSNs), which highly express DARPP-32 from an early stage of fetal development (Hemmings and Greengard, 1986; Kang et al., 2011; Onorati et al., 2014). Furthermore, MSNs are the most vulnerable type of neurons in Huntington's disease (HD) (Graveland et al., 1985; Vonsattel et al., 1985), an autosomal dominant monogenic disease. In recent years stem cell research has attempted to generate MSNs by differentiating human pluripotent stem cell (hPSC) in order to study HD pathogenesis, as well as to generate donor cells for cell replacement, and for drug screening. The use of DARPP-32 as specific MSN marker has been crucial to follow the generation of hPSC-derived MSNs (Aubry et al., 2008; El-Akabawy et al., 2011; Carri et al., 2012; Ma et al., 2012; Straccia et al., 2015; The HD iPSC Consortium, 2012; Victor et al., 2014). However, to our knowledge no information about striatal DARPP-32 isoform expression in human MSNs development is available in the literature.

In this study, we demonstrated that the expression of DARPP-32 transcript variants and the respective protein isoforms has a time-dependent regulation. In addition, we highlighted that the four antibodies are not equivalent in recognizing DARPP-32 isoforms in adult human putamen and caudate nuclei. In conclusion, we propose t-DARPP as more specific marker to identify fully mature MSNs.

EXPERIMENTAL PROCEDURES

Human brain tissue

Adult motor neocortex, hippocampus, cerebellum, caudate and putamen samples were obtained from the Neurological Tissue Bank of the Biobank-Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS; Barcelona, Spain; URL: www.clinicbiobanc.org), following the guidelines and approval of the local ethics committee. DARPP-32 positive cells are detected in human fetal forebrain, specifically in ganglionic eminence post-mitotic areas, in fetuses older than six post conception weeks (p.c.w.) (Naimi et al., 1996). Consequently fetuses (CRL of 22–54 mm) ranging in age from 7 to 9 p.c.w were collected by donation of the elective termination of pregnancy through the South Wales (UK) Initiative for Fetal Tissue Transplantation (''SWIFT'') program, with full ethical approval (02/4446), as source of MSNs progenitors (Kopyov et al., 1998; Hauser et al., 2002; Rosser et al., 2002). For mRNA studies, neocortical and striatal primordia were processed as previously published (Kelly et al., 2011). Further details on human tissue used in this study are in Table 1 and previously published (Straccia et al., 2015).

RNA isolation, retrotranscription and quantitative real time PCR

Total RNA was isolated using TRI Reagent (Sigma–Aldrich T9424) following the manufacturer's protocol. In brief, 1 μg of RNA for each condition was reverse transcribed using PrimeScript RT reagent kit (Takara, RR037A). cDNA was diluted to 5 ng/μL and 2 μL were used to perform quantitative real-time PCR (qPCR). PrimeTime qPCR assays for *PPP1R1B* isoforms (see Table 2) were used as recommended by provider (IDT technologies). *B2M* (NM_004048; Hs.PT.39a.22214845), *HPRT1* (NM_000194; Hs.PT.51.2145446) *RPL13A* (NM_012423; Hs.PT.51.21531404) and *HSP90AB1* (NM_007355; IDT Hs.PT.56a.38913643.gs) mRNA levels were not altered (data not shown) and were used as reference genes. QPCR was carried out with Premix Ex Taq (Takara, RR390A) in 6 μL of final volume using CFX384-C1000 Thermal Cycler equipment (Bio-Rad). Samples were run for 40 cycles (95 °C for 5 s, 60 °C for 20 s). Relative gene expression values were calculated using Bio-Rad CFX manager software (Bio-Rad).

Table 2. QPCR probes used to detect the specific DARPP-32 transcript variants

Description	IDT Technologies reference	Exons detection	Variants detection	Amplicon
DARPP-32	Hs.PT.56a.2727270	1-3	1	127 bp
t-DARPP	Hs.PT.56a.23169336	1b-5	2-3	138 bp
Total DARPP-32	Hs.PT.56a.40063116.g	5-6	1-2-3	135 bp

Table 3. List of the comprehensive revision of the anti-DARPP-32 antibodies used for immunodetection of human DARPP-32 in the literature. The antibodies used in this study are highlighted in bold. Ordered by provider name. (aa = aminoacids; FACS = Fluorescence-activated cell sorting; IHC = Immunohistochemistry; ICC = Immunocytochemistry; MSN = Medium spiny neurons; n.s. = not specified; hPSC = human pluripotent stem cells)

