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Nonyloxytryptamine mimics polysialic acid and modulates neuronal and glial functions in cell culture

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Abstract

Polysialic acid (PSA) is a major regulator of cell–cell interactions in the developing nervous system and in neural plasticity in the adult. As a polyanionic molecule with high water-binding capacity, PSA increases the intercellular space generating permissive conditions for cell motility. PSA enhances stem cell migration and axon path finding and promotes repair in the lesioned peripheral and central nervous systems, thus contributing to regeneration. As a next step in developing an improved PSA-based approach to treat nervous system injuries, we searched for small organic compounds that mimic PSA and identified as a PSA mimetic

5-nonyloxytryptamine oxalate, described as a selective 5-hydroxytryptamine receptor 1B (5-HT_{1B}) agonist. Similar to PSA, 5-nonyloxytryptamine binds to the PSA-specific monoclonal antibody 735, enhances neurite outgrowth of cultured primary neurons and process formation of Schwann cells, protects neurons from oxidative stress, reduces migration of astrocytes and enhances myelination *in vitro*. Furthermore, nonyloxytryptamine treatment enhances expression of the neural cell adhesion molecule (NCAM) and its polysialylated form PSA-NCAM and reduces expression of the microtubule-associated protein MAP2 in cultured neuroblastoma cells. These results demonstrate that 5-nonyloxytryptamine mimics

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Abbreviations used: 5-HT_{1B}, 5-hydroxytryptamine receptor 1B; 5-NOT, 2-(5-nonyloxy-1H-indol-3-yl)ethanamine; 5-nonyloxytryptamine oxalate; CGS12066B, 7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]-quinoxaline dimaleate; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; Erk, extracellular regulated kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCAM, neural cell adhesion molecule; nitrendipine, 5-O-ethyl-3-O-methyl-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate; PBS, phosphate buffered saline solution; PBST, phosphate buffered saline solution with 0.01% Tween-20; PSA, polysialic acid; SB224289, 1'-methyl-5-[[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine]hydrochloride.

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PSA and triggers PSA-mediated functions, thus contributing to the repertoire of molecules with the potential to improve recovery in acute and chronic injuries of the mammalian peripheral and central nervous systems.

Polysialic acid (PSA) is a linear homopolymer of α 2,8-linked sialic acid monomers (ranging from 8 to over 100 residues) attached to an N-linked carbohydrate core (Nakata and Troy 2005). Besides neuropilin-2 and SynCAM as less prominent PSA carriers, neural cell adhesion molecule (NCAM) is the most abundant carrier of PSA. PSA is attached to two N-glycosylation sites in the fifth immunoglobulin-like domain of NCAM and generates a bulky and highly negatively charged moiety with a large hydration shell. PSA thus increases the intercellular space and modulates cell–cell and cell–substrate interactions (Rutishauser 2008). PSA expression is most prominent during development, being more restricted in the adult to brain regions capable of structural and functional plasticity, as, for example, in the olfactory system and hippocampal dentate gyrus concomitant with neurogenesis (Bonfanti 2006). In adult mammals, a transient re-expression of PSA-NCAM in neurons and glial cells occurs in different lesion models (Brezun and Daszuta 2000; Bonfanti 2006). PSA-NCAM is also implicated in synaptic plasticity (Rutishauser 2008; Senkov *et al.* 2012), stem cell migration (Petridis *et al.* 2004; Rutishauser 2008), axonal path finding (Enriquez-Barreto *et al.* 2012), migration of luteinizing hormone-releasing neurons (Yoshida *et al.* 1999), neuron–glia plasticity at gonadotropin-releasing hormone neuron terminals (Kumar *et al.* 2012) as well as spatial learning and memory (Venero *et al.* 2006; Rutishauser 2008). PSA expressing Schwann cells improve functional recovery when engrafted in a mouse model of spinal cord injury (Papastefanaki *et al.* 2007; Ghosh *et al.* 2012).

To circumvent difficulties in generating purified PSA (colominic acid) from bacteria and to avoid degradation by active neuraminidases *in vivo*, we searched for small organic compounds that mimic PSA structurally and functionally, but do not have the disadvantages of native PSA. Molecular mimetics were shown to have superior affinity and metabolic stability (Magnani and Ernst 2009). Peptide mimics of PSA developed by phage display screening of large libraries with PSA monoclonal antibodies (Torregrossa *et al.* 2004; Mehanna *et al.* 2009) promoted functional recovery and plasticity after injury of the murine peripheral and central nervous systems (Marino *et al.* 2009; Mehanna *et al.* 2009, 2010). First attempts to construct PSA containing biomaterials for regeneration after nervous system injury made use of PSA or PSA mimetic coupled collagen fibers electrospun in fiber scaffolds which promoted proliferation of immortalized Schwann cells (Assmann *et al.* 2010). Grafted PSA mimetic collagen encouraged sensory and motor neuron outgrowth,

Keywords: 5-nonyloxytryptamine oxalate, migration, neural cell adhesion molecule, neurite outgrowth, polysialic acid, scratch injury.

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enhanced Schwann cell proliferation and process extension and stimulated peripheral nerve repair (Masand *et al.* 2012a, b), indicating the potential of mimetics to replace native PSA.

To convert PSA mimetics to even smaller and metabolically stable compounds, we identified 5-nonyloxytryptamine oxalate, a compound described as selective 5-HT_{1B} agonist, as novel PSA mimetic and tested its functions *in vitro* using cultures of murine primary neurons and glia from central and peripheral nervous system origin and a neuroblastoma cell line as model for easy manipulation (Gotti *et al.* 1987). Our results show that 5-nonyloxytryptamine binds to a PSA-specific monoclonal antibody and stimulates neuronal and glial cell functions.

Materials and methods

Mice

C57BL/6J mice of either sex were used as wild-type mice and obtained from the central breeding facility of the University Hospital Hamburg-Eppendorf. NCAM-deficient (–/–) mice (Cremer *et al.* 1994) on the C57BL/6J background were used for cell culture experiments. Mice were kept at standard laboratory conditions with food and water supply *ad libitum* and with an artificial 12 h light/dark cycle. All experiments were conducted in accordance with the ‘Principles of laboratory animal care’ (NIH publication No. 85-23, revised in 1985), the German and European Community laws on protection of experimental animals, and all procedures used were approved by the responsible committee of the State of Hamburg. The article was written in compliance with the ARRIVE guidelines for reports on animal research.

