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# Monoaminylation of Fibrinogen and Glia-Derived Proteins: Indication for Similar Mechanisms in Posttranslational Protein Modification in Blood and Brain

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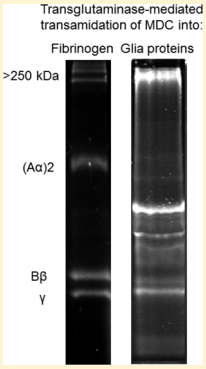
Supporting Information

**ABSTRACT:** Distinct proteins have been demonstrated to be posttranslationally modified by covalent transamidation of serotonin (5-hydropxytryptamin) to glutamine residues of the target proteins. This process is mediated by transglutaminase (TGase) and has been termed "serotonylation." It has also been shown that other biogenic amines, including the neurotransmitters dopamine and norepinephrine, can substitute for serotonin, implying a more general mechanism of "monoaminylation" for this kind of protein modification. Here we transamidated the autofluorescent monoamine monodansylcadaverine (MDC) to purified plasma fibrinogen and to proteins from a primary glia cell culture. Electrophoretic separation of MDC-conjugated proteins followed by mass spectrometry identified three fibrinogen subunits (A$\alpha$, B$\beta$, $\gamma$), a homomeric A$\alpha$2 dimer, and adducts of >250 kDa molecular weight, as well as several glial proteins. TGase-mediated MDC incorporation was strongly reduced by serotonin, underlining the general mechanism of monoaminylation.




**KEYWORDS:** *Serotonylation, transamidation, transglutaminase, fibrinogen, glia*

Serotonin (5-hydroxytryptamine, 5HT) was first discovered in the blood serum as a vasoconstrictor substance.[1] This monoamine is synthesized in enterochromaffin cells of the gastrointestinal tract and taken up into blood platelets by a serotonin transporter protein (SERT) and accumulated into vesicles by the vesicular monoamine transporter vMAT.[2,3] Contact of platelets to a site of vascular injury leads to exocytotic release of 5-HT, resulting in platelet adhesion and blood-clot formation.[4] This process involves transglutaminase (TGase)-mediated conjugation of serotonin into polypeptides, a posttranslational protein modification which has been termed "serotonylation"[5] and was first described in a model of hemostasis.[6] In this model, serotonylated proteins on "coated-platelets"[7] tightly bind to respective serotonin binding sites on fibrinogen and thrombospondin, leading to the formation of stable multivalent complexes and subsequently promoting blood-clot formation.[6,8,9]

Serotonylation has been shown to occur not only extracellularly but also inside cells. Here, serotonylation was demonstrated within platelets, in which small GTPases are constitutively activated upon serotonylation.[5] In recent years, it has been shown that the serotonylation of small GTPases as a regulatory mechanism to modulate the activity of small GTPases also occurs in pancreatic beta cells,[10] vascular smooth muscle cells,[11,12] and rat cortical neuronal cell lines.[13,14] In addition to serotonin, the catecholamines dopamine (DA) and norepinephrine (NE) have been reported to be selectively transamidated into proteins by TGase.[15,16] Histamine has also been demonstrated recently to be transamidated to small G proteins in a TGase-dependent manner.[17] Based on these findings, a general mechanism of TGase-mediated "monoaminylation" (including histaminylation, serotonylation, dopaminylation, and norepinephrinylation) has been proposed as a novel form of posttranslational protein modification (reviewed in ref 16).

In the central nervous system (CNS), the biogenic amines 5-HT, DA, and NE also serve as neurotransmitters, implicated in the control of numerous behavioral and physiological functions. Binding and transamidation studies with tritiated 5-HT, DA, and NE to whole brain homogenate have revealed that TGase transamidates these monoamines to neuronal and glial proteins, one of which has been identified as fibronectin.[18,19] Furthermore, we visualized the TGase-mediated incorporation of the fluorescent biogenic amine monodansylcadaverine (MDC) into fibronectin. MDC-transamidation was selectively inhibited by 5-HT, DA and NE, again confirming a more general process of "monoaminylation".[19] These findings suggest that monoaminylation also occurs in neural tissue. Considering the importance of serotonylation in blood clot formation, comparable tight interactions between serotonylated proteins on neurons and glia with particular binding proteins may also enhance cell−cell contacts in the CNS and contribute to the consolidation of neural networks.

