Regulation of Transglutaminase by 5-HT2A Receptor Signaling and Calmodulin

Ying Dai

Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_diss

Part of the Neuroscience and Neurobiology Commons

Recommended Citation
Dai, Ying, "Regulation of Transglutaminase by 5-HT2A Receptor Signaling and Calmodulin" (2009). Dissertations. 86.
https://ecommons.luc.edu/luc_diss/86

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2009 Ying Dai
LOYOLA UNIVERSITY CHICAGO

REGULATION OF TRANSGlutaminase BY
5-HT2A RECEPTOR SIGNALING AND CALMODULIN

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY
YING DAI
CHICAGO, IL
DECEMBER 2009
ACKNOWLEDGEMENTS

Over five years of study at two great Universities make it impossible for me to extend my gratitude to all of the people who have assisted me throughout my doctoral education. First of all, I wish to express my gratitude to all the members of my dissertation committee: Dr. George Battaglia, Dr. Thackery Gray, Dr. Nancy Muma, Dr. Edward J. Neafsey and Dr. Karie Scrogin. Their kind encouragement, perceptive criticism, and willing assistance made this dissertation possible. I sincerely thank my advisor, Dr. Nancy Muma, who has supervised my doctoral research, overseen the planning of the project and given my work minute consideration for the last five years. Without her mentoring and prodding, I would never have made it this far.

I appreciate the help of many KU faculties who have supported me during my dissertation studies. I would especially like to extend my gratitude to Dr. Qian Li and Dr. Stephen Fowler, for their invaluable guidance and friendship during this process.

I would also like to thank the faculty and staff of Neuroscience Program and my laboratory colleagues who have provided me with tremendous support in my graduate studies at Loyola University Chicago. In particular, I would like to thank Dr. Lydia DonCarlos, Peggy Richied, Nichole Dudek, Ju Shi, Cuihong Jia, Bozena Zemaitaitis and Fran Garcia.

Lastly, I want to take this opportunity to thank my loving parents, Yiquan Dai and
Zhiyang Song, who always support me and believe in me. Even though they are thousands of miles away, their constant love has given me much needed encouragement and inspiration throughout all of my work in graduate school.

I am grateful to the financial support from High-Q/CHDI Foundation and NIH (Grant# MH068612) that made the research in this dissertation possible.
Dedicated to my parents—

Your love and support have made me the person I am today.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ACKNOWLEDGEMENTS</th>
<th>iii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiv</td>
</tr>
</tbody>
</table>

## CHAPTER ONE: BACKGROUND

<table>
<thead>
<tr>
<th>INTRODUCTION</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>REVIEW OF RELEVANT LITERATURE</td>
<td>7</td>
</tr>
<tr>
<td>Transglutaminases (TGases), a family of enzymes</td>
<td>7</td>
</tr>
<tr>
<td>Physiological functions of TGases</td>
<td>8</td>
</tr>
<tr>
<td>Regulatory mechanisms and substrates of TGases</td>
<td>9</td>
</tr>
<tr>
<td>5-HT$_{2A}$ receptor signaling and small G protein transamidation</td>
<td>11</td>
</tr>
<tr>
<td>Small G proteins</td>
<td>18</td>
</tr>
<tr>
<td>Calmodulin (CaM)</td>
<td>23</td>
</tr>
<tr>
<td>Pathological role of TGases in neurodegenerative diseases</td>
<td>25</td>
</tr>
<tr>
<td>Alzheimer’s disease (AD) and TGase</td>
<td>25</td>
</tr>
<tr>
<td>Parkinson’s disease (PD) and TGase</td>
<td>26</td>
</tr>
<tr>
<td>Huntington's disease (HD) and TGase</td>
<td>27</td>
</tr>
</tbody>
</table>

## CHAPTER TWO: TRANSGLUTAMINASE-CATALYZED TRANSAMIDATION: A NOVEL MECHANISM FOR RAC1 ACTIVATION BY 5-HT2A RECEPTOR STIMULATION

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>35</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>37</td>
</tr>
<tr>
<td>RESULTS</td>
<td>44</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>66</td>
</tr>
</tbody>
</table>