Clone	Epitope	Species	Provider	Catalog n°	Assay	Human Sample	References
15	aa: 70-181	Human	BD	611520	IHC	Adult brain	Brito et al. (2013)
19A3	Glu160 region	Human	Cell Signaling	2306	ICC	hPSC-derived MSNs	The HD iPSC Consortium (2012)
n.s.	n.s.	n.s.	Cell Signaling	n.s.	IHC, FACS	Adult brain	Ernst et al. (2014)
n.s.	aa: 23-37	synthetic	Chemicon	AB1656	ICC	STROC05-derived MSNs	Ishikawa et al. (2007); El-Akabawy et al. (2011)
n.s.	n.s.	n.s.	Chemicon/ Merck Millipore	n.s	ICC	hPSC-derived MSNs	Ma et al. (2012)
EP720Y	N-terminus	Human	Epitomics/ Abcam	Ab40801	IHC; ICC	Fetal brain, hPSC-derived MSNs	Carri et al. (2012), Onorati et al. (2014)
H-62	aa: 134-196	Bovine	Santa Cruz	SC-11365	IHC	Fetal brain	The HD iPSC Consortium (2012), Pauly et al. (2013)
15	aa: 70-181	Human	Santa Cruz	SC-135877	IHC	Fetal brain	Onorati et al. (2014)
n.s.	n.s.	n.s.	Santa Cruz	n.s.	ICC	hPSC-derived MSNs; Fibroblast-derived MSNs	Aubry et al. (2008), Victor et al. (2014)
n.s.	n.s.	n.s.	Santa Cruz	n.s.	IHC	Adult brain	Kunii et al. (2011)

Table 4. Description of the four anti-DARPP-32 antibodies selected for the study. (n.a. = not available)

Manufacturer		Cell Signaling	Santa Cruz	BD Laboratories	Abcam
Reference		2306	SC-11365	611520	Ab40801
Clone		19A3	H-62	15/DARPP-32	EP720Y
Source		Rabbit IgG	Rabbit IgG	Mouse IgG1	Rabbit IgG
Peptide antigen		Glu160 region	aa 134-195	aa 70-181	N-terminus
Origin		Human	Bovine	Human	Human
Mono/Poly-clonal		Monoclonal	Polyclonal	Monoclonal	Monoclonal
WB Dilution (final titer)	Fetal	1/100	1/1000	1/100	1/5000
	Adult	1/1000 (n.a.)	1/10000 (200 ng/mL)	1/1000 (250 ng/mL)	1/50,000 (n.a.)

Protein extraction and Western Blot

Total protein extract was isolated from human tissue using TRI Reagent (Sigma–Aldrich T9424) according to the manufacturer's protocol. Total protein extracts were denatured using 2.5 mM dithiothreitol at 95 °C for 5 min and then 10–40 μg of each denatured samples was subjected to 12% SDS–PAGE and transferred to a nitrocellulose membrane (Millipore, IPVH00010) for 60 min at 2,5 mA/cm^2 as described previously (Straccia et al., 2013). Membranes were incubated with the DARPP-32 primary antibodies as indicated in Table 4 and monoclonal mouse anti-Tubulin (Sigma–Aldrich, T9026), diluted in immunoblot buffer (TBS containing 0.05% Tween-20 and 5% no-fat dry milk). For antibodies comparative analysis to minimize technical variability, 20 μg of total protein tissue extract from the same donor were loaded in each of the four lanes of the SDS–PAGE. To show similar signals, western blot exposures times were calibrated; for WGE samples, 19A3, EP720Y and H62 were exposed for about 10 s, while clone 15 (BD 611520) was exposed 3 min; for adult samples, 19A3, EP720Y, H62 and 15 anti-DARPP-32 antibodies clones were exposed for about 2 s, 10 s, 12 s and 3 min, respectively. For competition assay, 20 μg of WGE and striatum were separated by 10% SDS–PAGE and immunodetection was performed in parallel incubating the antibody with or without the blocking peptide. Anti-DARPP32 antibody (Abcam Ab40801) was diluted in immunoblot buffer with or without the presence of 1 μg/ml Blocking Peptide (Abcam, Ab189245). Anti-rabbit (Promega, W401B) and

Table 5. Immunohistochemical conditions tested with the four selected antibodies to detect DARPP-32 protein in adult human brain tissue. Antibodies anti-DARPP-32 have been tested in three different human brain tissue preparations (Paraffin-embedded, Cryoprotected and Frozen samples). Paraffin-embedded samples have undergone four different unmasking methods (antigen retrieval). Different antibody dilutions have been tested in all the previous conditions. (+ + = very good staining; + = good staining; n.d. = not detectable; pale = not well contrasted compared to the background; * = background signal interferes)

Samples preparation		Paraffin embedded								Cryoprotected			Frozen		
Antigen unmasking method		Citrate pH 6		Formic acid			EDTA pH9 ± Formic acid			no pre-treatment			no pre-treatment		
Dilution		1/50	1/250	1/30	1/250	1/500	1/30	1/100	1/250	1/500	1/30	1/250	1/500	1/30	1/250	1/500
Cell Signaling 2306	19A3	+ +	+ +			+ pale				+ + / + +			+ pale			+ pale
Santa Cruz sc-11365	H62	+ +	+ +		+ pale			* + / +				+			+	
BD 611520	15	+ +	+ +		+ pale				+ + / + +			+			+	
Abcam Ab40801	EP720Y	n.d.	n.d.	n.d.			n.d.				n.d.			n.d.		

* Background.

Anti-mouse (Promega, W402B) HRP-conjugated secondary antibodies were used at 1:5000 dilution. Chemiluminescent detection was performed incubating for 2 min with Luminata Classico western HRP substrate (Millipore, WBLUC500) and exposing for 1–10 s with Fuji Medical X-Ray Film Super RX-N (Fujifilm, 47410 19289).

We performed western blots quantification by ImageJ Gel Analysis plug-in on digital acquired films.