Antibodies, chemicals, and cell lines

In the following, purchased reagents are indicated with their companies in brackets: serotonin, colominic acid, Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St Louis, MO, USA); the PSA mimicking peptide (NTHTPYYPID; Mehanna *et al.* 2009) (Schafer-N, Copenhagen, Denmark); 5-nonyloxytryptamine oxalate [2-(5-nonyloxy-1H-indol-3-yl)ethanamine; 5-NOT], SB224289 hydrochloride (1’-methyl-5-[[2’-methyl-4’-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4’-piperidine]hydrochloride; SB224289), CGS12066B dimaleate (7-trifluoromethyl-4-(4-methyl-1-piperazinyl)pyrrolo[1,2-a]quinoxaline dimaleate; CGS12066B), and nitrendipine (5-O-ethyl-3-O-methyl-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate) (Tocris Bioscience, Bristol, UK). O-phenylenediamine dihydrochloride (Thermo Scientific, Waltham, MA, USA); Alexa or Cy coupled secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA or Invitrogen, Carlsbad, CA, USA); horse radish peroxidase coupled secondary

antibodies for western blots (Jackson ImmunoResearch or Bangalore Genei India Pvt. Ltd., Bangalore, India). PSA-specific monoclonal antibodies 735 (R. Gerardy-Schahn, Department of Biochemistry, Institute for Cellular Chemistry, Hannover Medical School, Hannover, Germany) and MenB (G. Rougon, Centre National de la Recherche Scientifique, Marseille, France); P61 anti-NCAM antibody (Christo Goridis, Département de Biologie, École Normale Supérieure, Paris, France); mouse monoclonal anti-phospho extracellular regulated kinase (Erk), rabbit polyclonal anti-Erk and goat polyclonal anti-myelin protein P0 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-PSA and anti-neurofilament antibodies (Millipore, Bedford, MA, USA); monoclonal anti- α -tubulin, anti-NCAM and anti-MAP2 antibodies (Sigma-Aldrich). The IMR-32 cell line was obtained from the National Center for Cell Science (NCCS, Pune, India).

Screening of a compound library and identification of PSA mimetics by ELISA; IMR-32 culture, treatments, cell cycle analysis, cytotoxicity and cell viability assays; protein assay and western blotting

For procedures see the Appendix S1.

Neurite outgrowth, neuronal survival, process formation and proliferation of Schwann cells, and *in vitro* myelination

Primary cultures of cerebellar neurons or Schwann cells were prepared from 7-day-old C57BL/6J or NCAM-deficient mice (Loers *et al.* 2005; Mehanna *et al.* 2009); motoneurons were prepared from E14 mice (Simova *et al.* 2006); hippocampal neurons were prepared from neonatal mice (Wang *et al.* 2011). Neurite or process lengths were quantified (Mehanna *et al.* 2009). Schwann cell processes and neurites with lengths of at least one cell body diameter were evaluated and total neurite or process lengths per cell were determined from 50 cells in each of two wells per experiment. At least three independent experiments were performed per condition. Cell survival was determined (Loers *et al.* 2005). The *in vitro* myelination was performed as described in the Supplemental methods.

Scratch injury assay

Astrocyte-enriched cultures from brains of neonatal mice were cultured for at least 1 week at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum on PLL-coated six-well plates until they had reached confluence (Kleene *et al.* 2007). Twelve hours before scratch injury, the medium was changed to serum-free DMEM.

The scratch injury assay with astrocytes and IMR-32 cells was performed according to Etienne-Manneville (2006) with modifications described in the Supplemental methods. Schwann cell motility was assayed with primary cultures of Schwann cells derived from sciatic nerves of 4- to 5-day-old mice (Lavdas *et al.* 2006) as described in the Supplemental methods.

Molecular modeling of PSA and 5-NOT

A model of PSA bound to the surface of antibody 735 was constructed using the information provided by Evans *et al.* (1995). The three-dimensional coordinates for this antibody were obtained from the Protein Data Bank (PDB id: 1PLG). Initially, a decamer of PSA was built using the program Discovery Studio (Accelrys Inc., San Diego, CA, USA) in helical conformation making approxi-

mately 1/2 of a turn within 17 Å ($n = 6$ residues per turn). Subsequently, program PYMOL (Schrödinger Inc., Portland, OR, USA) was used to produce a model of an eight-residue segment PSA docked onto antibody 735. The resulting model was manipulated in PYMOL to reproduce the specific inter-molecular contacts between PSA and antibody 735 identified by Evans *et al.* (1995).

A three-residue segment of the PSA structure in the antibody 735 model was selected to serve as a template for evaluating structure and chemical similarity to 5-NOT. The 2D structure of 5-NOT from PubChem and OpenEye OMEGA (<http://www.eyesopen.com/omega>) was used to enumerate all possible low-energy 3D conformations for this compound. We used OpenEye ROCS (<http://www.eyesopen.com/rocs>) to carry out shape- and chemistry-based matching of each conformation with the PSA template to identify the 3D conformation for 5-NOT that most closely matches the template. Finally, 5-NOT was superimposed in place of PSA in the antibody 735 structure using the ROCS alignment to identify putative interactions between 5-NOT and antibody 735.

Immunocytofluorescence staining

Cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.3% Triton X-100 in phosphate buffered saline solution, pH 7.4 (PBST). Cells were incubated with primary antibody [anti- α -tubulin (1 : 500) or anti-MAP2 (1 : 300) diluted in blocking solution (PBS with 2% bovine serum albumin)], for 24 h at 4°C. After three washes in PBS, secondary antibody (Alexa Fluor 488) was applied for 2 h at 25°C. Cells were also incubated with 4',6-diamidino-2-phenylindole (DAPI) for 10 min for nuclear staining, mounted with anti-fading reagent (Fluoromount; Sigma) and observed under a confocal microscope (Nikon A1RConfocal; Nikon Corporation, Tokyo, Japan).

For dual immunostaining cells were incubated together with antibodies against PSA and NCAM in blocking solution (1 : 250) at 4°C overnight. After three washes with PBS, cells were incubated with anti-mouse IgG 488 and anti-mouse IgG 546 antibodies in blocking solution for 2 h at 25°C and counterstained with DAPI. Relative immunofluorescence intensities were determined using NIS elements AR analysis software version 4.11.00 (Nikon Corporation).

For immunocytochemistry with cerebellar neurons see Supplemental methods.

Statistical analysis

The significance of values was determined by one-way ANOVA with Dunnett's *post-hoc* test. One-way ANOVA with Holm-Sidak *post hoc* test was also used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) assays and IMR-32 wound scratch assay. Paired *t*-test was used to analyze differences in western blots. Values are expressed as means $\pm$ SEM from at least three independent experiments and differences were considered significant at $p < 0.05$.