## CHAPTER THREE: PHOSPHOLIPASE C, CALCIUM AND CALMODULIN SIGNALING ARE REQUIRED FOR 5-HT2A RECEPTOR-MEDIATED TRANSAMIDATION OF RAC1 BY TRANSGLUTAMINASE
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Quantitative analysis of neuropathology</td>
<td>133</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Molecular switch of the small G-proteins</td>
<td>19</td>
</tr>
<tr>
<td>2.</td>
<td>Proposed involvement of TGase and CaM in the formation of htt-containing aggregates</td>
<td>33</td>
</tr>
<tr>
<td>3.</td>
<td>Serotonin-induced increase of Rac1 transamidation in A1A1v cells</td>
<td>52</td>
</tr>
<tr>
<td>4.</td>
<td>Serotonin receptor specificity on induction of Rac1 transamidation in A1A1v cells</td>
<td>53</td>
</tr>
<tr>
<td>5.</td>
<td>The effects of cell differentiation on Rac1 transamidation</td>
<td>55</td>
</tr>
<tr>
<td>6.</td>
<td>Rac1 activity was transiently increased after serotonin treatment</td>
<td>57</td>
</tr>
<tr>
<td>7.</td>
<td>Dose-dependent effects of cystamine on Rac1 transamidation</td>
<td>58</td>
</tr>
<tr>
<td>8.</td>
<td>Rac1 activity is inhibited by cystamine</td>
<td>59</td>
</tr>
<tr>
<td>9.</td>
<td>Knockdown of TGase2 by siRNAs prevents Rac1 transamidation</td>
<td>61</td>
</tr>
<tr>
<td>10.</td>
<td>Transamidation of serotonin to Rac1 is mediated by TGase</td>
<td>63</td>
</tr>
<tr>
<td>11.</td>
<td>DOI stimulates transamidation of Rac1 to serotonin to a similar extent as those treated with serotonin</td>
<td>65</td>
</tr>
<tr>
<td>12.</td>
<td>Effect of PLC inhibition on DOI-induced Ca$^{2+}$ signals and TGase-modified Rac1 in A1A1v cells</td>
<td>86</td>
</tr>
<tr>
<td>13.</td>
<td>Pre-incubation with a Ca$^{2+}$ chelator attenuated the DOI-mediated elevation of cytosolic Ca$^{2+}$ and TGase-modified Rac1</td>
<td>88</td>
</tr>
<tr>
<td>14.</td>
<td>The Ca$^{2+}$ ionophore mimiced the DOI-induced increase in cytosolic Ca$^{2+}$ and TGase-catalyzed transamidation of Rac1</td>
<td>90</td>
</tr>
<tr>
<td>15.</td>
<td>The CaM inhibitor W-7 caused a dose-dependent reduction in DOI-stimulated TGase-modified Rac1</td>
<td>92</td>
</tr>
</tbody>
</table>
16. Rac1 activity-related domains and potential TGase-targeted amino acid residues

17. Schematic representation showing 5-HT\textsubscript{2A} receptor signaling-mediated transamidation of Rac1 by TGase

18. Effect of CaM-fragment on body weight and survival

19. Video-based gait analysis on the treadmill

20. Expression of CaM-fragment enhanced the locomotor activity in R6/2 mice

21. CaM-fragment expression delayed the onset of the rotarod defects in R6/2 mice

22. TGase-modified htt in R6/2 mice striatum was reduced by CaM-fragment expression

23. Histological evaluation of neuropathology

24. Expression of CaM-fragment in mouse striatum did not significantly affect the activity CaM kinase II or TGase

25. Regulation of TGase by 5-HT\textsubscript{2A} receptor signaling and CaM
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>5-HT$_{2A}$</td>
<td>serotonin 2A receptor</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>AAV</td>
<td>adeno-associated virus</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca$_{2+}$</td>
<td>calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca$_{2+}$-calmodulin dependent kinase type II</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DOI</td>
<td>(-)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino-propane HCl</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GTPyS</td>
<td>Guanosine-5'-O-(thiotriphosphate)</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington's disease</td>
</tr>
<tr>
<td>HEK 293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>htt</td>
<td>huntingtin protein</td>
</tr>
<tr>
<td>IOD</td>
<td>integrated optical density</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidyl inositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TGase</td>
<td>transglutaminase</td>
</tr>
<tr>
<td>-------</td>
<td>------------------</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
ABSTRACT

Transglutaminase (TGase), nature’s biological glue, catalyzes the post-translational modification of proteins by formation of intra- and intermolecular protein cross-links or by primary amine incorporation. TGase has various physiological functions, such as skin-barrier formation and blood clot stabilization, whereas increasing evidence indicates they may also involved in neurodegenerative diseases including Huntington’s disease (HD), Alzheimer disease’s, and progressive supranuclear palsy. Mutant huntingtin (htt) and small G proteins (e.g. Rac 1) are potential substrates of TGases. The purpose of this dissertation was to characterize the mechanisms by which 5-HT_{2A} receptor signaling and calmodulin (CaM) regulate TGase-catalyzed transamidation of Rac1 and htt in cultured neuronal cells and HD transgenic animals, respectively.

5-HT_{2A} receptors are G-protein coupled receptors which are widely expressed in the brain, peripheral vasculature, platelets and skeletal muscle. They are involved in diverse physiological functions, from platelet aggregation to neuroendocrine release. In A1A1v cells, a rat cortical cell line, stimulation of 5-HT_{2A} receptor induces small G protein Rac1 transamidation and activation. An inhibitory agent or knockdown of TGase by siRNA revealed that TGase is responsible for transamidation and activation of Rac1. Moreover, serotonin was identified as an amine that becomes transamidated
to Rac1 by TGase. The classical signal transduction pathway of 5-HT$_{2A}$ receptors is Gq/11-coupled activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol. IP$_3$ mobilizes Ca$^{2+}$ from the endoplasmic reticulum and thereby increases intracellular Ca$^{2+}$ and consequently, it may enhance TGases enzymatic activity. Inhibition of PLC or manipulation of intracellular Ca$^{2+}$ by a chelating agent suppressed 5-HT$_{2A}$ receptor-mediated Rac1 transamidation, whereas an elevation in intracellular Ca$^{2+}$ via an ionophore can mimic 5-HT$_{2A}$ receptor-induced cytosolic Ca$^{2+}$ increases and is sufficient to induce TGase-catalyzed Rac1 transamidation. Moreover, a CaM inhibitor decreased 5-HT$_{2A}$ receptor-stimulated Rac1 modification by TGase in a dose-dependent manner. These results suggest that PLC, Ca$^{2+}$ and CaM signaling are required for 5-HT$_{2A}$ receptor-mediated transamidation of Rac1 by TGase.