Immunohistochemistry

Five-μm thick sections were obtained from deep-frozen tissue blocks (−80 °C), cryopreserved tissue (fixed for 24 h in 4% paraformaldehyde followed by immersion in 30% sucrose for 24 h and frozen at −80 °C), and 4% formalin-fixed and paraffin-embedded tissue blocks from the putamen and caudate nucleus of a neurologically healthy brain donor at the Neurological Tissue Bank of the Biobank-Hospital Clinic-IDIBAPS, after obtaining informed consent for the use of brain tissue for diagnostic and research purposes. Immunohistochemistry was performed on an automated immunostainer (DAKO autostainer plus) using the antibodies in Table 5. Immunoreaction was visualized using the DAKO Envision system. For antigen retrieval, paraffin sections were dewaxed and several pre-treatment methods were tested: immersion in 98% formic acid for 5 min, boiling of sections in a microwave for 20 min in citrate buffer at pH 6 or in EDTA at pH 9, or a combination of both (98% formic acid + citrate buffer pH 6 or EDTA pH 9). The best results were obtained by boiling the sections in a microwave for 20 min in citrate buffer at pH 6. The following lots of Anti-DARPP-32 EP720Y clone were used: GR96296-1 and GR96296-7 from Abcam Ab40801; Y1032103D2 from Epitomics cat. n°1710-1.

Sampling and statistics

Caudate and putamen samples from the same donor did not show significant differences in our analysis, for this reason they are considered as one biological sample after within-patient averaging. Data were analyzed using GraphPad Prism version 6.0c for Mac, GraphPad Software, La Jolla, California, USA (www.graphpad.com). Samples were tested for the normality and equality of variance. Unpaired two-tailed Mann–Whitney non-parametric test has been performed to test independent observations between two biological groups. Each biological group is composed of different donors' samples. Values of $p < 0.05$ were considered statistically significant.

RESULTS

DARPP-32 transcript variants in adult human brain regions

DARPP-32 expression has been largely characterized in mouse CNS development (Foster et al., 1988); however, its characterization in human development has been less explored due to specimens availability. In adult human post-mortem brain, DARPP-32 mRNA is highly expressed

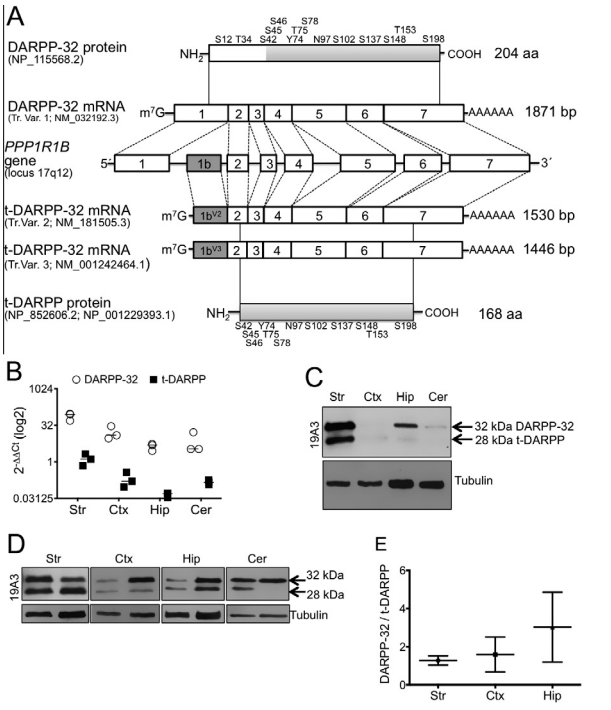

Fig. 1. (A) Schematic representation of *PPP1R1B* (DARPP-32) gene structure and its mRNA transcripts with the respectively translated protein isoforms. Human DARPP-32 and t-DARPP proteins are represented with their modification sites as predicted by PhosphoSitePlus (Hornbeck et al., 2015). (B) QPCR analysis of PPP1R1B splice variants expressed in human striatum (Str), motor neocortex (Ctx), hippocampus (Hip) and cerebellum (Cer). Individual values are plotted with median. (C) A representative western blot of three independent experiments is presented to show the relative DARPP-32 isoforms at protein level between the human brain regions under consideration (anti-DARPP-32 antibody, Clone 19A3). Ratio of DARPP-32/t-DARPP isoforms in human brain regions is analyzed by western blot (a representative one in D) for striatum (n = 4), motor neocortex (n = 4), hippocampus (n = 3) and cerebellum (n = 3). Quantification is showed as scatter dot plot in E.

in basal ganglia and choroid plexus than in neocortex and hippocampus, with a specific expression also in the Purkinje layer of cerebellum (Brené et al., 1994). DARPP-32 gene, namely PPP1R1B, has eight exons and codes for three different transcript variants (Fig. 1A). Full length DARPP-32 mRNA (transcript variant 1; NM_032192.3) does not include exon 1b and is translated into full length DARPP-32 protein of 204 aminoacids (aa). Transcript variants 2 (NM_181505.3) and 3 (NM_001242464.1) include the exon 1b with exon 1 being skipped. The two transcript variants differ on their Transcription Start Site in exon 1b ($1b^{V2}$ versus $1b^{V3}$). However both variants translation begins in the same ATG located in exon 2, giving rise to the same truncated DARPP-32 protein (t-DARPP) of 168 aa. The expression levels for DARPP-32 transcript variants in adult human brain have been investigated in dorso-lateral prefrontal neocortex, hippocampus and caudate (Kunii et al., 2014). However, a direct comparison between the DARPP-32 transcripts or protein isoforms was not described as we characterize in the present work.

