

Identification of a Molecular Target of Psychosine and Its Role in Globoid Cell Formation

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Abstract. Globoid cell leukodystrophy (GLD) is characterized histopathologically by apoptosis of oligodendrocytes, progressive demyelination, and the existence of large, multinuclear (globoid) cells derived from perivascular microglia. The glycosphingolipid, psychosine (D-galactosyl-β-1,1' sphingosine), accumulates to micromolar levels in GLD patients who lack the degradative enzyme galactosyl ceramidase. Here we document that an orphan G protein-coupled receptor, T cell death-associated gene 8, is a specific psychosine receptor. Treatment of cultured cells expressing this receptor

with psychosine or structurally related glycosphingolipids results in the formation of globoid, multinuclear cells. Our discovery of a molecular target for psychosine suggests a mechanism for the globoid cell histology characteristic of GLD, provides a tool with which to explore the disjunction of mitosis and cytokinesis in cell cultures, and provides a platform for developing a medicinal chemistry for psychosine.

Key words: psychosine • G protein-coupled receptor • cytokinesis • leukodystrophy • sphingolipid

Introduction

Globoid cell leukodystrophy (GLD¹; also known as Krabbe's disease) is a hereditary metabolic disorder of infants, characterized morphologically by almost total absence of myelin, severe gliosis, and the presence of characteristic, multinucleated globoid cells in the white matter (Suzuki and Suzuki, 1978). The deficiency of the catabolic enzyme galactosyl ceramidase results in accumulation of psychosine (PSY; D-galactosyl-β-1,1'-sphingosine) in the brain (Suzuki and Suzuki, 1978). This accumulation of PSY in the white matter of children with GLD correlates temporally with apoptosis of oligodendrocytes and globoid cell formation by microglia (Tanaka and Webster, 1993; Cho et al., 1997). The nature of a causal relationship between globoid cell formation and disappearance of oligodendrocytes, if any, is not understood. The course of the human disease is mimicked by the galactosyl ceramidase

(GALC)-deficient twitcher mouse (Igisu and Suzuki, 1984). The homozygous *GALC*⁻/*GALC*⁻ twitcher mice are phenotypically normal at age 22 d but afterwards exhibit head twitching, progressive paralysis, and death by age 45 d (Matsushima et al., 1994). These *GALC*⁻/*GALC*⁻ mice accumulate high levels (120 μM in brain at age 31 d) of PSY (Shinoda et al., 1987).

Although PSY has been long suspected as a molecular agent in GLD and its mouse model, the mechanism of action of PSY is not understood (Suzuki, 1998). Recently, Xu et al. (2000) demonstrated that sphingosylphosphorylcholine (SPC) is a ligand for an "orphan" (i.e., previously unknown ligand) G protein-coupled receptor named ovarian cancer G protein-coupled receptor (OGR1). Due to our long-standing interest in lysophospholipid mediators such as sphingosine 1-phosphate, we began studying additional orphan G protein-coupled receptors that are similar to OGR1. One of these, named T cell death-associated gene 8 (TDAG8; so named because it is one of the genes expressed to high levels during the programmed cell death of immature T lymphocytes [Choi et al., 1996]), shares 41% identical amino acids with OGR1. In the course of testing a set of putative and known lipid signaling molecules, we found that TDAG8 is a specific receptor for PSY and several related gly-

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¹Abbreviations used in this paper: GFP, green fluorescent protein; GlcPSY, D-glucosyl-β-1,1' sphingosine; GLD, globoid cell leukodystrophy; OGR1, ovarian cancer G protein-coupled receptor; PSY, psychosine; PTX, pertussis toxin; SPC, sphingosylphosphorylcholine; TDAG8, T cell death-associated gene 8.