Results

Nonyloxytryptamine binds to anti-PSA antibody 735 and mimics PSA

To identify PSA-mimicking compounds, the NIH Clinical Collection 1 Library was screened for compounds that inhibit binding of the PSA receptor site of antibody 735 to the PSA

mimetic peptide. This screen resulted in the identification of 5-NOT as PSA mimetic. To confirm these results, a competition ELISA was performed with different concentrations of 5-NOT and nitrendipine as negative control. 5-NOT inhibited binding of antibody 735 to catalase carrying colominic acid, the bacterial analog of PSA, as well as of the PSA peptide mimetic (data not shown) in a dose dependent manner, with maximal inhibition at approximately 100 nM. Nitrendipine did not impede antibody binding to colominic acid nor PSA peptide at any tested concentration (Figure S1). When cerebellar neurons were stained with PSA antibodies 735 or MenB in the absence or presence of 5-NOT, binding of these antibodies was markedly reduced by 5-NOT [by ~ 80% (735) or ~ 60% (MenB)], showing that 5-NOT reduces PSA antibody binding to native PSA at the cell surface (Figure S1).

To investigate how the small organic molecule 5-NOT could act as a mimetic for the large and negatively charged PSA, we compared the structure of the PSA 8mer predicted helical conformation (Evans *et al.* 1995) (Fig. 1a) and the 5-NOT structure by a rapid overlay of chemical structures (ROCS) and identified a conformation of 5-NOT that matched the PSA shape from an antibody 735 structural model based on previous crystallographic and biochemical data (Evans *et al.* 1995). The overall similarity score was 0.42 on a scale of 0 to 2, indicating that although 5-NOT does not contain a high degree of similarity with PSA it can adopt a shape compatible with the van der Waals volume of

the PSA-binding conformation within antibody 735. We then generated a putative 5-NOT/antibody 735 model structure by superimposing 5-NOT to PSA in the antibody 735 model structure using the ROCS alignment. A comparison of the models of the PSA/antibody 735 and 5-NOT/antibody 735 complexes (Fig. 1b) suggests that 5-NOT binds in the region of antibody 735 that contains numerous hydrophobic residues that are critical for interaction with PSA, including Y54 and F60 on the light chain and Y181 on the heavy chain of the antibody.

Nonyloxytryptamine induces neurite outgrowth and Schwann cell process formation

To investigate if 5-NOT also functionally mimics PSA, we determined the process outgrowth of PSA-responsive neurons and glial cells (representative images are shown in Figure S2). Application of 0.1 to 100 nM 5-NOT to cerebellar granule neurons led to a concentration dependent increase in neurite length from 10% (0.1 nM) to 100% (100 nM) of the neurite length of control cells only treated with vehicle control (Fig. 2a). The stimulatory effects of 5-NOT and the PSA analog colominic acid were similar, reaching 205% of control value with colominic acid and 200% with 5-NOT (Fig. 2b). Since 5-NOT was identified as agonist for the rodent 5-hydroxytryptamine (5-HT_{1B}, serotonin) 1B (5-HT_{1B}) receptor and the human 5-HT_{1Dβ} receptor (K_i at 5-HT_{1B}/5-HT_{1Dβ} = 1–16 nM with 300-fold selectivity over 5-HT_{1A}; Glennon *et al.* 1994) and neurons in the deep

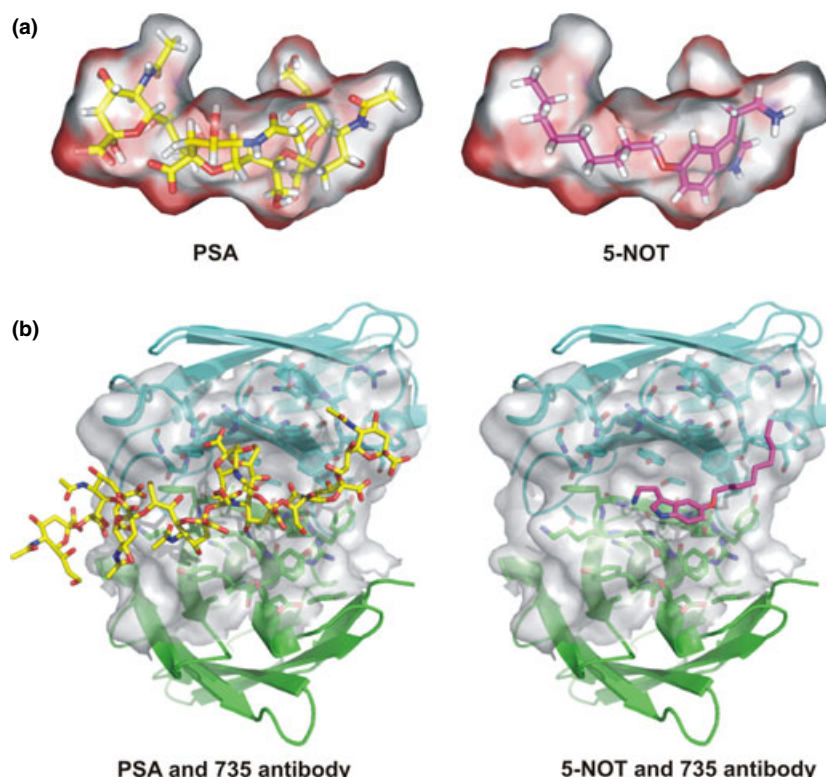


Fig. 1 Structure models of polysialic acid (PSA) and 5-nonyloxytryptamine oxalate (5-NOT). (a) Shape and chemistry of PSA, shown as a surface, superimposed with the 3D structure of PSA (left) and 5-NOT (right) shows that 5-NOT can adopt a conformation compatible with the shape of PSA as it is bound to antibody 735. (b) Structure models of PSA/PSA antibody 735 (left) and 5-NOT/antibody 735 (right) complexes suggest that 5-NOT binds to a hydrophobic region in antibody 735 that is critical to interactions with PSA.