To analyze the levels of mRNAs that are translated in the two different DARPP-32 protein isoforms, we have used three specific qPCR probe-based assays that recognize transcript variant 1 (DARPP-32), transcript variants 2-3 (t-DARPP) and all transcript variants (total DARPP-32), respectively (Table 2). Hence we show the levels of the DARPP-32 transcript variants in adult human motor neocortex, hippocampus, striatum (caudate-putamen nuclei) and cerebellum (Fig. 1B). No statistical differences in the expression of both transcript variants were observed between the adult caudate and putamen regions, indicating similar regulation of PPP1R1B gene expression. For this reason we refer them as striatum from now onward. Motor neocortical (DARPP-32 = 17,16 ± 10,85; t-DARPP = 0,21 ± 0,14), hippocampal (DARPP-32 = 4,45 ± 1,42; t-DARPP = 0,05 ± 0,01) and cerebellar (DARPP-32 = 7,80 ± 7,59; t-DARPP = 0,15 ± 0,03) samples displayed similar gene expression levels for both DARPP-32 transcript variants. On the other hand, striatum showed higher mRNA levels for DARPP-32 transcripts (DARPP-32 = 79,31 ± 25,63; t-DARPP = 1, 38 ± 0,72). To verify this result at protein levels, we determine the relative contribution of DARPP-32 protein isoforms by western blot analysis (Fig. 1C). Striatum showed higher expression of both DARPP-32 isoforms compared to the other regions mirroring the gene expression analysis. We extended the DARPP-32 protein isoforms analysis in order to determine the relative ratio (t-DARPP/DARPP-32) in each region, since it could be important for the cellular signaling integration (Fig. 1D and E). Striatum showed a consistent ratio of about 1:1 between isoforms compared to the other brain regions, where DARPP-32 isoform ratio was more variable. Only in one out of three cerebellar samples we could detect some t-DARPP.

The t-DARPP isoform is highly induced in human adult striatum

Next we characterized total *DARPP-32* gene expression in human striatal development, specifically in whole ganglionic eminence (WGE) at 49 and 63 days post-conception (pcd) and in post-mortem striatum of individuals between 31 and 71 years old. Human WGE showed detectable mRNA expression of total DARPP-32 (Fig. 2A) as already demonstrated elsewhere by immunocytochemistry (Onorati et al., 2014). In addition, we showed that total *DARPP-32* mRNA levels were significantly increased (p < 0,0001; two-tailed unpaired Mann–Whitney test) of about 18 folds in the adult striatum (n = 6) relative to WGE (n = 12) (Fig. 2A).

We also compared the mRNA levels of full length DARPP-32 (tr. var. 1) and t-DARPP variants (tr. var. 2-3) in human WGE versus adult striatum (Fig. 2B), using a probe recognizing all DARPP-32 mRNAs (tr. var. 1-2-3) as reference for normalization (Table 2). DARPP-32 transcript variant was significantly up-regulated (p = 0,0002; two-tailed unpaired Mann–Whitney test) in adult striatum (n = 6) compared to WGE (n = 12), which showed low expression of DARPP-32 gene. Interestingly t-DARPP transcripts expression is proportionally more highly induced (fetal mean Ct = 33,9 ± 4,0; adult mean Ct = 26,0 ± 1,9) in adult

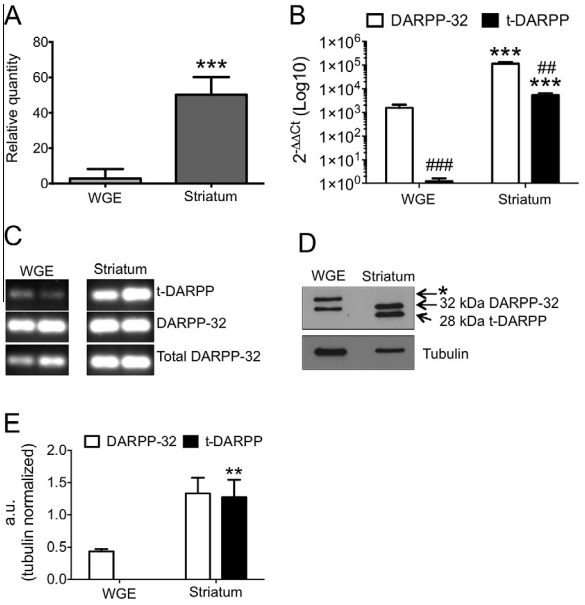

Fig. 2. QPCR analysis of total PPP1R1B gene expression in human WGE and adult striatum (A); $^{***}p < 0{,}001$ when comparing total DARPP-32 gene expression between WGE and striatum. Analysis of expression of the specific DARPP-32 transcript variants by qPCR in WGE and striatum (B); $^{***}p < 0{,}001$ when comparing the same transcript variant between WGE and striatum; $^{\#\#}p < 0{,}01$ $^{\#\#\#}p < 0{,}001$ when comparing DARPP-32 versus t-DARPP in the same stage. Representative semi-quantitative PCR analysis of PPP1R1B splice variants in two samples per each developmental stage (C). A representative western blot of four independent experiments shows protein expression of DARPP-32 isoforms in human WGE and adult striatum (D). Cell Signaling anti-DARPP-32 antibody (Clone 19A3, catalog n°2306) also detected an unidentified reactive (*) band in WGEs. Tubulin was used as loading control to perform relative quantification (E), $^{**}p < 0{,}01$ when comparing t-DARPP between WGE and striatum. Error bars are presented as standard error of the mean (SEM).