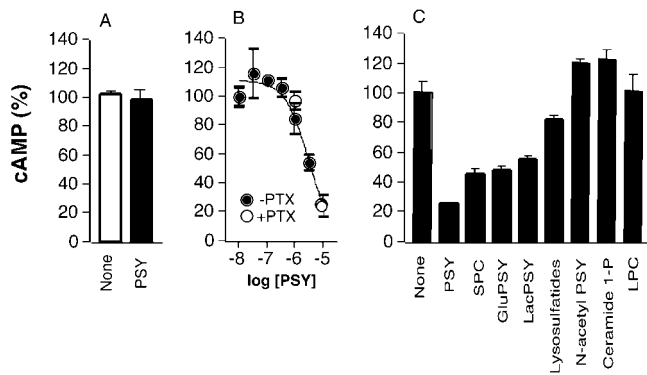


Figure 1. Inhibition of forskolin-evoked cAMP accumulation in TDAG8 DNA-transfected RH7777 cells. Clonal populations of RH7777 cells transfected with the TDAG8 receptor DNA (B and C) or plasmid vector (A) were treated with forskolin and isobutylmethylxanthine and challenged with varying concentrations of PSY. After 15 min, cells were solubilized and the cAMP content was determined. The cAMP content per well in the absence of PSY was 115.3 ± 15.8 pmol. (A) Lack of response in mock-transfected RH7777 cells treated with $10 \mu\text{M}$ PSY. (B) Dose-response curve of PSY for inhibition of cAMP accumulation (open circles, PTX [100 ng/ml for 24 h] treated; filled circles, PTX untreated). (C) Activity of various sphingoid-based lipids ($10 \mu\text{M}$) in this assay.

cosphingolipids. Further, the PSY/TDAG8 pair evokes a multinuclear phenotype in cultured cells reminiscent of the globoid cell formation that is the neurohistologic fingerprint of Krabbe's disease.

Materials and Methods

Materials

GluPSY, LacPSY, N-acetyl PSY, and lysosulfatide were from Matreya, Inc.; PSY and SPC were obtained from Avanti Polar Lipids. [α - ^{32}P]CTP was obtained from ICN Biochemicals; pcDNA3 plasmid was from Invitrogen; RH7777 cells (CRL 1601) and HEK293 cells (CRL-1573) were from the American Type Culture Collection; and human multiple tissue expression array and the pEGFP-N1 plasmid were from CLONTECH Laboratories, Inc. All other chemicals were from Sigma-Aldrich.

Cloning and Stable Transfection of TDAG8

Human TDAG8 was cloned from a genomic DNA library by PCR with two primers, forward primer 5'-AGACTTCTCTGTTTACTTTCT and reverse primer 5'-CTTCCCTCAAACATCCTTG, subcloned into the pcDNA3 expression vector, and its nucleotide sequence was verified. RH7777 or HEK293 cell monolayers were transfected with the TDAG8/pcDNA3 plasmid DNA using the calcium phosphate precipitate method, and clonal populations expressing the neomycin phosphotransferase gene were selected by addition of geneticin (G418) to the culture media. The RH7777 or HEK293 cells were grown in monolayers at 37°C in a 5% CO_2 /95% air atmosphere in growth media consisting of 90% MEM, 10% fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate.

Construction of TDAG8-GFP DNA and Confocal Microscopy

Human TDAG8 DNA was subcloned into the pEGFP-N1 vector at EcoRI-XhoI sites and transiently expressed in HEK293T cells by transfection using the calcium phosphate precipitation method. Cells were allowed to express the transgene for 2 d and then cultures were plated onto coverslips for an additional day. Indicated concentrations of lipid were added for 2 h at 37°C and then coverslips were washed with PBS at

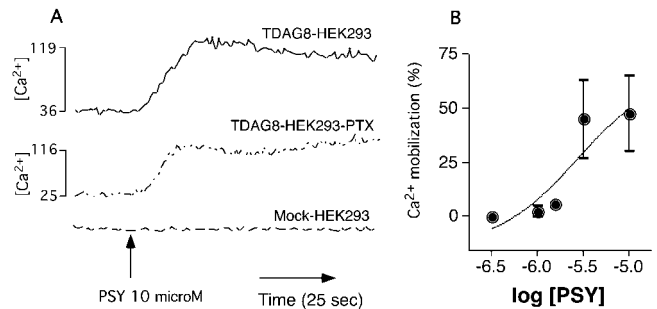


Figure 2. PSY induced Ca^{2+} mobilization in TDAG8-HEK293 cells. Cells were placed in suspension by treatment of monolayers with trypsin/EDTA, loaded with the calcium-sensitive fluorophore, INDO-1/AM, and $2\text{--}4 \times 10^6$ cells were placed in a temperature-controlled cuvette. (A) Representative traces in response to $10 \mu\text{M}$ PSY by TDAG8/HEK293 or mock-transfected HEK293 cells. (B) Concentration-response curve for PSY in evoking Ca^{2+} mobilization by TDAG8/HEK293 cells.