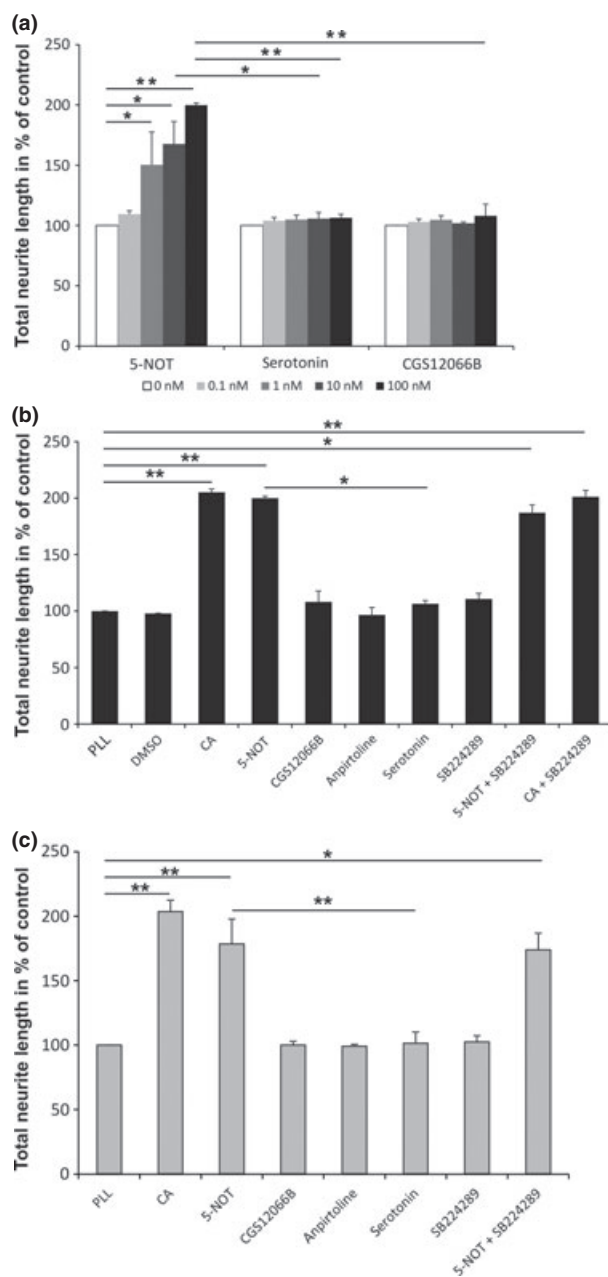


Fig. 2 Nonyloxytryptamine stimulates neurite outgrowth of wild-type cerebellar neurons and less so of neural cell adhesion molecule (NCAM)-deficient cerebellar neurons. (a) Concentration dependent stimulation of neurite outgrowth is induced by 5-nonyloxytryptamine oxalate (5-NOT) but not by serotonin or the 5-HT_{1B} agonist CGS12066B. (b) Comparison of the outgrowth of cerebellar neurons in the presence of colominic acid (CA), dimethyl sulfoxide (solvent control), 5-NOT, serotonin and the 5-HT_{1B} agonists anpirtoline and CGS12066B (all compounds at 100 nM concentration). The 5-HT_{1B} antagonist SB224289 did not inhibit the stimulatory activity of 5-NOT or colominic acid. (c) Neurite outgrowth from NCAM-deficient neurons. Data represent mean values of neurite lengths per cell $\pm$ SEM as compared with PLL only from three independent experiments. Asterisks denote significant differences from control. * $p < 0.01$, ** $p < 0.001$.

cerebellar nuclei express the 5-HT_{1B} receptor (Sari *et al.* 1999) and granule neurons receive serotonergic input (Nichols 2011), we wanted to rule out that the observed effect of 5-NOT on neurite outgrowth was due to 5-NOT's action as 5-HT_{1B} agonist. Therefore, other 5-HT_{1B} agonists and serotonin were tested for their effects on neuritogenesis of cerebellar granule neurons (Fig. 2a and b). Neither anpirtoline nor CGS12066B as 5-HT_{1B} agonists (K_i of 28 nM or 51 nM, respectively; Neale *et al.* 1987; Schlicker *et al.* 1992) nor serotonin stimulated neuritogenesis. Application of 5-NOT together with the 5-HT_{1B} inverse agonist SB224289 (pK_i 8.2 nM) (Gaster *et al.* 1998) did not reduce 5-NOT induced neuritogenesis (Fig. 2b), indicating that 5-NOT stimulates neuritogenesis of cerebellar neurons independently of its activity as serotonin receptor agonist. To elucidate if 5-NOT or colominic acid act via NCAM at the neuronal cell surface, the two compounds were applied to NCAM-deficient neurons and stimulated neuritogenesis to 80% of the level seen in wild-type neurons, while neither CGS12066B nor serotonin stimulated neuritogenesis above control levels (Fig. 2c).

To determine if similar effects of 5-NOT can be seen in other PSA-responsive neurons, hippocampal and motor neurons were cultured in the presence of colominic acid, 5-NOT, serotonin, anpirtoline, CGS12066B, and SB224289 (Fig. 3). Colominic acid and 5-NOT stimulated neuritogenesis of hippocampal and motor neurons by two- and 2.5-fold, respectively, whereas anpirtoline, CGS12066B and serotonin did not (Fig. 3a and b). Application of SB224289 together with 5-NOT only slightly reduced 5-NOT induced stimulation. As for cerebellar neurons colominic acid and 5-NOT stimulated neuritogenesis from NCAM-deficient hippocampal neurons, although lesser than wild-type neurons (Fig. 3a). However, addition of colominic acid and 5-NOT to NCAM-deficient motor neurons did not stimulate neuritogenesis (Fig. 3b).

Similarly, colominic acid and 5-NOT increased process formation of Schwann cells, whereas serotonin, anpirtoline and CGS12066B were ineffective (Fig. 3c). Application of SB224289 together with 5-NOT did not inhibit 5-NOT's stimulatory effect on process formation. Colominic acid and 5-NOT did not stimulate process formation in NCAM-deficient Schwann cells (Fig. 3c).

Nonyloxytryptamine modulates migration of Schwann cells, astrocytes, and IMR-32 cells

Since over-expression of PSA by Schwann cells leads to enhanced migration (Luo *et al.* 2011; Ghosh *et al.* 2012), we examined if 5-NOT affects migration of cultured Schwann cells after scratch injury (Fig. 4a and b). Colominic acid, PSA peptide mimetic and 5-NOT enhanced migration of Schwann cells, whereas serotonin and CGS12066B were ineffective. Schwann cells treated with colominic acid and 5-NOT migrated distances of $390 \pm 15 \mu\text{m}$ and