striatum compared to full-length DARPP-32 variant (fetal mean Ct = 27,5 ± 3,7; adult mean Ct = 21,5 ± 1,4) (Fig. 2B and C) ($p = 0{,}0001$; two-tailed unpaired Mann–Whitney test). However the relative quantity of DARPP-32 (tr. var. 1) was significantly higher compared to t-DARPP (tr. var. 2-3) mRNAs levels in both WGE ($p < 0{,}0001$; two-tailed unpaired Mann–Whitney test) and adult striatum ($p = 0{,}0022$; two-tailed unpaired Mann–Whitney test). To further study if these transcriptional changes during human striatal development were mirrored at protein level, we analyzed DARPP-32 isoforms by western blot (Fig. 2D). The t-DARPP isoform was not detected in any WGE sample (Fig. 2D), while in adult human striatal samples its expression was significantly induced ($p < 0{,}0091$; two-tailed unpaired Mann–Whitney test; Fig. 2E). T-DARPP has no significant differences with the full-length DARPP-32 protein isoform levels in adult striatum (Fig. 2D and E). Furthermore DARPP-32 isoform was not significantly increased ($p = 0{,}1$; two-tailed unpaired Mann–Whitney test) in adult human striatum ($n = 9$) compared to WGE ($n = 4$) (Fig. 2D and E), discordantly to its transcript up-regulation.

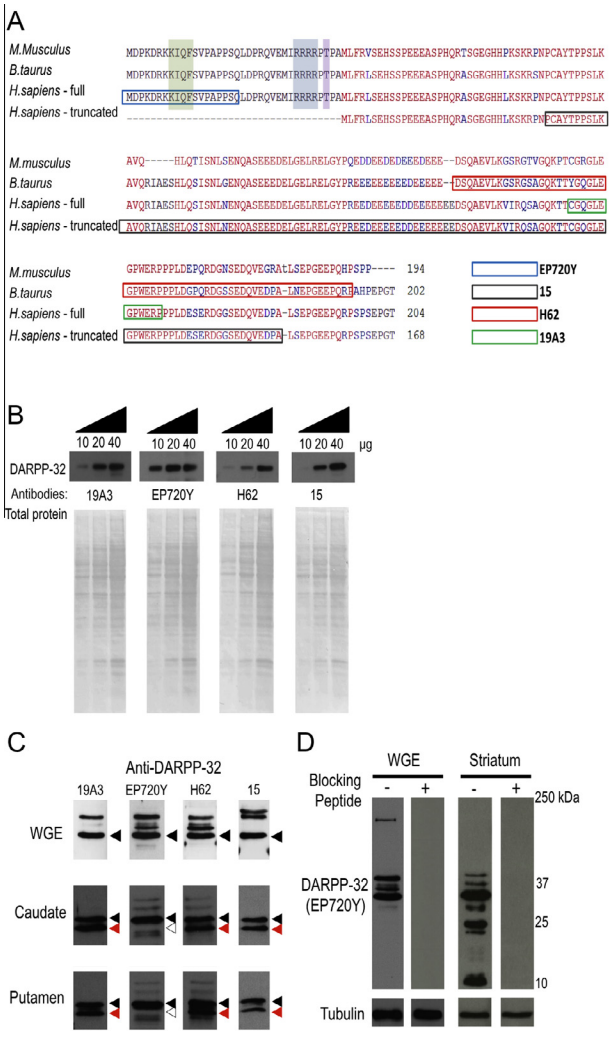

Fig. 3. Tested antibodies are not equivalent for the detection of both DARPP-32 protein isoforms. The alignment of mouse, bovine and human protein sequences shows at the N-terminus the conserved RVxF domain (shaded green), the potential PP-1 interaction domain (shaded blue) and the Thr 34 (shaded violet) (A). Orthologs conserved aa are depicted in red, while the different aa between species are in blue. Blue box shows the epitope for the Abcam Ab40801 antibody at the N-terminus of DARPP-32 full-length isoform. BD 611520 (black box), Santa Cruz SC-11365 (red box) and Cell Signaling 2306 (green box) epitopes are localized in the shared aminoacidic sequence of DARPP-32 and t-DARPP (A). Immune-detection signals by Western blot (B) of different amounts of loaded protein (10–40 μg) from the same original human adult cerebellar sample with the four different antibodies anti-DARPP-32 under analysis. Antibodies incubation conditions are indicated in Table 4. Ponceau-S staining of blotted membrane showed the total amount of protein transferred from each lane of the 12% SDS–PAGE. (C) Western blot comparative analysis of the four antibodies under consideration in WGE or adult striatal sample. Representative of five independent experiments. Adult caudate nuclei and putamen express both full-length DARPP-32 (red arrow head) and t-DARPP (black arrow head). However Abcam Ab40801 clone EP720Y was not able to identify t-DARPP (white arrowhead). Competition assay for EP720Y clone (Abcam Ab40801) (D); the presence of the blocking peptide abrogated any bands immunoreactivity due to anti-DARPP-32 incubation. Tubulin immunodetection has been used as control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Antibodies against DARPP-32 are not equivalent for detection of DARPP-32 isoforms in adult human neostriatum