room temperature twice and fixed with cold 70% ethanol for 45 min. Coverslips were then dried and mounted onto slides using Vectashield with DAPI (Vector Laboratories). Confocal microscopy was performed using a Micro Systems LSM (ZEISS) and Axiovert 100 inverted scope at an excitation wavelength of 488 nm with $63\times$ magnification for green fluorescent protein (GFP).

cAMP Accumulation and Ca^{2+} Mobilization

For assay of cAMP, cells were plated on 24-well dishes as subconfluent populations. After 24 h, they were washed with PBS twice and incubated in Hepes-Krebs-Ringer buffer for 10 min. Cells were stimulated with different concentrations of lipid in the presence of $1 \mu\text{M}$ forskolin and 1 mM isobutylmethylxanthine for 15 min. The reaction was stopped by adding HCl to 0.1 N final concentration. After centrifugation to remove cell debris, the cAMP in the supernatant fluid was measured in an automated immunoassay (Gamma flow). Assay of calcium mobilization was performed as described previously by us (Im et al., 2000b). In brief, intracellular calcium fluxes were measured on cell populations ($2\text{--}4 \times 10^6$ cells) that had been loaded with the calcium-sensitive fluorophore, INDO-1, in the presence of 2 mM probenecid. Responses were measured using a temperature-controlled fluorimeter (Aminco SLM 8000C; SLM Instruments). Lipids were delivered as aqueous solutions containing 0.1% (wt/vol) fatty acid-free BSA; this vehicle was determined to elicit no response.

Northern Blot Analysis

For hybridization, a ^{32}P -labeled human TDAG8 cDNA fragment was used. The human RNA master blot (CLONTECH Laboratories, Inc.) was hybridized and washed according to the protocol supplied by the manufacturer.

DAPI Staining

For DAPI staining, cells were grown on coverslips and treated with $10 \mu\text{M}$ PSY for 6 (RH7777 cultures) or 4 d (HEK293 cells). After treatment, cells were washed with PBS at room temperature twice and fixed with cold 70% ethanol for 45 min. Coverslips were then dried and mounted onto slides using Vectashield with DAPI (Vector Laboratories) to display nuclei. Images are obtained using a fluorescence microscope (ZEISS) and Openlab v2.0 software on a Macintosh G3 computer.

Flow Cytometry

Cells were treated with $10 \mu\text{M}$ PSY for 6 d, harvested, and then fixed with 70% ethanol. Cells were treated with RNase A (0.1 mg/ml in PBS) at 37°C for 30 min, stained with propidium iodide ($50 \mu\text{g/ml}$ in PBS), and then subjected to flow cytometry with a FACScan™ flowcytometer (Becton Dickinson) for measurement of the DNA content.

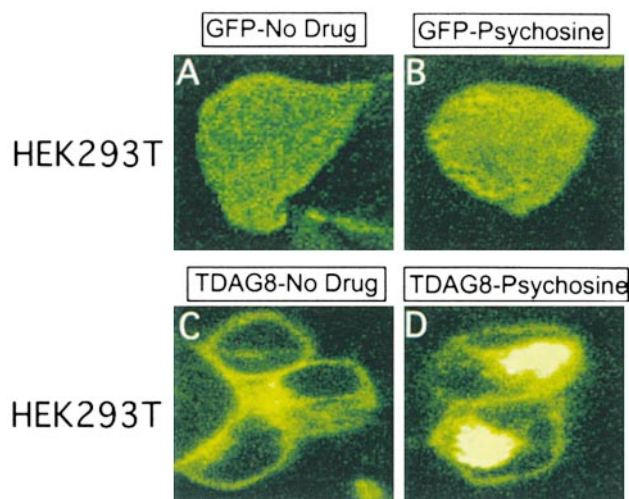


Figure 3. Internalization of TDAG8-GFP fusion protein in response to PSY treatment. (A and B) HEK293T cells expressing GFP fusion protein alone. (C and D) HEK293T cells expressing TDAG8/GFP fusion protein. The cells depicted in A and C were untreated, and the cells in B and D were treated with 10 μ M PSY for 2 h at 37°C.