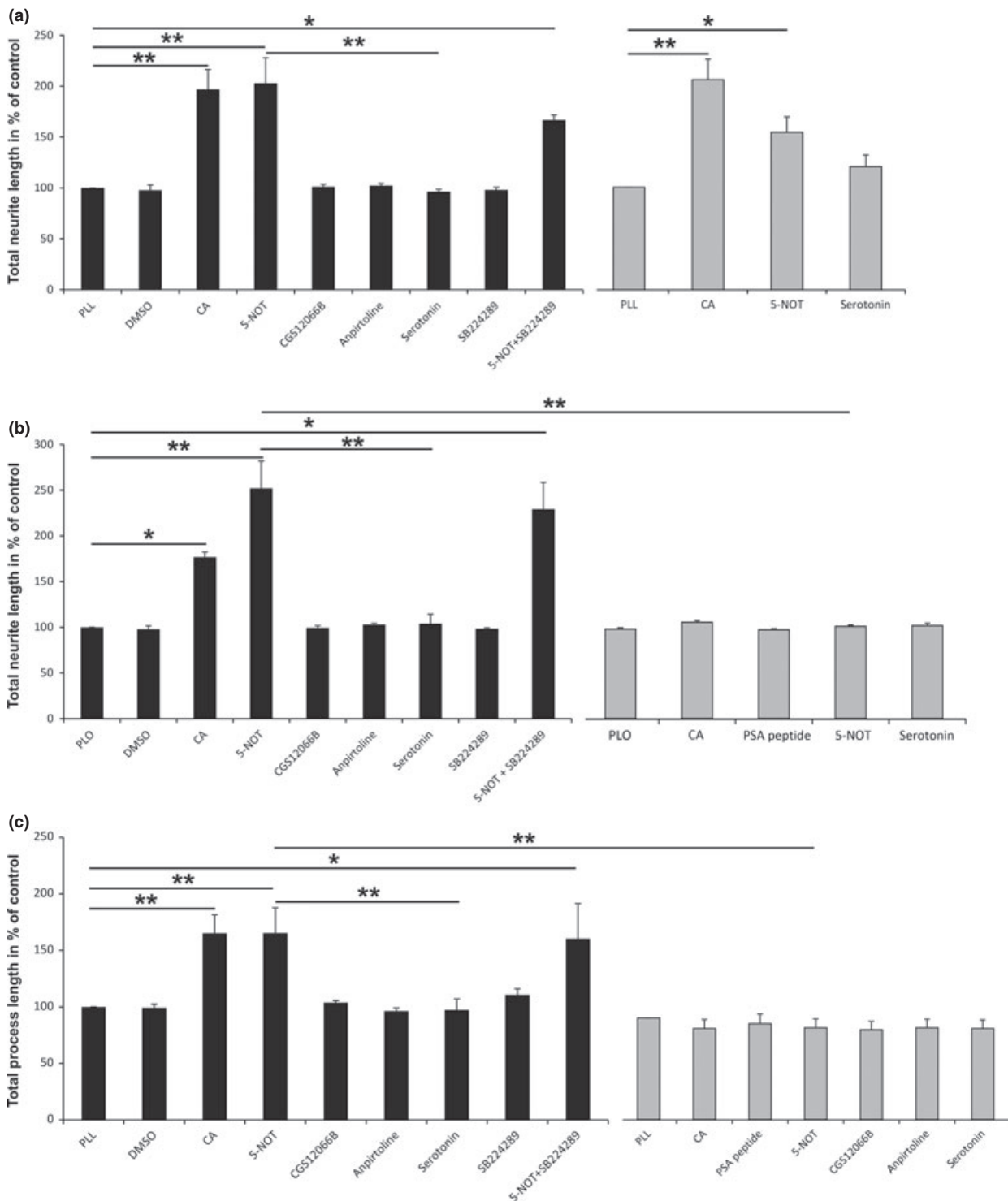


Fig. 3 Nonyloxytryptamine stimulates neurite outgrowth of hippocampal neurons in a neural cell adhesion molecule (NCAM) independent manner, but it stimulates neurite outgrowth of motoneurons and process formation of Schwann cells in an NCAM-dependent manner. Neurite outgrowth of wild-type (black bars, left) and NCAM-deficient (gray bars, right) hippocampal neurons (a), motoneurons (b) or process formation of Schwann cells (c) was determined in the presence and

absence of colominic acid (CA), 5-nonyloxytryptamine oxalate (5-NOT) and 5-HT_{1B} agonists and antagonists (compounds at 100 nM concentration). Data represent mean values of neurite lengths/process length per cell $\pm$ SEM from three independent experiments as compared with PLL only. Asterisks denote significant differences from control. * $p < 0.01$, ** $p < 0.001$.

379 ± 18 µm, respectively (Fig. 4b), indicating that colominic acid and 5-NOT similarly enhance migration.

Migration of cultured astrocytes was reduced by the PSA peptide mimetic and 5-NOT, but not by serotonin and 5-HT_{1B} agonists and antagonists (Fig. 4c and d). PSA peptide mimetic and 5-NOT-treated astrocytes did not close the scratch gap after 72 h in culture and importantly these compounds did not reduce proliferation of these cells (data not shown). Treatment with the 5-HT_{1B} antagonist SB224289 did not abolish the reduced migration induced by 5-NOT (Fig. 4d). In addition, IMR-32 cell migration was increased by treatment with 5-NOT (Fig. 4e and f).

These results indicate that both PSA and 5-NOT enhance Schwann cell and IMR-32 cell migration, but reduce astrocyte migration.

Nonyloxytryptamine stimulates myelination

Since PSA enhances myelination after peripheral nerve injury (Charles *et al.* 2000; Jungnickel *et al.* 2009), we investigated the effects of colominic acid and 5-NOT on myelination of dorsal root ganglion axons by Schwann cells *in vitro*. Colominic acid and 5-NOT enhanced myelination, whereas serotonin was ineffective (Figure S3).

Nonyloxytryptamine treatment leads to Erk phosphorylation and enhanced PSA and NCAM expression in cultured neurons

Treatment of cerebellar granule neurons with 5-NOT led to rapidly increased Erk phosphorylation which was reduced to control values after 2 h (Fig. 5). Levels of total Erk protein were unchanged. Interestingly, 5-NOT treatment also increased PSA and NCAM expression. Treatment with serotonin as control also enhanced phospho-Erk levels, whereas levels of PSA and NCAM remained unchanged (Fig. 5).

Cytotoxicity and apoptosis

Cell viability of IMR-32 cells (MTT and LDH test; Figure S4ai and ii) was significantly decreased after exposure to 5-NOT for 72 h at 5 µM but not at 1 µM concentration, with the percentage of viable cells being reduced to 43% by 5 µM 5-NOT. An increase in LDH release was observed at 5 µM, but not at 1 µM 5-NOT concentration. Treatment with up to 200 nM 5-NOT did not lead to cell death of cerebellar neurons (data not shown).

To elucidate if treatment of IMR-32 cells with 5-NOT induces apoptosis or necrosis, cells were treated with 10 nM 5-NOT and the percentage of live, apoptotic, and necrotic cells was determined using the Annexin V-FITC assay. The numbers of living cells in control cultures (without any treatment) and in treated groups were not significantly different (Figure S4bi). Similarly, no significant difference was observed in the number of apoptotic and necrotic cells in both groups. Based on these observations, 10 nM 5-NOT was chosen for further studies on IMR-32 cells.

Nonyloxytryptamine decreases the number of cells in the G2.M phase

PSA has been shown to modulate growth and differentiation of neuroblastoma cells and PSA levels change during differentiation of neuroblastoma cells, stem cells and neurons (Hildebrandt *et al.* 1998; Decker *et al.* 2002; Poongodi *et al.* 2002). Therefore, we analyzed if 5-NOT has an influence on the cell cycle of neuroblastoma cells (Figure S4bii). Treatment with 10 nM 5-NOT significantly decreased the number of cells in the G2.M phase. These results indicate that 5-NOT may prevent cells from accumulating in the G2.M phase and subsequent apoptosis.