T-DARPP is highly induced in adult human striatum as we show in Fig. 2B. In view of this we tested different antibodies to verify their efficacy in detecting DARPP-32 isoforms. We reviewed the literature searching for anti-DARPP-32 antibodies used in human specimens (Table 3). We found only five antibodies properly referenced (Carri et al., 2012; The HD iPSC Consortium, 2012; Brito et al., 2013; Pauly et al., 2013; Onorati et al., 2014); two of them were raised against the same epitope, so we finally chose four anti-DARPP-32 antibodies from different providers to test (Table 4). The four antibodies that we selected are generated using different antigenic peptides (Tables 3 and 4), one of them being a bovine sequence (Clone H62, Santa Cruz). The alignment of mouse, bovine and human protein sequences with different color boxes (Fig. 3A) indicates the distinct epitopes that are recognized by the four antibodies under consideration. Anti-DARPP-32 clone EP720Y (Ab40801, Abcam) recognizes the N-terminus of DARPP-32 and therefore, it cannot detect the t-DARPP.

We first compared the anti-DARPP-32 antibodies' sensitivity (Fig. 3B). Anti-DARPP-32 clone EP720Y showed the highest sensitivity detecting 10 micrograms of total protein extract with strong signal, followed by clone 19A3, H62 and 15, respectively. Thereafter, we tested the four antibodies by western blot in human total protein tissue extract of WGE and adult caudate and putamen nuclei. WGE samples ($n = 5$ of different donors) did not show detectable t-DARPP protein (Fig. 3C), while all four antibodies detected DARPP-32 protein in fetal samples (Fig. 3C). Adult human caudate and putamen nuclei showed protein expression of both DARPP-32 and t-DARPP (Fig. 3C). However, as predicted by the alignment (Fig. 3A), clone EP720Y did not detect t-DARPP neither in caudate nor in putamen total protein extract.

Several other protein bands were immune detected with a similar pattern by the four antibodies in all different samples we tested, with the EP720Y clone being the antibody with the greatest number of unidentified immunoreactive signals (Fig. 3D). We next examined if those signals could be due to secondary antibody cross-reaction. In order to verify this possibility we performed a competition assay for the fetal WGE and the adult human striatal tissues. The presence of the immunogenic peptide completely blocked the immunodetection of any protein bands (Fig. 3C), suggesting that the EP720Y antibody detects the other protein signals as specific by western blot. Further experiments are required to determine the identity of these proteins.

Anti DARPP-32 antibodies are not equivalent for immunostaining detection of DARPP-32 in adult human striatal MSNs

So far we have demonstrated that adult human striatal tissues express high levels of both DARPP-32 protein isoforms in contrast with WGE. Furthermore, we showed that the four antibodies against DARPP-32 are not equivalent by western blot. Next, we tested the efficacy of these antibodies in identifying MSNs in adult human striatum in several different conditions by immunohistochemistry (Table 5). EP720Y anti-DARPP-32 antibody failed to stain MSNs or any other type of cells in all the tested conditions in adult post-mortem brain samples. To confirm this result and to exclude any other technical issue related to the specific batch of EP720Y antibody, we tested three different batches with the same negative outcome. On the other hand, the other three DARPP-32 antibodies specifically stained MSNs showing clear staining of MSNs' soma and fibers through-out the striatal parenchyma. Finally, we chose as the best condition human paraffin-embedded samples treated with citrate buffer at pH = 6 (Fig. 4A, B and Table 5). With these conditions, clones 19A3, H62 and 15 of anti-DARPP-32 antibodies stained with different efficacy the adult human striatal MSNs (Fig. 4A). All three antibodies neatly identified DARPP-32 on the cell bodies of MSNs in both putamen and caudate nuclei (Fig. 4B). Clone 19A3 staining is more intense and sharp, allowing to perfectly distinguishing projections and processes in continuity with the MSN soma. Clones H62 and 15 show more similar staining patterns, less intense and contrasted with the background that allows appreciation of the nucleus within the neuronal body.

DISCUSSION

Deciphering the differential spatio-temporal expression of DARPP-32 isoforms helps to better understand the integration of glutamate and dopamine signaling during human neurodevelopment. At the cellular level, it is important to understand how DARPP-32 isoforms affecting cell physiology, especially in MSNs where DARPP-32 is highly expressed. In recent years, stem cell research has attempted to generate *in vitro* human GABAergic MSNs from hPSC, with the identification of hPSC-derived MSNs mainly based on DARPP-32 immunostaining (Aubry et al., 2008; El-Akabawy et al., 2011; Ma et al., 2012). This is colocalized with CTIP2 (*BCL11B*) and GABA by some authors (Danjo et al., 2011; Carri et al., 2012), based on rodents striatal biology (Arlotta et al., 2008) and on human fetal brain data (Carri et al., 2012; Onorati et al., 2014). However, striatal mRNAs and proteins expression could be different in the adult human brain.