In the next study, we propose that interrupting CaM interactions with htt is therapeutically beneficial in HD. This hypothesis was based on the observations that CaM regulates TGase-modification of mutant htt in cells and co-localizes with TGase and htt in intranuclear inclusions in HD cortex. Furthermore the association of CaM with mutant htt was demonstrated by affinity purification and coimmunoprecipitation approaches. Our previous studies demonstrated that in HEK293 and SH-SY5Y cells, expression of a CaM-fragment, consisting of amino acids 76-121 of CaM, decreased binding of CaM to mutant htt, decreased TGase-modified htt, decreased cytotoxicity associated with mutant htt and normalized intracellular calcium release. In this study,
an adeno-associated virus (AAV) that expresses the CaM-fragment was injected into the striatum of HD transgenic R6/2 mice and littermate control mice. The HD mice with CaM-fragment expression had significantly reduced body weight loss and improved motor function compare to HD control mice. Without affecting the activity of CaM-dependent enzymes such as CaM-dependent kinase II, CaM-fragment specifically reduced TGase-modified htt, the percentage of htt-positive nuclei and the size of intranuclear htt aggregates in HD mouse striatum. Thus, disrupting CaM htt interaction with CaM-fragment may provide a new therapeutic strategy for HD patients.

The data presented here support our hypothesis that stimulation of 5-HT$_{2A}$ receptors induces TGase-catalyzed Rac1 transamidation and activation by PLC and Ca$^{2+}$/CaM signaling, whereas disruption of CaM htt interaction inhibits TGase-catalyzed modification of htt and provides neuronal protection in HD. These results further suggest that CaM regulates the TGase transamidation reaction and TGase modification of htt is involved in the formation and stabilization of htt-aggregates and may play a role in the pathogenesis of HD.
CHAPTER ONE
BACKGROUND

INTRODUCTION

Transglutaminases (TGases, EC 2.3.2.13) are a family of calcium (Ca\textsuperscript{2+})-dependent enzymes that catalyze the formation of a covalent bond between peptide-bound glutamine residues and either peptide-bound lysine residues or mono- or polyamines (Folk and Finlayson, 1977, Folk et al., 1980, Walther et al., 2003). TGases are widely distributed in the human body with various physiological functions, such as extracellular matrix organization, skin-barrier formation and blood coagulation (Griffin et al., 2002; Lorand and Graham, 2003). TGases are subject to transcriptional regulation by retinoic acid and steroid hormones (Fujimoto et al., 1996; Ou et al., 2000), and require the binding of Ca\textsuperscript{2+} for their activity (Burgoyne and Weiss, 2001).

Serotonin signaling may also regulate TGases activity possibly by increasing cytosolic Ca\textsuperscript{2+} (Walther et al., 2003, Guilluy et al., 2007). Stimulation of 5-HT\textsubscript{2A} receptors activate phospholipase C (PLC) through Gq/11, leading to an accumulation of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and consequent release of Ca\textsuperscript{2+} from the endoplasmic reticulum. In platelets, activation of 5-HT\textsubscript{2A} receptors stimulate TGase-catalyzed transamidation of serotonin to small G proteins, RhoA and Rab4,
making them constitutively active (Walther et al., 2003). TGase-dependent RhoA transamidation and activation by serotonin stimulation was also observed in vascular smooth muscle cells (Guilluy et al., 2007). Neuronal differentiation of SH-SY5Y cells induced by retinoic acid increases the expression and activation of TGases, resulting in transamination of putrescine to RhoA and activation of RhoA (Singh et al., 2003).

Small G proteins are a family of monomeric 20–30 kDa GTP-binding proteins, which are homologous to the alpha subunit of heterotrimeric G-proteins. Small G proteins regulate a wide variety of processes in the cell, including growth, cellular differentiation, cell movement and lipid vesicle transport (Matozaki et al., 2000). Rac1 belongs to the Rho family of small G proteins, a subgroup of the Ras superfamily. Members of the Rho family are involved primarily in the regulation of cytoskeletal organization (Etienne-Manneville and Hall, 2002). Bearing 5 glutamine residues (Gln 2, 61, 74, 141, 162) and 17 lysine residues in the amino acid sequence (Matos et al., 2000), Rac1 might serve as a suitable substrate of TGases. TGase-catalyzed transamination of small G proteins may result in constitutive activation of the proteins (Walther et al., 2003). Investigating the regulatory mechanisms of TGase-modification of small G proteins and the functional consequences in a neuronal cell line may further explore the role of TGase in neuronal differentiation and neurite outgrowth.

Calmodulin (CaM), another Ca^{2+}-binding protein, has also been shown to play a role in regulating TGase. CaM is a 17 kDa protein that activates a host of enzymes
during Ca\(^{2+}\) binding (Cheung, 1982). CaM coimmunoprecipitated with TGase in transfected cells (Zainelli et al., 2004) and increased TGase activity in human erythrocyte (Billett and Puszkin, 1991), platelets and chicken gizzard (Plank et al., 1983). Recently, a series of studies showed that the activity of TGase is critical for the pathology of a number of neurodegenerative diseases, such as Huntington's disease (HD) (Karpuj and Steinman, 2004; Violante et al., 2001). CaM may also be involved in HD based on its interaction with both TGase and huntingtin (htt) (Bao et al., 1996, Zainelli et al., 2004).