Results and Discussion

When expressed in RH7777 hepatoma cells, human TDAG8 mediated PSY-induced inhibition of forskolin-driven cAMP rise in a concentration-dependent manner ($EC_{50} = 3.4 \mu\text{M}$) (Fig. 1, A and B). This response was evoked also by structurally related lysolipids, e.g., *D*-glucosyl- β -1,1' sphingosine (GlcPSY), LacPSY, and lysosulfatide, but not by *N*-acetyl PSY, sphingosine 1-phosphate, lysophosphatidic acid, ceramide 1-phosphate, or lysophosphatidylcholine (Fig. 1 C). Similar results were found using the orthologous mouse TDAG8 DNA (data not shown). The PSY response was not blocked by pretreatment of RH7777 cultures with pertussis toxin (PTX; 100 ng/ml for 24 h), suggesting the involvement of PTX-insensitive G proteins, perhaps $G_{\alpha z}$ (Kozasa and Gilman, 1995). SPC also was active in this assay, but this response, which was PTX sensitive (not shown), probably proceeds through an endogenous receptor in RH7777 cells (Im et al., 2000a). This suspicion was confirmed when SPC failed in further assays (see below). The ability of PSY to activate TDAG8 was confirmed by this lipid evoking Ca^{2+} transients in TDAG8/HEK293 cells (Fig. 2) and the PSY-driven internalization of a TDAG8/GFP fusion protein in HEK293T cells (Fig. 3). Both actions were mimicked by related lysoglycolipids (e.g., GlcPSY and lysosulfatide), but not by SPC or *N*-acetyl PSY at concentrations up to 10 μ M (data not shown).

Recently, the generation of large, multinuclear (globoid) cells characteristic of Krabbe's disease was reproduced in vitro by treating human U937 monocyte cells with PSY (Kanazawa et al., 2000). We replicated this finding (not shown) and further used reverse transcriptase PCR to discover that TDAG8 is expressed in these cells, but not in other cell lines (e.g., HEK293 and K562) that do not form globoid cells in response to PSY treatment (Im, D.S., and K.R. Lynch, unpublished data). To discover the human tissues that express TDAG8, we probed a human

Table I. PSY Induces Multinuclear Cells in TDAG8-expressing RH7777 Cultures

Treatment	Multinuclear cells (mean \pm SEM)	Multinuclear cells/total cell count
	%	
None	2.18 \pm 0.48	32/1,437 (3)*
PSY	39.32 \pm 3.38 [‡]	378/984 (6)
GlcPSY	44.08 \pm 4.08 [§]	392/890 (6)
Lysosulfatide	21.16 \pm 3.24 [§]	243/1,138 (3)
LacPSY	4.10 \pm 0.29	67/1,617 (3)
SPC	6.27 \pm 0.74	57/900 (3)

*Numbers in parentheses indicate total number of experiments.

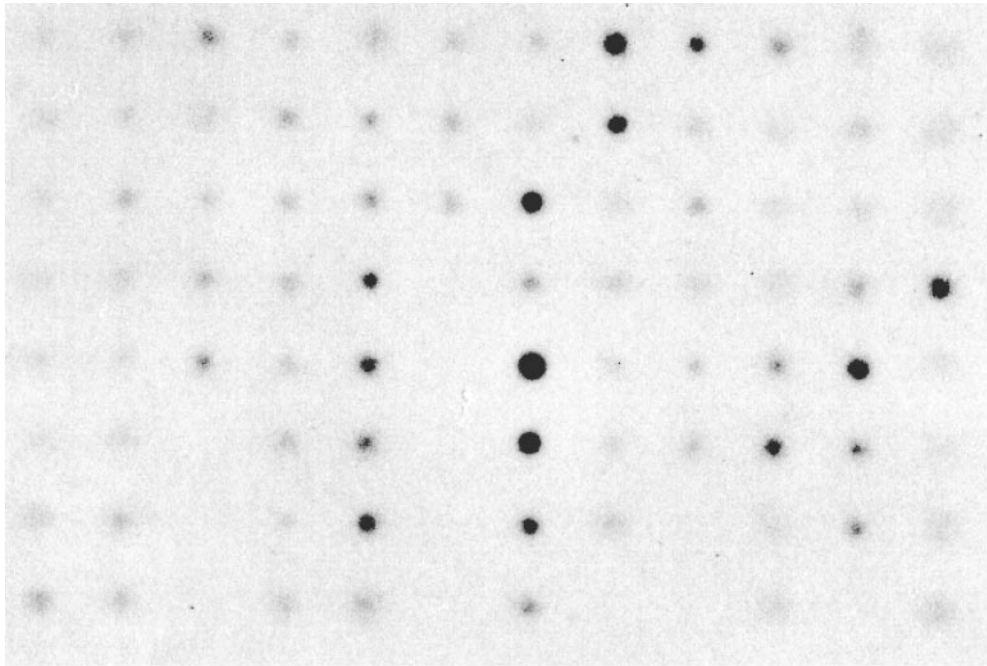
[‡] $P > 0.005$.