Here we focus our analysis on DARPP-32 expression in human brain, especially in fetal and adult human striatum. We show that *DARPP-32* total gene expression is highly increased in human adult striatum and that its transcripts' levels are higher compared to other adult brain regions, as also shown by transcriptomic results in Human Brain Atlas [www.hbatlas.org; (Kang et al., 2011)] and Allen Human Brain Atlas (Hawrylycz et al., 2012; Sunkin et al., 2013). Furthermore, we demonstrate that t-DARPP is markedly increased in adult caudate-putamen nuclei, suggesting a

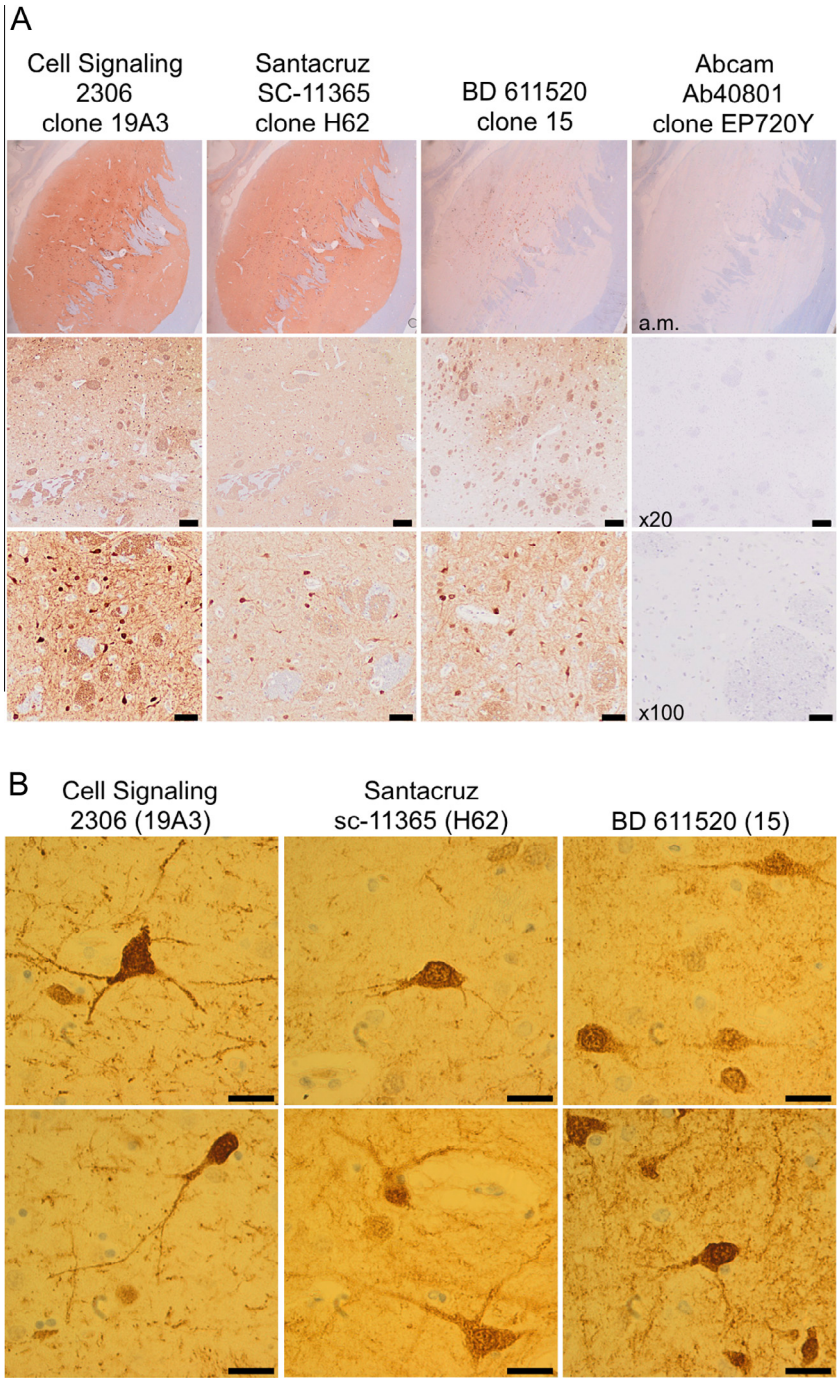

Fig. 4. Immunohistochemical comparative analysis of anti-DARPP-32 antibodies in human adult striatum. Representative microphotographs of paraffin-embedded samples (same donor) unmasked with citrate buffer pH = 6 are shown at different magnifications (A). Antibodies were tested at different dilutions, here 1:250 dilution is shown, except for Abcam Ab40801 (EP720Y), which shows the condition of 1:50 dilution. The whole striatal section is shown at arbitrary magnification (a.m.) to show the tissue integrity. Photo magnification of ×20 (bars = 200 μm) and ×100 (bars = 50 μm). (B) Representative MSNs' microphotographs (bars = 20 μm).

specific role for this isoform in human MSNs physiology during adulthood. Interestingly we found a difference in the ratio DARPP-32/t-DARPP between mRNAs and proteins. At gene expression level t-DARPP transcripts are relatively low expressed compared to DARPP-32 transcript, whereas at protein level isoforms are equally expressed. Future studies with larger sample population could better clarify this observation. However discrepancies between mRNA and protein levels were studied and reported in human cell biology (Guo et al., 2008; Gry et al., 2009).