HD is an autosomal dominant neurodegenerative disorder with progressive neurological symptoms and mental decline such as chorea, rigidity, gait disturbance, abnormal posturing, seizure, depression and dementia (Harper, 1991). HD occurs in individuals whose HTT gene has more than 36 CAG repeats, resulting in a mutant protein with abnormal expansions of the polyglutamine domain in the N-terminus. The pathological hallmark of HD is intranuclear inclusions and cytoplasmic aggregates composed of mutant htt protein (DiFiglia et al., 1997; Ross et al., 1998). In vitro and in cell culture, mutant htt is an excellent substrate for TGases (Gentile et al., 1998; Kahlem et al., 1998; Karpuj et al., 1999). The mRNA, protein, and enzymatic activity of TGases are elevated in HD brain (Karpuj et al., 1999; Lesort et al., 1999; Zainelli et al., 2003). Increasing evidence indicates that TGases may contribute to the formation of mutant htt aggregates in HD (Cooper et al., 1999). Htt protein colocalizes with TGase2 and its product, \(\varepsilon-(\gamma\text{-glutamyl})\) lysine covalent bond in
intraneural inclusions in the frontal cortex of HD (Zainelli et al., 2004). On the other hand, our lab and others reported CaM associates with mutant htt as demonstrated by coimmunoprecipitation (Zainelli et al., 2004) and affinity purification (Bao et al., 1996). Furthermore, CaM colocalizes with TGase2 and with htt in HD intraneural inclusions (Zainelli et al., 2004). Taken together, these studies suggest that mutant htt may bind to CaM resulting in increases TGase-modification to htt. This modification may stabilize htt monomers or polymers and contribute to htt-containing aggregate formation and cytotoxicity.

Both TGases and CaM have been explored as therapeutic targets for HD. Administration of the TGase inhibitor, cystamine, extended survival, improved motor performance and increased neuroprotective gene transcription in HD transgenic mice (Dedeoglu et al., 2002, Karpuj et al., 2002a). TGases2 ablation in HD transgenic mice results in a drastic reduction in ε-(γ-glutamyl) lysine bond levels and neuronal death in the cortex and striatum (Mastroberardino et al., 2002). CaM inhibitor, W5, can decrease TGases-catalyzed cross-linking of htt in cells transfected with TGase2 and mutant htt (Zainelli et al., 2004). However, direct inhibition of CaM or TGase may not be the best therapeutic approach, since both enzymes have a lot of other biological functions and long-term inhibition might cause untoward effects. A new strategy is to identify the htt-binding domain in CaM, and express this CaM fragment in HD models. It might disrupt CaM htt interaction by competing with endogenous CaM, and thereby decrease TGase-modified htt, htt-aggregates and cell death. This
therapeutic strategy has been tested in cell models of HD and will next be tested in an HD transgenic mouse model.

We hypothesize that stimulation of 5-HT<sub>2A</sub> receptors induces TGase-catalyzed Rac1 transamidation and activation by increasing intracellular Ca<sup>2+</sup>, whereas disruption of CaM-htt interaction inhibits TGase-catalyzed modification of htt and provides neuronal protection in HD. Three specific aims are proposed to test this hypothesis.

**Specific Aim 1:** To determine if the activity and transamidation of Rac1 by TGase are increased by 5-HT<sub>2A</sub> receptor signaling.

The first specific aim is to determine if the activity and transamidation of Rac1 are increased by 5-HT<sub>2A</sub> receptor signaling in a rat cortical cell line, A1A1v cells. We will use either a TGase inhibitor or siRNA targeting TGase to determine if Rac1 transamidation and activation are mediated TGase. In addition, we will determine if serotonin is incorporated into Rac1 by TGase following 5-HT<sub>2A</sub> receptor stimulation. Lastly, we will test if DOI, a 5-HT<sub>2A/2c</sub> receptor agonist, selectively increases TGase-catalyzed transamination of Rac1 in vivo.

**Specific Aim 2:** To determine if 5-HT<sub>2A</sub> receptor-regulated transamination of Rac1 by TGase is dependent on an increase of intracellular Ca<sup>2+</sup> mediated by PLC signaling, and if calmodulin regulate TGase-modification of Rac1.

The second specific aim designed is to explore the downstream mechanistic pathways in 5-HT<sub>2A</sub> receptor-mediated Rac1 transamidation. We will determine if
inhibition of PLC prevents the increase of intracellular Ca\(^{2+}\) and transamination of Rac1 in response to activation of 5-HT\(_{2A}\) receptors. A Ca\(^{2+}\) chelator or Ca\(^{2+}\) ionophore will be used to manipulate intracellular Ca\(^{2+}\) and determine if the increase in intracellular Ca\(^{2+}\) is necessary and sufficient to induce Rac1 transamination. We will also determine if inhibiting calmodulin prevents 5-HT\(_{2A}\) receptor-stimulated transamidation of Rac1.

**Specific Aim 3:** To determine if disrupting the interaction of calmodulin and huntingtin decreases TGase-modified huntingtin and huntingtin-aggregates, improves motor function and increases survival of HD transgenic mice.