Nonyloxytryptamine induces sprouting of processes and enhances PSA and NCAM expression by IMR-32 cells

5-NOT treatment of IMR-32 led to sprouting of small extensions as seen by immunolabeling for α-tubulin (Figure S4aiii). To correlate 5-NOT induced morphological changes of IMR-32 cells with expressions of PSA and NCAM, triple immunofluorescence staining for PSA (green), NCAM (red) and nuclear staining (DAPI, blue) was performed (Fig. 6a). Increased expression of PSA and NCAM was observed by treatment with 5-NOT as compared to untreated cells or cells treated with nitrendipine (Fig. 6b). This result was confirmed by western blot analysis (Fig. 6c).

Similarly, expression of MAP2, a marker of differentiated neurons, was decreased in compound treated groups as compared to untreated and nitrendipine treated groups, as seen by immunofluorescence staining and western blot analysis (Figure S5a–c).

The combined results indicate that 5-NOT acts as a PSA mimetic and stimulates PSA and NCAM expression by neurons.

Discussion

Recent studies have investigated the potential of glycomimetics for drug design and therapy (Mehanna *et al.* 2010; Irintchev *et al.* 2011; Masand *et al.* 2012b; Prost *et al.* 2012). One important drug target is PSA, which is involved in invasive meningococcal diseases, influenza virus infections (Rameix-Welti *et al.* 2009), cancer (Falconer *et al.* 2012), schizophrenia and depression (Senkov *et al.* 2012), synaptic plasticity (Bonfanti 2006; Bonfanti and Theodosis 2009; Senkov *et al.* 2012), and neuroregeneration (Mehanna *et al.* 2010; Ghosh *et al.* 2012; Masand *et al.* 2012b). PSA also regulates the development of the nervous system, regeneration after injury and synaptic plasticity (Parkash and Kaur 2005; Bonfanti 2006; Rutishauser 2008; Bonfanti and Theodosis 2009; Franceschini *et al.* 2010; Kumar *et al.* 2012). Thus, manipulation of PSA functions has an important therapeutic value in nervous system disorders.

In search for novel PSA mimetics, we identified 5-NOT as PSA mimicking compound. 5-NOT competed with the PSA

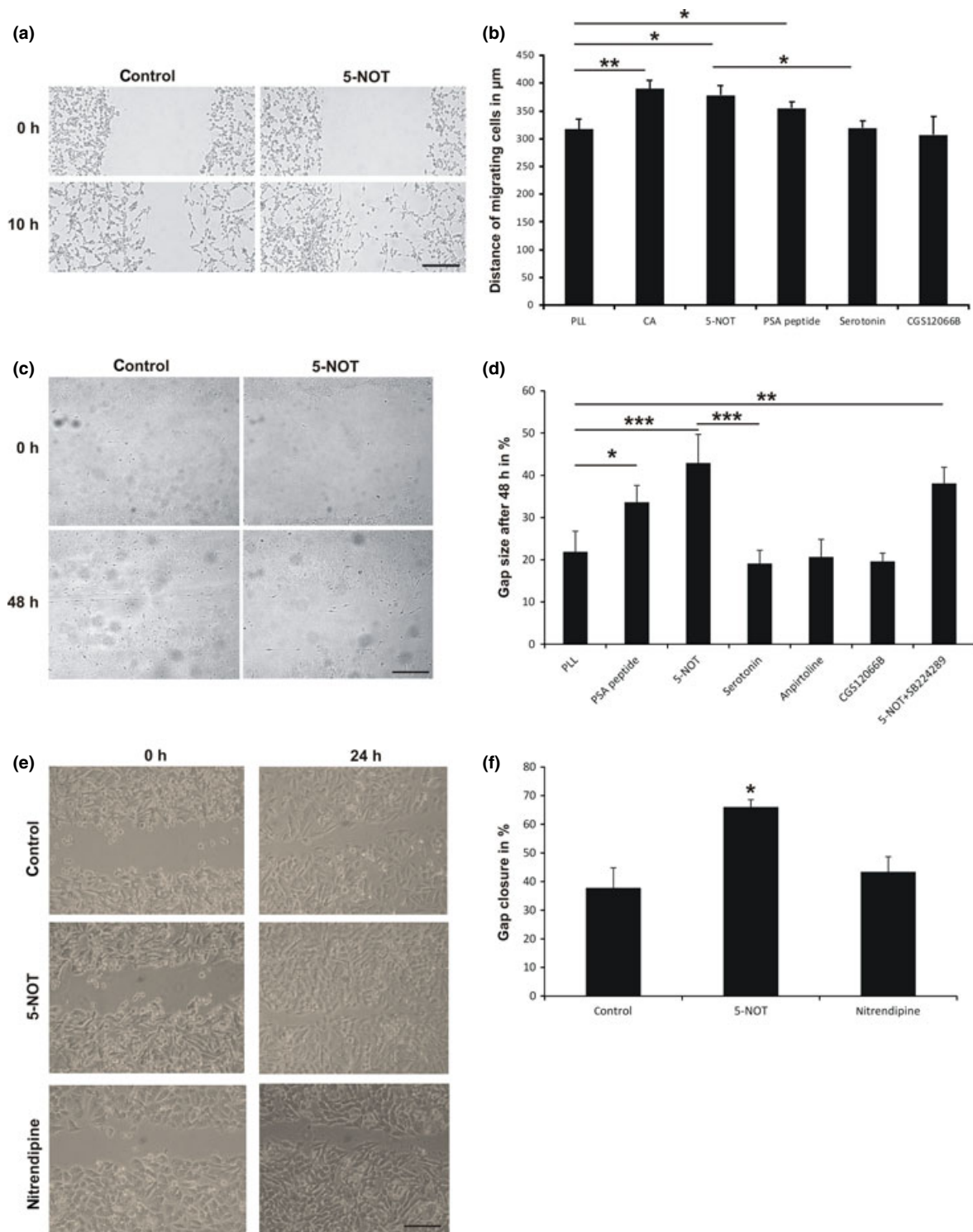


Fig. 4 Nonyloxytryptamine stimulates migration of Schwann cells and IMR-32 cells after scratch injury but inhibits migration of astrocytes into a wound area. Confluent monolayers of wild-type Schwann cells (a, b) and astrocytes (c, d), or IMR-32 cells (e, f) were scratched resulting in a gap of around 800 μm in the cell layer (gap width 100%). Recolonization of the wounded areas (closure of the gap) was studied using an inverted phase-contrast microscope from

0 to 10 h (Schwann cells), 0 to 24 h (IMR-32 cells) or 0 to 48 h (astrocytes). (a, c, e) Representative images of Schwann cells (a), astrocytes (c) or IMR-32 cells (e) 0 h after injury and 10, 24 or 48 h after injury. Scale bars, 200 μm. (b, d, f) Histograms show data representing mean values from three independent experiments. Asterisks denote significant differences from control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