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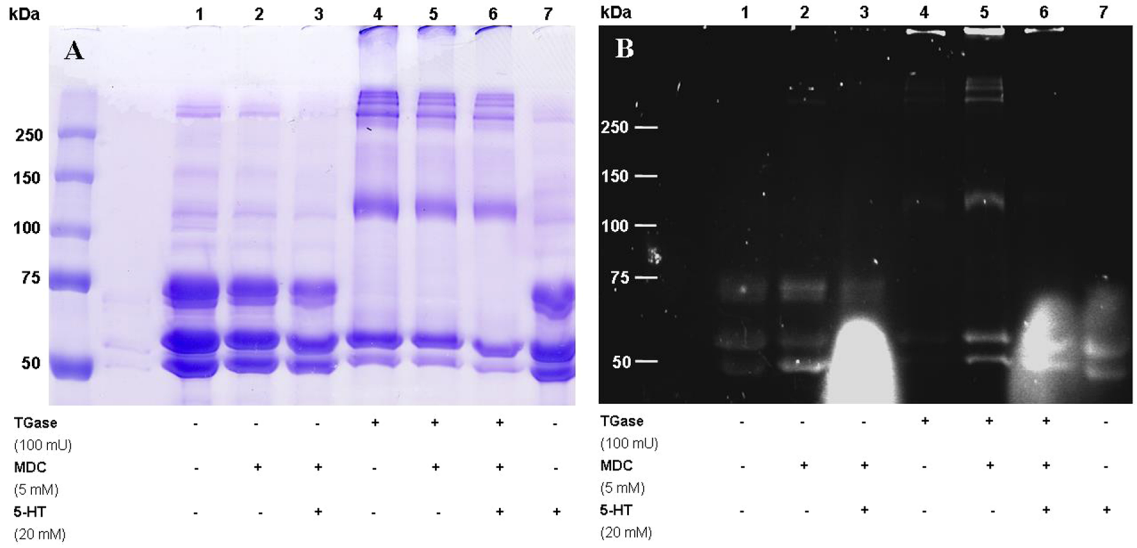


**Figure 1.** Treatment of human plasma fibrinogen with TGase and monoamines. A total of 50 $\mu$g of purified fibrinogen was incubated in the absence or presence of TGase, MDC, and 5-HT as indicated and described in Methods. Subsequently, the proteins were subjected to PAGE and stained with Coomassie Brilliant Blue (A) and visualized under UV light (B). Bands migrating at 47, 56, 120, and >250 KDa were excised and analyzed using MS.

In order to identify further possible glial target proteins for TGase-mediated monoaminylation, in the present study, we exploited the approach of visualizing "MDC-monoaminylated" protein bands combined with mass spectrometry. As a control for the validity for this experimental design, we used plasma fibrinogen, as this protein, when isolated from COAT-platelets, has been shown to contain covalently linked serotonin[6] and thus should also serve as a target for TGase-mediated conjugation with MDC. As a source for possible new glial target proteins for monoaminylation, we used protein homogenates prepared from mouse primary glial cell cultures.