Abnormal expression of t-DARPP transcripts in human brain are associated with schizophrenia and affective disorders (Kunii et al., 2014). However, little is known about t-DARPP subcellular localization and physiological function in human CNS. The t-DARPP protein

lacks the first 36 residues of N-terminus, which include the phosphorylation site Thr 34 and the PP-1 interacting domain. For this reason t-DARPP predicted molecular weight is 18,7 kDa (www.uniprot.org). However we detected its specific immune-signal migrating around 28 kDa, as previously described (Belkhiri et al., 2008), which may be most likely due to post-translational modification similarly to full length isoform DARPP-32. The consistent detection of this protein signal by three different antibodies in several adult biological samples without clear differences due to sex, age and post mortem delay reinforces previous observations from El-Rifai's research group in cancer cells (Belkhiri et al., 2008).

T-DARPP cannot inhibit PP-1, but it could target CREB mediating an alternative integration of dopaminergic-glutamatergic inputs in human adult MSNs, as suggested by observations in cancer cells (Gu et al., 2009; Yger and Girault, 2011). In this view, t-DARPP up-regulation in mature MSNs would increase the complexity of signaling integration of this hub protein in the human striatum.

Interestingly, the stimulation of D1R in the direct pathway and A_{2A} receptor activation in the indirect pathway triggers the phosphorylation of DARPP-32 on the Thr34 (Lindskog et al., 2002; Shen et al., 2013), determining an enrichment of DARPP-32 in MSNs nuclei *in vitro* and the consequent inhibition of nuclear PP-1 with pleiotropic effects (Stipanovich et al., 2008). One effect is that Histone 3 phosphorylation on Ser 10 is not removed, determining long-term effects on transcription through chromatin decondensation (Stipanovich et al., 2008). Although we cannot determine t-DARPP protein levels in between the discrete fetal and adult stages here analyzed, we can hypothesize that the increase in t-DARPP during striatal maturation could imply less DARPP-32 to the nucleus and more condensed chromatin. And this could represent a switch from the highly active transcription of neuronal development toward a mature and functional MSNs transcriptional program. Furthermore, DARPP-32 into the nucleus has been described to directly interact with the splicing factor tra2-beta1 in competition with PP-1 in non-striatal cells (Benderska et al., 2010). This nuclear DARPP-32 interaction with tra2-beta1 could modify the alternative splicing program as it was suggested elsewhere (Benderska et al., 2010). In a neurodevelopmental perspective, alternative splicing determines the specifications of the different neuronal subtypes and creates variability, which is needed for brain plasticity (Li et al., 2007; Zheng and Black, 2013). The increase in t-DARPP isoform in mature MSNs could tune the striatal alternative splicing highly required for specification and connectivity establishment, but less needed in mature circuitry.

Although from a practical point of view t-DARPP seems to be a better marker to follow MSNs maturation, it is not possible to generate antibodies against the truncated-DARPP-32 based on the aminoacidic sequence and ignoring its 3D structure, since its peptidic sequence is completely shared by the full-length isoform. Several anti-DARPP-32 antibodies have been reported in the literature for their efficacy to stain human striatal specimens at different developmental stages. However, anti-DARPP-32 antibodies are not always properly specified in research manuscripts (i.e. commercial catalog number), preventing the proper identification of the specific antibody used. Considering this, we undertook the immunohistochemical comparison of the four antibodies on post-mortem adult human striatum. Although Abcam Ab40801 clone EP720Y antibody was successfully used in previous immunohistochemical analysis of human fetal telencephalon (Carri et al., 2012; Onorati et al., 2014) and we also show that it works for western blot, we could not detect any staining in adult human post-mortem brain in any of the tested conditions. This negative result could be due to a sensitivity problem of this antibody, since here we show that about 50% of total DARPP-32 protein content is the t-DARPP, which cannot be recognize by this specific antibody. The remaining antibodies we tested did specifically recognize MSNs in adult human caudate-putamen with subtle technical differences. Finally, specific t-DARPP detection through new antibodies, based on any conformational difference of this isoform compared to the full-length, would allow to better following the maturation of MSNs, but also will allow the biochemical dissection of its function inside the cell.

CONCLUSIONS

To date, information on DARPP-32 isoforms expression during human striatal development is limited. Here we show an increase in DARPP-32 gene expression in human adult striatum compared to WGE, with the t-DARPP isoform being the most induced in the human adult MSNs. The augmented level of t-DARPP in adult striatum suggests an altered integration of neurotransmission signals inside the MSNs and/or a change in the mature MSN transcriptional program.

CONFLICT OF INTEREST

The authors confirm no potential conflict of interest.

CONTRIBUTORS

M.S. designed the study, performed the majority of the experiments and drafted the manuscript. J.C. participated in western blot analysis. A.E.R. provided human fetal samples and participated in manuscript preparation. J.M.C. coordinated the study and drafted the manuscript. All authors critically revised and approved the final manuscript.

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