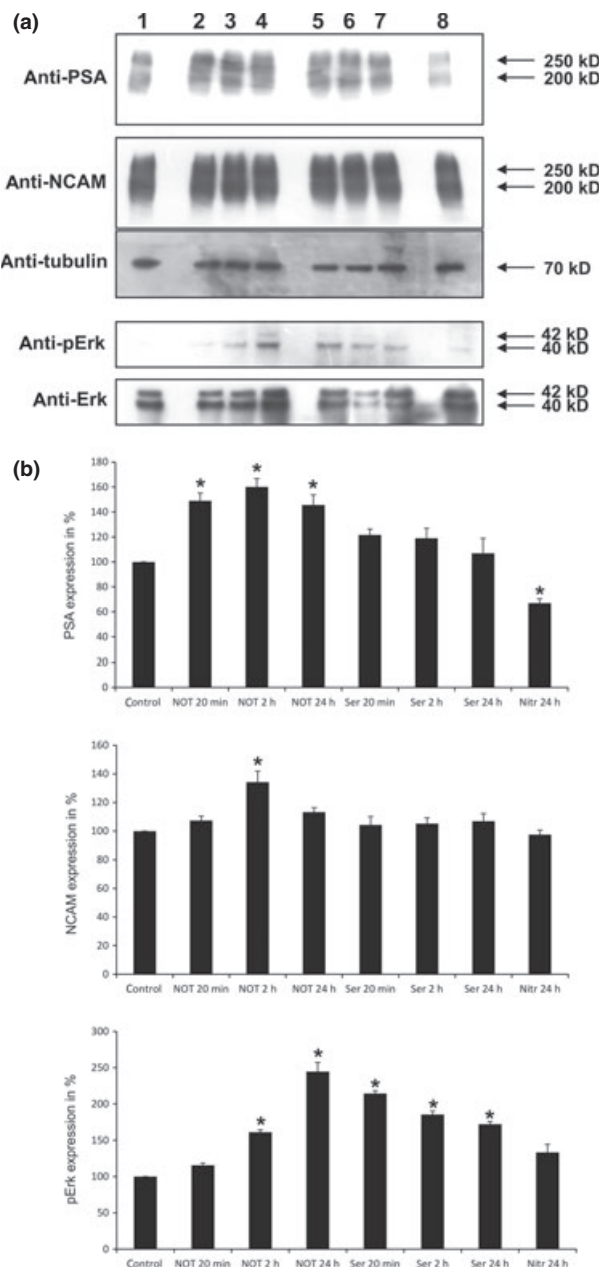


Fig. 5 Nonyloxytryptamine stimulates phosphorylation of Erk and an increase in polysialic acid (PSA) and neural cell adhesion molecule (NCAM) expression. Cerebellar neurons were treated with 5-nonyloxytryptamine oxalate (5-NOT), serotonin or solvent control for 20 min, 2 h, and 24 h. Afterward, cells were lysed and proteins from the lysates were subjected to western blot analysis using anti-phospho-Erk, anti-PSA, and anti-NCAM antibodies. Loading control: anti-tubulin antibody or anti-total Erk antibody. (a) Representative western blots are shown. Lane 1: vehicle control; lane 2: 5-NOT 20 min; lane 3: 5-NOT 2 h; lane 4: 5-NOT 24 h; lane 5: serotonin 20 min; lane 6: serotonin 2 h; lane 7: serotonin 24 h; lane 8: nitrendipine 24 h. (b) Graphs show quantitation of western blot signals for PSA, NCAM, and phospho-Erk from three independent experiments. Mean + SEM values for control-treated probes normalized to the values of loading control set to 100% are shown. * $p < 0.05$.

mimetic peptide, PSA or colominic acid for binding to the PSA-specific monoclonal antibody 735. Modeling of PSA and 5-NOT suggested that 5-NOT can adopt a conformation that is compatible with the structure of PSA as it is bound to the PSA-binding region of antibody 735. 5-NOT stimulated neuritogenesis of cerebellar neurons, hippocampal neurons, and motor neurons as well as process formation of Schwann cells to a similar extent as colominic acid/PSA (Figs 2 and 3). Maximal outgrowth was achieved at 100 nM 5-NOT, which is 50–100 times lower than the optimal concentration of colominic acid/PSA needed to induce maximal stimulation.

Since 5-NOT has been described as 5-HT_{1B} agonist (Glennon *et al.* 1994), it had to be ruled out that stimulation of neurite outgrowth and Schwann cell process formation was because of activation of this receptor. Neither serotonin, CGS12066B nor anpirtoline stimulated neuritogenesis and process formation by Schwann cells, and application of 5-NOT together with the 5-HT_{1B} receptor antagonist SB224289 did not reduce the effect of 5-NOT. Furthermore, the main serotonin receptors expressed by early post-natal cerebellar granule neurons, hippocampal neurons, motoneurons, and Schwann cells are not HT_{1B} receptors, but 5-HT₃, 5-HT₂, 5-HT₄, or 5-HT₇ receptors (Yoder *et al.* 1997; Robert *et al.* 2001; Heckman *et al.* 2009; Oostland *et al.* 2011; Berumen *et al.* 2012) which are not stimulated by 5-NOT. These observations validate 5-NOT as a true PSA mimetic compound.

Interestingly, 5-NOT did not affect NCAM-deficient motoneurons and Schwann cells, suggesting that 5-NOT acts on motoneurons and Schwann cells via NCAM or indirectly via molecules that are dysregulated in expression in the constitutive absence of NCAM and which may react to 5-NOT. However, 5-NOT stimulated neuritogenesis of NCAM-deficient cerebellar and hippocampal neurons up to 80% of the value seen in wild-type cells, suggesting that 5-NOT acts via the PSA-receptors histone H1 and myristoylated alanine-rich C kinase substrate known to mediate PSA-stimulated neurite outgrowth (Mishra *et al.* 2010; Theis *et al.* 2013). In addition, 5-NOT and similarly serotonin enhanced Erk phosphorylation, while only 5-NOT treatment enhanced the expression of PSA in primary neurons and IMR-32 cells. These results show that serotonin and 5-NOT stimulate Erk phosphorylation via different pathways and that only 5-NOT, and not serotonin, interacts with PSA-NCAM and stimulates PSA-NCAM expression and downstream signaling via phosphorylation of Erk.