[§] $P > 0.05$.

multiple tissue RNA array with radiolabeled TDAG8 DNA. As documented in Fig. 4, TDAG8 RNA is found most prominently in extracts of spleen (fetal and adult), lymph node, and peripheral blood leukocytes, although a low level signal was found in virtually all human tissue extracts. This expression pattern, coupled with our observation that THP1 and HL60 cell cultures express TDAG8 (not shown), is consistent with TDAG8 gene expression in monocytes and macrophages, including tissue macrophages such as microglia.

To test the hypothesis that PSY acting via TDAG8 mediates the disjunction of mitogenesis and cytokinesis characteristic of globoid cells, we treated cell cultures transfected with TDAG8 DNA with PSY and quantified nuclear DNA content. When TDAG8-expressing RH7777 cells were treated with 10 μ M PSY, multinuclear globoid cells were observed by DAPI staining (Fig. 5 A, and Table I). Neither expression of TDAG8 without PSY treatment nor PSY treatment in the absence of TDAG8 DNA transfection resulted in the appearance of multinuclear, globoid cells. Likewise, TDAG8/HEK293 cells became multinuclear in response to PSY treatment (Fig. 5, B and C) and both receptor and ligand were required to generate the globoid cell phenotype. In concert with the structure activity profile found with inhibition of cAMP and calcium mobilization (see above), GlcPSY and lysosulfatide mimicked the action of PSY in evoking globoid cell formation, whereas *N*-acetyl PSY and SPC did not (data not shown). The requirement for both members of the ligand receptor pair to be present, the long time course required for globoid cell formation, and a previous report using U937 cells (Kanazawa et al., 2000) all suggest that this phenomenon involves a disjunction of mitosis and cytokinesis rather than simple cell fusion.

Our identification of a PSY receptor and the role of this ligand receptor pair in evoking globoid cell formation has several implications. First, TDAG8 is the second member of the OGR1 receptor cluster found to have a lipid ligand; OGR1 is activated by SPC (but not PSY), whereas two other members of the cluster, G2A (Weng et al., 1998) and GPR4 (Heiber et al., 1995), remain to be paired with a ligand. Our identification of a PSY receptor confirms the prescient observations of Okajima and Kondo (1995) and Himmel et al. (1998), both of whom suggested that PSY acts through a specific G protein-coupled receptor. Second, the PSY receptor, presumably acting through un-



	1	2	3	4	5	6	7	8	9	10	11
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, descending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen
F	temporal lobe	hippocampus		ventricle, right	ileocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus
G	p. g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary		colorectal adenocarcinoma, SW480	fetal lung
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549	

* paracentral gyrus

Figure 4. Autoradiogram of a human multiple tissue array RNA dot blot (CLONTECH Laboratories, Inc.) hybridized with ³²P-labeled human TDAG8 DNA.

known heterotrimeric G proteins, blocks cell division, but not nuclear division, and thus provides a tool that might be useful in exploring mechanisms of cytokinesis. It is noteworthy that two tissues that contain multinuclear cells, placenta (trophoblasts) and lung (macrophages), are prominent in expressing TDAG8 RNA (Fig. 4). Third, the structure activity profile of PSY receptor ligands suggests that TDAG8 might be involved in the pathogenesis of related lipid storage disorders, such as Gaucher's disease (accumulation of GlcPSY), which is characterized by hepatomegaly, splenomegaly, and osteoporotic erosion

(Brady, 1978), and metachromatic leukodystrophy (lysosomal sulfatide accumulation), which is characterized by myelin degeneration (Moser and Dulaney, 1978). However, neither of these diseases is associated with the globoid cell formation characteristic of Krabbe's disease. Finally, the identification of a molecular target for PSY and related lipids provides a platform on which a medicinal chemistry, including the discovery and optimization of receptor blockers, can be built. PSY antagonists might prove useful clinically in altering the course of some lipid storage disorders. Currently, only palliative care is available to