## RESULTS AND DISCUSSION

We previously demonstrated that the monoamines 5-HT, DA, NE, and the fluorescent MDC are selectively transamidated by TGase to fibronectin from both glia C6 cells and purified from blood plasma.[18,19] In the present study, MDC was transamidated to purified plasma fibrinogen and the resulting products were visualized by Coomassie-staining and UV light as described in Methods. Fibrinogen is a 340 kDa glycoprotein which is composed of two sets of three homologous polypeptide chains known as A$\alpha$, B$\beta$, and $\gamma$ chains that assemble to form an hexameric structure (A$\alpha$B$\beta\gamma$)2.[20−22] As shown in Figure 1, under reducing conditions, purified plasma fibrinogen appears in three major bands with molecular weights of 63, 56, and 47 kDa, resembling the A$\alpha$, B$\beta$, and $\gamma$ chains, respectively. The addition of MDC and/or 5-HT did not alter this subunit composition. The addition of TGase, however, diminished the intensity of the 47 kDa band and eliminated the 63 kDa band; instead a new band of about 120 kDa and higher adducts of >250 kDa were now observed in both the running gel and in the sample wells of the stacking gel (Figure 1, lanes 4−6).

In parallel, we monitored MDC-labeling in fibrinogen by UV illumination of the protein gels. As shown in Figure 1, the addition of TGase induced significant incorporation of MDC into the B$\beta$ and $\gamma$ fibrinogen subunits, as well as into the 120 kDa band and the higher adducts of >250 kDa. Fluorescence of the 120 kDa and the >250 kDa adducts was not observed in the absence of TGase or in the presence of 5-HT, indicating TGase-dependent transamidation into these polypeptides. The fainter and more diffused UV staining of the enriched A$\alpha$, B$\beta$, and $\gamma$ chains also seen in the absence of TGase may reflect nonspecific protein staining of these strongly Coommassie-stained protein bands. Indeed, UV staining of large amounts of proteins due to the excitation of tryptophan residues in polypeptide chains has been previously described, and this knowledge has also been been exploited by others to rapidly visualize proteins in polyacrylamide gels soaked in trichloroacetic acid or chloroform followed by illumination with UV light.[23] The tryptophan moiety of serotonin added in lanes 3, 6, and 7 in Figure 1 explains the strong UV illumination in these lanes.

For mass spectrometry, the fluorescent bands were excised and treated as described in Methods. Mass spectrometric analyses of the B$\beta$ and $\gamma$ bands confirmed the sequence identity of respective MDC-labeled peptides. Examples of MS and MS/MS spectra of the MDC modified fibrinogen beta peptide (AAATqK) are shown in Figure 2. The 120 kDa band was shown to contain MDC-modified peptides of the A$\alpha$ subunit, thus most likely representing an A$\alpha$2 dimer. This finding is consistent with an immunoelectrophoretic study in which the formation of homologous A$\alpha$2 dyads were demonstrated upon cross-linking of fibrinogen by TGase.[24] The higher molecular weight adducts >250 kDa contained MDC-labeled peptides of the A$\alpha$ and B$\beta$ subunits as well as peptides of the $\gamma$-subunit, which, however, did not contain covalently bound MDC. All MDC-labeled peptides were identified in bands isolated from two independent gels and are summarized in Table 1.

Taken together, these findings suggest that the recombinant TGase2 used for this set of experiments covalently transamidates MDC into all three fibrinogen subunits and cross-links the subunits to higher adducts. TGase-mediated cross-linking of the A$\alpha$ subunits into A$\alpha$2 dyads and higher adducts seems to be very efficient, as it eliminates the existence of A$\alpha$ monomers. Thus, our results emphasize the dual functions of TGases to modify proteins by the formation of isopeptide bonds through protein cross-linking via $\varepsilon$-($\gamma$-glutamyl)lysine bonds and/or through incorporation of biogenic amines at