5-NOT treatment not only reduced the expression of unphosphorylated MAP2 in IMR-32 cells but also reduced the number of cells in the G2.M phase of the cell cycle. These results are in agreement with previous studies, which reported that enhanced expression of MAP2 causes G2.M phase cell cycle arrest and apoptosis (Sánchez *et al.* 2000). Erk mediated phosphorylation of MAP decreases their affinity for microtubules and hence weakens the microtubule

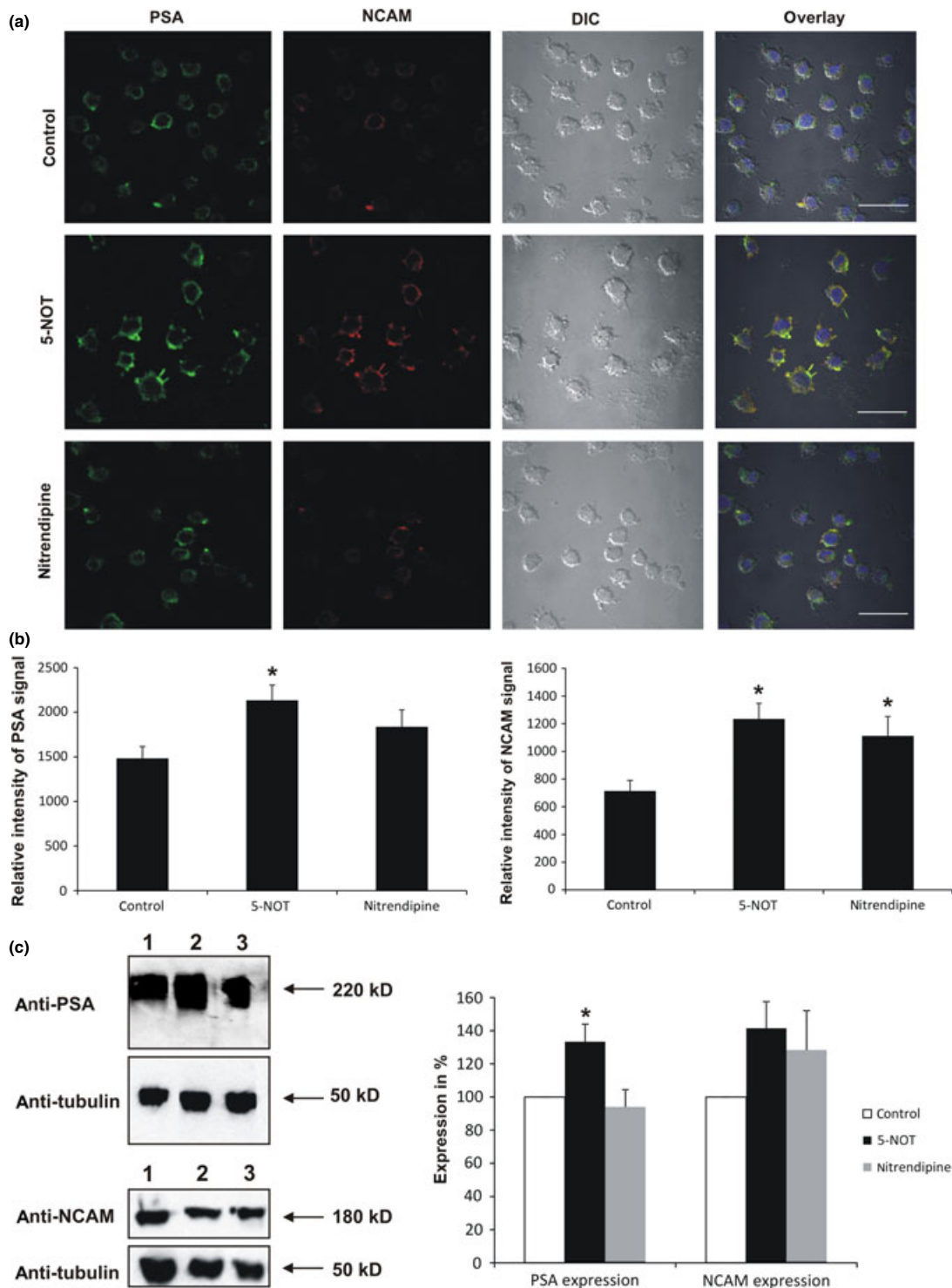


Fig. 6 Nonyloxytryptamine enhances the expression of polysialic acid (PSA)-neural cell adhesion molecule (NCAM) on IMR-32 cells. (a) IMR-32 cells were treated with 5-nonyloxytryptamine oxalate (5-NOT) or nitrendipine (10 nM) or vehicle control (control) for 72 h and stained for PSA (green) and NCAM (red). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Drug treatment increases expression and co-localization of PSA and NCAM. Scale

bar, 50 μ m. (b) Histograms representing the intensity of PSA and NCAM staining in 5-NOT (10 nM) and nitrendipine (control compound; 10 nM) treated cells compared to control cells. * $p < 0.05$. (c) Representative immunoblots of PSA and NCAM showing change in expression in cells treated with vehicle control (control; lane 1), 10 nM 5-NOT (lane 2), or 10 nM nitrendipine (control compound; lane 3).

stabilizing effect of MAPs (Mollinedo and Gajate 2003), which in turn may result in reducing the accumulation of cells in the G2.M phase.

A compound able to stimulate neuroregeneration after trauma is expected not only to trigger beneficial neuronal but also glial functions. Adequate migration of Schwann cells as observed in this study to be induced by 5-NOT is crucial for regeneration after peripheral nerve injury (Schlosshauer *et al.* 2003; Corfas *et al.* 2004). In contrast, proliferation and migration of astrocytes following central nervous system injuries are not beneficial for regeneration (Sofroniew and Vinters 2010), since reactive astrocytes contribute to the glial barrier and release inflammatory cytokines (Barreto *et al.* 2011). Having these considerations in mind, a therapeutic compound should promote Schwann cell migration and inhibit astrocyte migration, as could be shown for 5-NOT.

Identification of novel compounds from synthetic and natural libraries mimicking PSA functions may offer new therapeutic perspectives in acute and chronic neurological impairments. This study suggests that the small organic compound 5-NOT, as PSA mimetic, has the potential to induce neuroplasticity, stimulate Schwann cell migration, process formation and myelination as well as inhibit astrocyte migration after acute injury, thus raising hopes for this molecule to contribute to therapy.

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Competing interests

The authors have declared that no competing interests exist. The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the US Army or the US Department of Defense. This article has been approved for public release with unlimited distribution.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supplementary Materials and methods.

Figure S1. 5-Nonyloxytryptamine oxalate (5-NOT) competes with colominic acid for binding to the PSA-specific antibody 735 (mAb 735).

Figure S2. Representative images of (a) cerebellar neurons, (b) hippocampal neurons, (c) motor neurons and (d) Schwann cells bearing neurites/processes.

Figure S3. Nonyloxytryptamine stimulates myelination of dorsal root ganglion neurons *in vitro*.

Figure S4. Nonyloxytryptamine treatment does not influence the viability of IMR-32 cells.

Figure S5. MAP2 expression is down-regulated in IMR-32 cells after 5-NOT treatment.

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