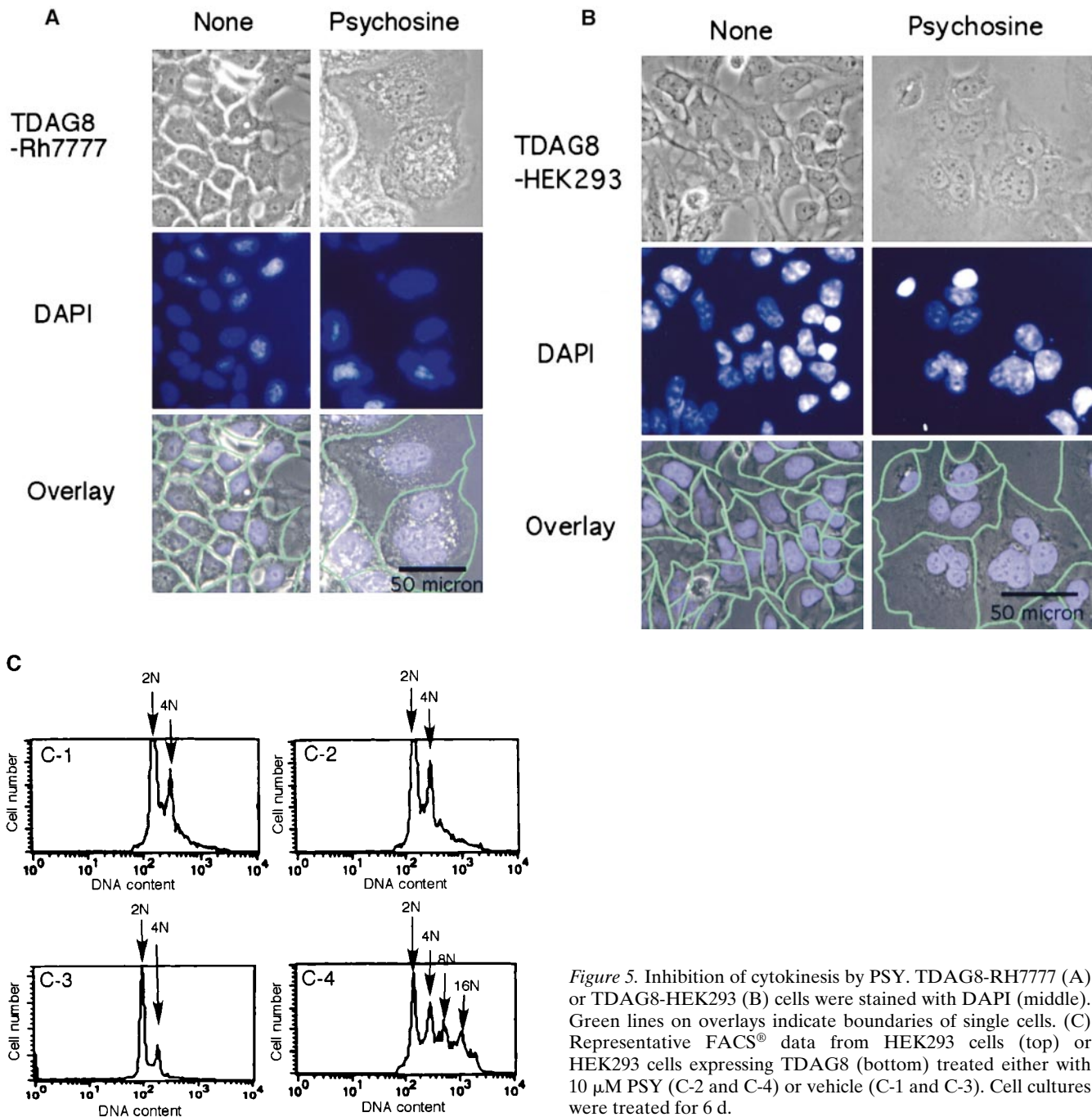


Figure 5. Inhibition of cytokinesis by PSY. TDAG8-RH7777 (A) or TDAG8-HEK293 (B) cells were stained with DAPI (middle). Green lines on overlays indicate boundaries of single cells. (C) Representative FACS[®] data from HEK293 cells (top) or HEK293 cells expressing TDAG8 (bottom) treated either with 10 μ M PSY (C-2 and C-4) or vehicle (C-1 and C-3). Cell cultures were treated for 6 d.

Krabbe's disease and metachromatic leukodystrophy patients, whereas patients suffering from the often more indolent Gaucher's disease have available enzyme replacement therapy (albeit at enormous cost). The twitcher mouse provides a convenient model for testing potential receptor blockers. In addition, if a mouse with its TDAG8 genes ablated proves fertile, crossing this genotype onto a twitcher background could prove informative.

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