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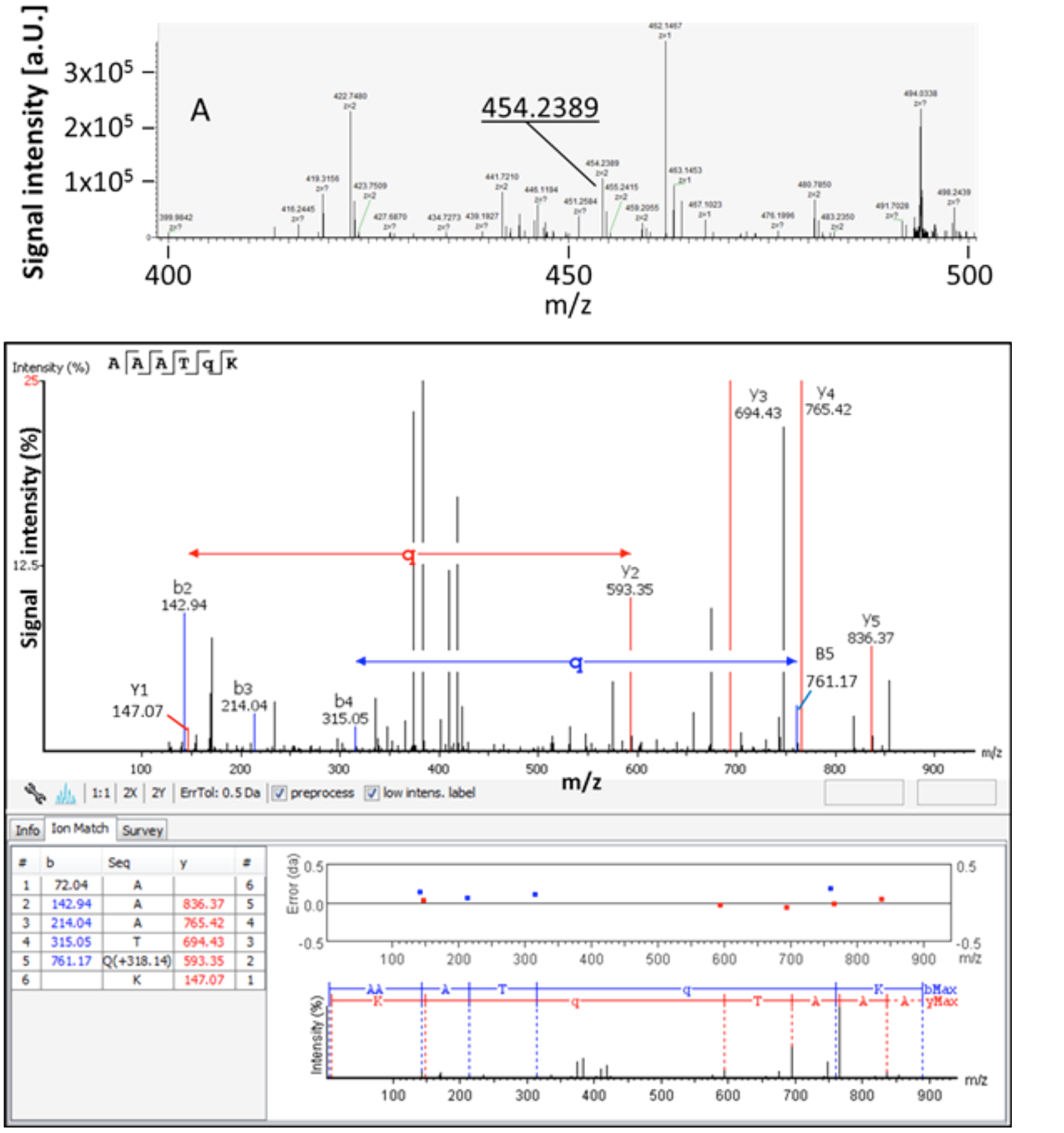


**Figure 2.** MS and MS/MS spectra of the MDC-modified fibrinogen $\beta$-subunit. (A) MS spectrum containing the MDC modified fibrinogen beta peptide (AAATqK) with $m/z$ 454.2389. (B) MS/MS spectrum of the peptide AAATqK and its corresponding peptide fragments as a spectrum and in table format. The MDC modified Gln residue (q) was identified in the b (blue q) as well as the y (red q) fragment ion series. The MDC modification induces a peptide mass shift of 318.14 Da.

selected peptide-bound glutamine residues.[25,26] The fact that the addition of 5-HT strongly reduces TGase-mediated MDC transamidation to fibrinogen together with the recent report on TGase-mediated histaminylation of fibrinogen[27] provides further evidence for a general posttranslational protein modification by "monoaminylation".