We will use a viral vector to deliver a calmodulin-fragment, which can interrupt calmodulin and mutant huntingtin binding, to the striatum of R6/2 HD transgenic mice, then monitor survival and body weight, evaluate motor performance by behavioral tests such as DigiGait, Actometer and Rotarod. We will also measure neuropathological changes such as intranuclear htt-aggregates and striatal atrophy by immunofluorescence and Nissl staining. The specificity of calmodulin-fragment will be tested by measuring calmodulin-dependent protein kinase II (CaMKII) and TGase activity.
REVIEW OF RELEVANT LITERATURE

In the literature review, the family of TGases will be introduced first, followed by their physiological functions. The next section will focus on regulatory mechanisms and substrates of TGases. The regulation of TGases-mediated transamidation of small G protein by 5-HT$_{2A}$ receptor signaling will be highlighted. Rac1, as a potential substrate of TGases, will be presented. Regulation of TGases by calmodulin will also be discussed in this section. Lastly, the pathological role of TGases in neurodegenerative diseases including Alzheimer disease, Parkinson disease and especially Huntington’s disease will be reviewed.

**Transglutaminases (TGases), a family of enzymes**

TGases (EC 2.3.2.13) are a family of Ca$^{2+}$-dependent enzymes. Once activated, TGases catalyze the cross-linking of proteins via the γ-carboxamide group of peptide-bound glutamine and the ε-amino group of peptide-bound lysine, forming a inter- or intramolecular isodipeptide bond (Griffin et al., 2002). The enzyme can also covalently link biogenic amines and polyamines, such as spermine or serotonin (5-Hydroxytryptamine; 5-HT), to a peptide-bound glutamine residue (Dale et al., 2002; Folk et al., 1980).

To date, nine isoforms of transglutaminase have been identified, each encoded by different but structurally and functionally related related gene. Family members include keratinocyte transglutaminase (TGase1), tissue transglutaminase (TGase2), epidermal transglutaminase (TGase3), prostate transglutaminase (TGase4),
transglutaminase X (TGase5), transglutaminase Y (TGase6), transglutaminase Z (TGase7), factor XIII-A subunit (fibrin-stabilizing factor) and ATP-binding erythrocyte membrane protein band 4.2 (B4.2) (Lorand and Graham, 2003).

At least 4 isoforms, TGase1, 2, 3 and TGase7, are expressed in normal human brain tissue (Kim et al., 1999, Grenard et al., 2001) and mutant huntingtin protein is identified as a substrate of the first three TGase isoforms (Zainelli et al., 2005). However, of the nine known human isozymes, tissue TGase (TGase2) is ubiquitously expressed and most well characterized.

**Physiological functions of TGases**

TGases are widely distributed in various tissues and are involved in various physiological functions such as blood clot formation, wound healing, skin barrier formation, extracellular matrix assembly, cell death, and cell differentiation (Griffin et al., 2002, Lorand and Graham, 2003).

For example, following exposure to thrombin and Ca$^{2+}$, fibrin stabilizing factor (factor XIII) is activated and converted to factor XIII-A containing only the A subunit that, in turn, catalyzes the formation of $\text{N}^\varepsilon(\gamma$-glutamyl)lysine protein-to-protein side chain bridges within the clot network. Introduction of these covalent crosslinks greatly stabilize the fibrin and augments its resistance to fibrinolytic enzymes (Lorand, 2001).

In addition to cytoplasmic and nuclear localization, a significant part of the TGase2 protein pool is present on the cell surface. Cell surface TGase2 acts as a
coreceptor of integrins, coordinating binding of extracellular matrix (ECM) proteins (Zemskov et al., 2006). It also crosslinks the fibronectin (as well as other ECM proteins) to improve cell adhesion (Aeschlimann and Thomazy, 2000). TGase2 functions to stabilize the ECM by forming large polymeric structures that are resistant to proteolytic/chemical degradation and mechanical stresses (Griffin et al., 2002).

The homozygous null TGase2 mice are viable and have normal size and weight with no severe phenotype (De Laurenzi and Melino, 2001, Nanda et al., 2001), suggesting that other TGase isoforms may compensate for the lack of TGase2 to some extent. However, TGase2-/- mice do have altered fibroblast function, defective wound healing (Nanda et al., 2001), decreased phagocytic clearance of apoptotic cells (Szondy et al., 2003, Rose et al., 2006), mild glucose intolerance and hyperglycemia likely due to reduced insulin secretion (Bernassola et al., 2002). Therefore, it is plausible that under disease or stress, TGase2 play an essential role in tissue repair or healing and that further testing of these mice will reveal how TGase2 is critical in other physiologic states.

**Regulatory mechanisms and substrates of TGases**

TGases are $\text{Ca}^{2+}$-dependent enzymes containing a cysteine in their active site that is unmasked only in the presence of $\text{Ca}^{2+}$ (Hand et al., 1993) and $\text{Ca}^{2+}$ activation of TGase is further regulated by other signal modulators such as GTP (Monsonego et al., 1998), phospholipids (Griffin et al., 2002, Beninati and Piacentini, 2004), tumor necrosis factor alpha (Chen et al., 2000), nitric oxide (Bernassola et al., 1999) and
An elevation in intracellular Ca\(^{2+}\) is the required element for activation of TGases. Elevations in intracellular Ca\(^{2+}\) needed to activate TGases may arise via extracellular influx, release from intracellular stores, or decreased levels of other Ca\(^{2+}\) binding proteins (Lorand and Conrad, 1984). The binding of Ca\(^{2+}\) to TGases induces conformational changes and exposes the active site to substrate proteins (Hand et al., 1993, Casadio et al., 1999).