In order to detect other neural target proteins for monoaminylation, we used whole protein homogenates from mouse primary glial cells cultures. This culture was prepared according to ref 28 and is thought to better reflect the true nature of astroglial cell populations as compared to the immortalized C6 glioma cell line. Protein homogenates were treated with TGase comparable to fibrinogen described in Methods. As shown in Figure 3, Coomassie staining revealed that treatment of the glia protein preparation with recombinant TGase in the presence or absence of MDC and 5-HT did not alter the protein pattern in the SDS gel as strongly as did the same treatment of fibrinogen (see Figure 1A). This suggests that in our glial protein preparation the addition of recombinant TGase does not efficiently increase the cross-linking of target proteins via $\varepsilon$-($\gamma$-glutamyl)lysine bonds.


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**Table 1. Identified Fibrinogen Subunits with their MDC-Labeled Peptides[a]**

| protein | peptide |
|---|---|
| human fibrinogen A$\alpha$ | R.HQSACK.D |
| | R.EVDLKDYEDQQK.Q |
| | K.ALTDMPQMR.M |
| | K.QFTSSTSYNR.G |
| | K.NSLFEYQK.N |
| human fibrinogen B$\beta$ | K.IRPFFPQQ |
| | K.AAATQK.K |
| | R.YYWGGQYTWDMAK.H |
| human fibrinogen $\gamma$ | R.LTIGEGQQHHLGGAK.Q |

[a]Peptides were identified using PEAKS (Bioinformatics Solutions Inc.). Modified Glutamine residues are shown in bold. Detailed information on all identified peptides is provided as Table 1A in the Supporting Information.

UV illumination revealed four distinct fluorescent bands not seen in the absence of TGase, thus suggesting TGase-mediated transamidation of MDC into the respective proteins (Figure 3B). Again, the slight background fluorescence observed is most likely due to nonspecific protein fluorescence. In contrast to the transamidation experiments shown for purified fibrinogen in Figure 1, we did not observe the strong UV illumination in the lanes in which 5-HT was added. This is most likely due to the different sample preparation (fibrinogen was diluted directly from the buffer provided by MERCK into the Laemmli sample buffer, whereas the glial proteins were first dissolved in lysis buffer before mixing with the sample buffer; see Methods section).

MDC-labeled bands were then excised from the gel under UV light and further processed for analysis with mass spectrometry, as described in Methods. Peptides were identified using PEAKS (Bioinformatics Solutions Inc.). Modified Glutamine residues are shown in bold. Detailed information on all identified peptides is provided as Table 1A in the Supporting Information. MDC-labeled peptides and the respective proteins that were identified using a mouse protein data bank are listed in Table 2. All peptides shown were identified in two independent experiments.

**Table 2. Identified Proteins with their MDC-Labeled Peptides[a]**

| protein | peptide |
|---|---|
| vimentin | R.QQYESVAAK.N |
| | R.DGQVINETSQHHDDLE |
| filamin A | K.AGVAPLQVK.V |
| actin cytoplasmic | K.DSYVGDEAQSK.R |
| histone H1.2 | K.AASGEAKPQAK.K |
| Histone 1.3 | K.GTLVQTK.G |
| heat shock cognate 71 kDa protein | K.NQVAMNPTNTVFDAK.R |
| | R.FDDAVVQSDMK.H |
| | R.MVQEAEK.Y |
| | K.VEIIANDQGNR.T |
| pyruvate kinase M1 | R.SAHQVAR.Y |
| | R.QAHLYR.G |
| V-type proton ATPase subunit B_ brain isoform | R.SGQVLEVSGSK.A |

[a]Peptides were identified using PEAKS (Bioinformatics Solutions Inc.). Modified Glutamine residues are shown in bold. Detailed information on all identified peptides is provided as Table 2A in the Supporting Information.