TGase2 has been reported to be a GTP-binding protein with GTPase activity (Achyuthan and Greenberg, 1987). GTP binding to the enzyme can inhibit its transamidation activity at physiological Ca\(^{2+}\) levels (Bergamini et al., 1987). Binding of GTP to TGase keeps the enzyme in a conformation such that the C-terminus blocks the enzyme's catalytic core (Monsonego et al., 1998, Casadio et al., 1999). Interestingly, an elevation in cytosolic Ca\(^{2+}\) will overcome GTP inhibition and activate TGase (Bergamini, 1988).

Other regulatory effects may also occur through the interaction of TGase with phospholipids, nitric oxide and CaM. When phospholipid vesicles are in the crystalline-liquid transition state, they can interact with and inhibit TGase activity. The inhibition may be due to the hydrophobic environment provided by the phospholipid hydrocarbon chain when the enzyme is inserted into the bilayer (Fesus et al., 1983). Exposure to tumor necrosis factor alpha causes fibronectin multimerization in lung endothelial matrix by increasing TGase activity (Chen et al.,
Nitric oxide has an inhibitory effect on both TGase2 and coagulation factor XIII, probably via S-nitrosylation of their crucial thiol groups (Bernassola et al., 1999). CaM increased TGase activity in human erythrocyte (Billett and Puszkin, 1991), platelets and chicken gizzard (Plank et al., 1983). A CaM inhibitor can decrease TGases-catalyzed cross-linking of htt in cells transfected with TGase2 and mutant htt (Zainelli et al., 2004).

Small G proteins have emerged as the new substrates of TGase. It has been reported that neuronal differentiation of SH-SY5Y cells induced by retinoic acid increases the expression and activation of TGases, resulting in transamidation of putrescine to RhoA and activation of RhoA (Singh et al., 2003). In platelets and aortic smooth muscle cells, TGases can catalyze the incorporation of serotonin into small GTPases ("transamidation"), such as RhoA and Rab4. This process of transamidation renders these small GTPases constitutively active (Walther et al., 2003, Guilluy et al., 2007). Activation of 5-HT2A receptors, which increases Ca²⁺ availability, induces serotonin transamidation to small GTPases (Walther et al., 2003). Therefore, the next section will introduce the 5-HT2A receptor, including its structure, signal transduction, distribution and pathophysiological roles.

5-HT2A receptor signaling and small G protein transamidation

Overview and classification of 5-HT receptors

As one of the most complex families of neurotransmitter receptors, 5-HT receptors have at least 16 distinct members cloned, identified and classified into 7
different subfamilies according to their ligand recognition profiles, signal transduction mechanisms, and structural characteristics (Humphrey et al., 1993, Hoyer et al., 1994, Hoyer et al., 2002). Except for the 5-HT3 receptor, which is a ligand-gated ion channel, the rest of 5-HT receptors belong to the G protein-coupled receptor (GPCR) superfamily.

The 5-HT1 receptor class includes 5 members, 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1F and 5-HT1F, which are classically coupled to the Gi/o proteins that negatively regulate adenylyl cyclase and induces the opening of K+ channels. They are expressed both pre and post-synaptically and involved in the regulation of serotonin release (Hoyer et al., 2002).

The 5-HT2 family consists of 5-HT2A, 5-HT2B and 5-HT2C receptors that primarily activate phospholipase C (PLC) via coupling with Gq/11. Activation of 5-HT2A receptors also mediates neuronal depolarization, a result of the closing of K+ channels (Aghajanian and Marek, 1999, Lambe and Aghajanian, 2001).

The 5-HT3 receptor is unique among the currently known subtypes not only because it is one of the first 5-HT receptors identified, corresponding to the M receptor (Gaddum and Picarelli, 1957), but also because it belongs to the ligand-gated channel superfamily instead of coupling to G proteins or second messenger systems. The 5-HT3 receptor mediates the rapid excitatory electrophysiological response due to a transient inward current, subsequent to the opening of nonselective cation channels (Na+, Ca2+ influx, K+ efflux) (Peters et al., 1992, Hoyer et al., 2002).
The 5-HT4, 5-HT6 and 5-HT7 receptors are single members in different families that mainly stimulate adenyl cyclase through the activation of Gαs proteins (Raymond et al., 2001).

The 5-HT5 family (5-HT5A and 5-HT5B receptors) is neither coupled to adenyl cyclase nor PI-PLC. The effector systems of this family remain unclear, but some evidence indicates 5-HT5A receptor is implicated in the action of LSD (Grailhe et al., 1999, Nelson, 2004).

**5-HT2A receptor distribution and pathophysiological roles**

5-HT2A receptors are widely distributed in central and peripheral tissues. In the central nervous system (CNS), these receptors have been found mainly in the cortical areas (neocortex, entorhinal and pyriform cortex, claustrum), caudate nucleus, nucleus accumbens, olfactory tubercle and hippocampus (Hoyer et al., 1986, Pazos et al., 1987). In the cortex the great majority of labeled cells were pyramidal neurons (Wright et al., 1995, Willins et al., 1997). In the periphery, 5-HT2A receptors are located in platelets (de Chaffoy de Courcelles et al., 1987), vascular smooth muscle (Cohen et al., 1981), and uterine smooth muscle (Wilcox et al., 1992), inducing vasoconstriction and platelet aggregation. Studies that have investigated the subcellular location of the 5-HT2A receptor indicate that the majority of 5-HT2A receptors in the human cerebellum and rat CNS are in cytosol, rather than the cellular membrane (Cornea-Hebert et al., 1999, Eastwood et al., 2001).

The 5HT2A receptor has been shown to be involved in a number of different
processes including endocrine modulation (Van de Kar et al., 2001), pain modulation (Sommer, 2004), thermoregulation (Ootsuka and Blessing, 2006), cognitive function and memory (Meneses, 2002, Williams et al., 2002). In addition, 5-HT$_{2A}$ receptor signaling is implicated in a number of disorders including hypertension, atherosclerosis, and different psychiatric diseases such as anxiety, stress, suicide behavior and schizophrenia (Roth, 1994, Weisstaub et al., 2006).

Serotonin is one of many neurotransmitters that influence the activation of hypothalamic-pituitary-adrenal (HPA) axis and stimulate secretion of oxytocin, adrenocorticotrophic hormone (ACTH), corticosterone (Van de Kar et al., 2001, Hemrick-Luecke and Evans, 2002, Zhang et al., 2002). Serotonergic terminals from the medial raphe nucleus make synaptic interaction with the corticotrophin releasing factor (CRF) synthesizing neurons in the paraventricular nucleus of rat hypothalamus (Liposits et al., 1987). Moreover, 5-HT$_{2A}$ receptors are found in hypothalamic paraventricular nucleus and in both CRF synthesizing neurons and oxytocin-containing neurons (Van de Kar et al., 2001, Zhang et al., 2002). When CRF is released, it induces ACTH secretion from the anterior pituitary gland, which in turn stimulates the release of corticosterone from the adrenal cortex. Administration of DOI, a 5-HT$_{2A/2C}$ receptor agonist increases plasma levels of oxytocin, ACTH, corticosterone in rats. The 5-HT$_{2A}$ receptor selective antagonist, MDL 100,907, but not the 5-HT2C receptor antagonist SB242084 can dose-dependently inhibit the neuroendocrine effects of DOI (Van de Kar et al., 2001, Zhang et al., 2002).
5-HT$_{2A}$ receptors are the target of some therapeutic agents for a variety of psychiatric disorders. Atypical antipsychotic drugs such as clozapine display potent 5-HT$_{2A}$ receptors antagonism with relatively weak blockade at D2 dopamine (DA) receptors, various effects at D1 receptors (Meltzer et al., 1989, Arora and Meltzer, 1994). Moreover, the clinical response to the selective serotonin reuptake inhibitors (SSRI) in treatment-resistant patients is augmented by atypical antipsychotic drugs and some antidepressants (Ostroff and Nelson, 1999, Carpenter et al., 2002, Marangell et al., 2002). The common feature of these agents is that they are able to occupy 5-HT2 receptors and to block the responses mediated by those receptors, in particular by 5-HT$_{2A}$ receptors (Marek et al., 2003). These reports support a role for 5-HT$_{2A}$ receptors in antipsychotic drug action.

5-HT$_{2A}$ receptors not only mediated the effects of some antipsychotic drugs, but also are involved in pathophysiology of psychiatric disorders. Abnormalities in 5-HT$_{2A}$ receptors have been proposed to be implicated in the pathology of schizophrenia (Woolley and Shaw, 1954, Dean, 2003). Direct evidence supporting this hypothesis came from radioligand binding studies in post-mortem schizophrenia patients, which suggest there is a decrease in the density of cortical 5-HT$_{2A}$ receptors in those patients (Arora and Meltzer, 1991, Dean and Hayes, 1996). The levels of mRNA encoding the 5-HT$_{2A}$ receptors were also reduced in the superior temporal gyrus of schizophrenic subjects (Hernandez and Sokolov, 2000). In depressed patients and suicide victims, the postmortem studies indicated an elevated level of 5-HT$_{2A}$
receptor in the cortex, amygdala and hippocampus (Arango et al., 1990, Hrdina et al., 1993, Pandey et al., 2002). However, positron emission tomography (PET) and radioligand binding studies detected a significant decrease in 5-HT2A receptor density in hippocampus of both young and old depressed patients (Mintun et al., 2004, Sheline et al., 2004). Furthermore, 5-HT2A receptors are also involved in modulating anxiety-related behaviors in humans and rodents. For example, global disruption of 5-HT2A receptor signaling in htr2a−/− mice reduces the inhibition in conflict anxiety paradigms, whereas selective restoration of 5-HT2A receptor signaling to the cortex normalized conflict anxiety behaviors (Weisstaub et al., 2006).

5-HT2A receptor structure and signal transduction

The serotonin 5-HT2A receptor gene is located on human chromosome 13q14-q21 and comprises 471 amino acid in rats, mice, and humans (Hoyer et al., 2002). It contains three exons, separated by two introns, with the coding region of the gene spanning 1.4 kb (Sanders-Bush et al., 2003).

The 5-HT2A receptor is coupled via the Gq/11 proteins to the phospholipase C (PLC) signaling cascade. PLC hydrolyzes phosphatidyl-inositol 4,5-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 mobilizes Ca2+ from the endoplasmic reticulum and thereby leads to an increase of cytosolic Ca2+ concentration (Roth et al., 1998).

In addition to the major pathways mentioned above, extensive evidence suggests that 5-HT2A receptors couple to other effector pathways. 5-HT2A receptor stimulation
also induces the activation of phospholipase A2 and the release of the second messenger arachidonic acid, which is involving Gβγ-mediated ERK1/2 activation and Ga12/13-coupled, Rho-mediated p38 activation (Felder et al., 1990, Kurrasch-Orbaugh et al., 2003). Through interaction with ADP-ribosylation factor 1 (ARF1) via its C-terminal domain, 5-HT2A receptor is able to signal through the phospholipase D (PLD) pathway (Mitchell et al., 1998, Robertson et al., 2003).