In our earlier studies,[19] we discussed the possibility that, in the CNS, the monamines serotonin, dopamine, and norepinephrine not only play important roles as neurotransmitters, but may also be important TGase substrates. Considering the nonsynaptic release of 5-HT in the brain and the role of 5HT in thrombus formation, we also hypothesized that, in the CNS, TGases may transamidate extracellular serotonin to neuronal and glial cell surface-exposed proteins and/or proteins of the extracellular matrix (ECM). Comparable to the blood clotting

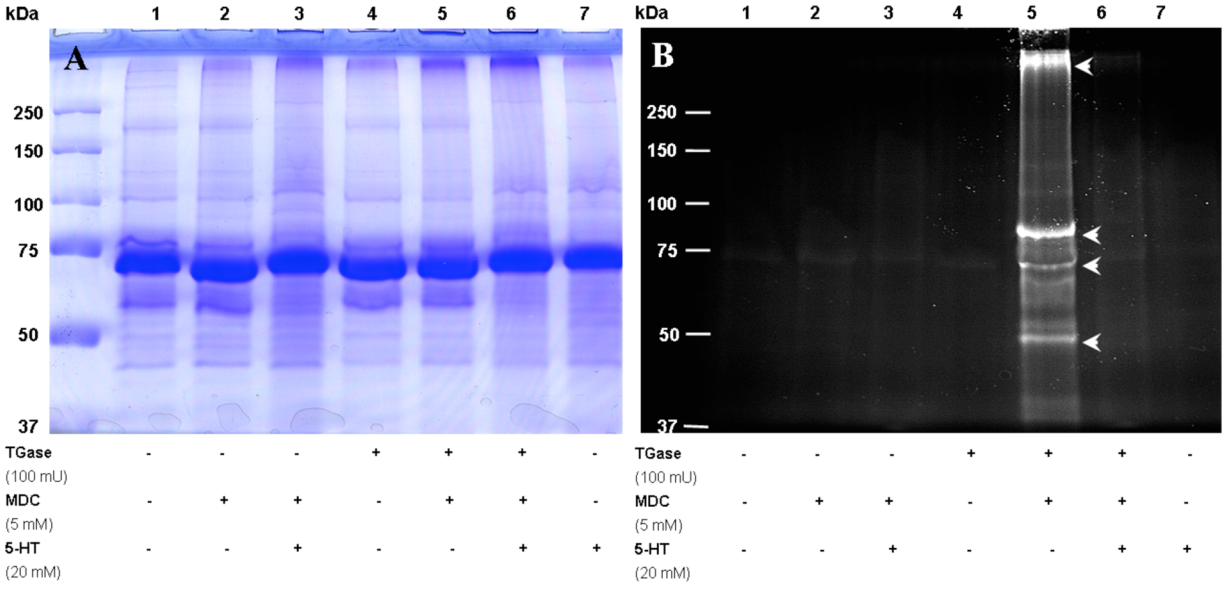


**Figure 3.** Treatment of protein preparations from mouse primary glial cell cultures with TGase and monoamines. A total of 50 $\mu$g protein homogenate was incubated in the absence or presence of TGase, 5-HT, and MDC as indicated and described in Methods. Subsequently, the proteins were subjected to PAGE and stained with Coomassie Brilliant Blue (A). In addition, MDC-labeled bands were visualized under UV light. The bands marked with arrowheads were excised under UV light and further processed for mass spectrometry as described in Methods.



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process, tight interactions between serotonylated proteins and 5HT binding proteins may be important for the formation of proteinaceous networks. Consistent with this hypothesis, we identified the ECM protein fibronectin as a target for monoaminylation.[19]

Interestingly, none of the identified MDC-labeled peptides belong to a protein of the ECM. This is most likely due to the fact that in this study, we first prepared a total protein homogenate from glia cells, which was then subjected to TGase-mediated MDC transamidation, whereas in our earlier study, intact C6 cells were first MDC-labeled and then subjected to SDS PAGE. Furthermore, despite using protein homogenate from a primary glia cell culture, none of the identified polypeptides was solely of neural identity, with the exception of the brain isoform of V-type proton ATPase. Actin, vimentin, heatshock proteins and histones have already been shown to be TGase 2 targets by others (for a detailed overview, see ref 25).

In addition, our data confirm findings by the group of Watts, who very elegantly demonstrated serotonylation of actin and filamin A in smooth muscle cells using biotinylated 5-HT as a TGase substrate. Based on those findings, a new mechanisms of action for 5-HT in muscle contraction had been proposed.[12] At this point, it should mentioned that by transamidating biotinylated 5-HT, Watts and colleagues clearly identified "serotonylated" proteins, whereas we used fluorescent MDC as a substrate and therefore here identify "monoaminylated" proteins. Recently we showed that TGase-mediated incorporation of the monoamine MDC into fibronectin as well as into living C6 glioma cells is blocked by the amines serotonin, dopamine, norepinephrine, and cystamine.[18,19] Furthermore, we also showed that TGase-mediated transamidation of [$^3$H]5-HT is inhibited by the amines dopamine, noradrenaline and cystamine and that TGase-mediated transamidation of [$^3$H]-dopamine and [$^3$H]norepinephrine is completely blocked by all nonradioactive amines listed above.[19] These findings strongly suggest that all Gln residues that are accessible for monoaminylation (i.e., transamidation of serotonin, dopamine, norepinephrine, and MDC, repectively) are identical.

Taken together, the findings of our present study have validated the approach of visualizing "MDC-monoaminylated" proteins combined with mass spectroscopy to be successful in identifying target proteins for TGase-mediated monoaminylation in a homogeneous cell population. Extending this methodology into other cellular systems (e.g., primary hippocampal neurons or homogeneous serotonergic neurons differentiated from embryonic stem cells[29]) will allow for the identification of brain-specific substrate proteins for monoaminylation. Differential MDC-transamidation, such as whole cellular protein homogenates versus cell-surface transamidation of intact cells, should further allow for distinguishing between intracellular and cell surface-exposed target proteins for monoaminylation.

## METHODS

**Materials.** Serotonin (5-HT) hydrochloride (H-9523) and monodansylcadaverine (30432) were obtained from SIGMA Aldrich. Human plasma fibrinogen (341576) was obtained from Merck Millipore, Merck KGaA Germany. All other chemicals were of analytical grade.

**Experimental Methods.** Mouse Primary Glial cells cultures. Mouse purified primary glial cultures were prepared from brains of wild type NMRI mouse (Cr;NMRI (Han) from Charles River Laboratories International, Inc. Germany) newborn mouse brains (Days P0) as described previously.[28] Briefly, mice were sacrificed and brains were isolated and cortices prepared. Meninges were removed, cortices were minced, and cells were dissociated. Cells were suspended in Dulbecco's modified Eagle's medium, including 10% fetal calf serum (FCS, Life Technologies, Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin (Life Technologies, Gibco), plated on T75 (Sarstedt, Germany), and incubated at 37 C in a humid atmosphere with 5% $CO_2$.

Preparation of mouse whole glia protein homogenate. Cultured glia cells were scraped at a confluence of about 80% with ice cold lysis buffer (50 mM Tris-HCl, 150 mM $NaCl_2$ pH 7,4) and then sonicated on ice (one time for 30 s at 200 W) followed by hand homogenizing with 20 strokes in a potter. The protein concentration of whole glia protein homogenate was 2.3 mg/mL as determined according to Laemmli.[30] After addition of 5% glycerol, the homogenates were aliquoted and directly frozen and stored at −20 °C.

Expression and purification of active recombinant TGase. For expression of the guinea pig TGase, we applied the method using the chemical chaperone betaine as described in ref 31. Briefly, we grew *Escherichia coli* in the presence of 2.5 mM betaine and 1 M sorbitol. The expression of tissue TGase was induced by addition of 1 mM IPTG at 28 °C for 20 h as described. For purification, the culture (500 mL) was centrifuged at 1500*g* for 30 min at 4 °C and the pellet resuspended in 8 mL of lysis buffer containing 10 mM imidazole and 4 mg/mL lysozyme. After incubation for 30 min, the culture was then sonicated on ice (three times for 30 s at 200 W) and centrifuged (2000*g*, 30 min, on ice). The resulting supernatant was incubated with 2 mL of equilibrated Ni-NTA agarose (QIAGEN) for 1 h at 4 °C with shaking. The agarose solution was then transferred onto a column, and the lysate was run through. The column was washed twice with wash buffer containing 20 mM imidazol. The TGase was then eluted four times with 1 mL of elution buffer containing 250 mM imidazol following by desalting with Econo-Pac 10-DG columns (Bio-Rad) according to the manufacturer's protocol. Each fraction was then analyzed via SDS PAGE and Western blotting using the antibody against TGase2 (H-237). The first two elution fractions were pooled, and enzyme activity was determined as described.[31] Specific activity was routinely between 10 and 15 U/mg protein. Desalted elutions were stored at 4 °C.

TGase-mediated incorporation of monodansylcadaverine (MDC) into fibrinogen and whole glia protein homogenate. For visualization of TGase-mediated conjugation of MDC to distinct proteins, 50 $\mu$g of fibrinogen and 50 $\mu$g of whole glia protein homogenate were incubated in the presence and absence of 5 mM MDC, 100 mU recombinant TGase, and 20 mM 5-HT in a final volume of 100 $\mu$L of enzyme buffer (250 mM Tris-HCl, 8.75 mM $CaCl_2$ pH 7.5) with gentle shaking at 37 °C overnight.

*Electrophoresis and Fluorography.* SDS polyacrylamide gelelectrophoresis (SDS-PAGE) in 9% gels was performed according to the method of Laemmli.[32] For visualization of TGase-mediated conjugation of MDC incorporation, 50 $\mu$g of fibrinogen or 50 $\mu$g of whole glia protein was incubated with 5 mM MDC as described above. In all experiments, the samples were heated to 95 °C for 5 min prior the subjection to SDS-PAGE. MDC labeled protein bands were visualized by fluorescence under UV light followed by Coomassie staining of the same SDS-PAGE. All experiments were performed at least three times.

*Mass Spectrometry.* The major fluorescent bands of MDC-labeled fibrinogen and glia proteins were excised from two independent gels, and protein samples were in-gel digested with trypsin as described previously.[19] Extracted peptides were redissolved in 0.1% trifluoroacetic acid and loaded on a C18 precolumn (Acclaim; Dionex, Idstein, Germany) using a RSLCnano HPLC system (Dionex, Idstein, Germany). Peptides were then eluted with an aqueous−organic gradient, resolved on a C18 column (Acclaim; Dionex, Idstein, Germany) with a flow rate of 300 nL/min, and electrosprayed into a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany). A Triversa Automate instrument (Advion biosciences, Ithaca, NY) was used as the ion source. Each scan cycle consisted of one FTMS full scan and up to seven ITMS dependent MS/MS scans of the seven most intense ions. Dynamic exclusion (30 s), mass width


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(10 ppm), and monoisotopic precursor selection were enabled. All analyses were performed in positive ion mode. Extracted MS/MS spectra were searched against the Uniprot/Swissprot database (human or mouse) using the PEAKS search engine (Bioinformatics Solutions Inc.) accepting common variable modifications and one missed tryptic cleavage. Peptide tolerance was ±10 ppm, and MS/MS tolerance was ±0.5 Da. The MDC modification was manually edited and integrated in the database searches.

## ■ ASSOCIATED CONTENT

### Supporting Information

Tables 1A and 2A with detailed information on all peptides detected including the measured mass over charge ($m/z$), their amino acid positions, accuracy of measurement in ppm, and indication of the gel band in which the peptides were detected. This material is available free of charge via the Internet at http://pubs.acs.org/.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

5-HT, 5-hydroxytryptamine; DA, dopamine; MDC, monodansylcadaverine; NE, norepinephrine; TGase, transglutaminase

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