5-HT2A receptor is known to trigger MAPK activation via PKC/Raf-1 pathway (Hershenson et al., 1995, Watts, 1996). The JAK/STAT signaling pathway is activated by a number of G protein coupled receptors including 5-HT2A receptors (Guillet-Deniau et al., 1997, Singh et al., 2009).

Depending on the cell types, 5-HT2A receptor is able to increase or diminish cyclic adenosine monophosphate (cAMP) accumulation. It increases cAMP in a cell line derived from embryonic rat cortex (A1A1 cells) by protein kinase C-dependent and calcium/calmodulin-dependent mechanisms (Berg et al., 1994a) and in FRTL-5 thyroid cells through a pertussis toxin-sensitive mechanism (Tamir et al., 1992). However, in rat renal mesangial cells, 5-HT2A receptor activation can inhibit adenylyl cyclase activity and forskolin-stimulated cAMP accumulation (Garnovskaya et al., 1995).

Because transamidation of small G proteins (e.g. RhoA and Rab4) mediated by 5-HT2A receptor signaling leads to platelet α-granule release and aggregation (Walther et al., 2003), and TGase-dependent Rho A transamidation also inhibits contraction of
vascular smooth muscle cells, a brief introduction of small G proteins especially Rac1 whose transamidation is extensively studied in this dissertation will be presented in the next section.

Small G proteins

Small G-proteins, short for small guanine nucleotide-binding proteins, are monomeric G proteins with molecular masses of 20–30 kDa. They are homologous to the alpha subunit of heterotrimeric G-proteins, but they can function on their own by cycling between an inactive GDP-bound state and an active GTP-bound state. The GTP/GDP cycling is controlled by many regulator molecules such as GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and GDP dissociation inhibitors (GDIs) (Hakoshima et al., 2003). GTP hydrolysis is accelerated by GAPs, resulting in inactivation of the cognate G-protein. GAP-dependent signaling is antagonized by GEF, which activate small G proteins by promoting the nucleotide exchange of GTP for GDP. Furthermore, small G proteins are regulated by GDIs, which maintain small G proteins in their inactive GDP-bound form, in addition to preventing their association to the plasma membrane (Figure 1).
Figure 1. Molecular switch of the small G-proteins. The activation of small G protein is mediated by three types of regulatory proteins: GDI (guanine nucleotide dissociation inhibitor), GAP (GTPase activating protein), and GEF (guanine nucleotide exchange factors).
Biological functions of Rac1

Rac1, short for Ras-related C3 botulinum toxin substrate 1, belongs to the Rho family of small G proteins, a subgroup of the Ras superfamily. Binding to a variety of downstream target proteins (effectors), members of the Rho family (e.g. RhoA, Rac1, Cdc42) are associated with a wide array of cellular processes such as cytoskeletal organization, vesicular transport, cell cycle progression, cell adhesion and migration, neuronal differentiation and a variety of enzymatic activities (Etienne-Manneville and Hall, 2002, Burridge and Wennerberg, 2004).

Rac1 is best known as a regulator for the assembly of the actin cytoskeleton, thereby playing a role in neurite outgrowth and neuronal differentiation. Expression of a constitutively active mutant of Rac1 in neuroblastoma cells leads to the formation of neurites (Leeuwen et al., 1997), and production of filopodia and lamellipodia in the developing growth cone (Kozma et al., 1997). In contrast, over-expression of dominant negative Rac1 in SH-SY5Y cells blocks retinoic acid (RA)-induced neurite outgrowth and expression of neuronal markers, suggesting that activation of Rac1 regulates neuronal differentiation (Pan et al., 2005). Rac1 play an essential role in fibroblasts cell growth in response to mitogenic stimulation since microinjection of Rac1 stimulated cell cycle progression through G1 and subsequent DNA synthesis. This effect can be blocked by microinjection of dominant negative forms of Rac1 (Olson et al., 1995). In growth factor-stimulated fibroblasts and neuronal cells, Rac1 was transiently activated in a broad area of the plasma membrane, followed by a
localized activation at nascent lamellipodia (Kurokawa et al., 2005). Consistent with this finding, Rac1 is required to stimulate the formation of lamellipodia and membrane ruffles.

Rac1 is also involved in multiple cell death pathways. Depending on the interaction with other signaling molecules and on the type of cells, it becomes pro-apoptotic (Embade et al., 2000, Aznar and Lacal, 2001, Harrington et al., 2002) or anti-apoptotic (Nishida et al., 1999, Deshpande et al., 2000, Murga et al., 2002). For example, neurotrophin receptor p75 activates Rac1, which in turn activates c-Jun N-terminal kinase (JNK) and causes apoptosis in neuronal cells (Harrington et al., 2002). On the other hand, Rac1 protects endothelial cells from tumor necrosis factor-alpha-induced apoptosis (Deshpande et al., 2000) and promotes COS7 cell survival by the activation of phosphatidylinositol 3-kinase (PI3K) and Akt (Murga et al., 2002).

Structure of Rac1

The Rho family are monomeric globular proteins consisting of less than 300 amino acids (Wennerberg and Der, 2004). Like all members of the Rho family, Rac1 functions as a conformational switch by cycling active GTP and inactive GDP-bound forms. Rho GTPases share a common GTPase domain, which carries out the basic function of nucleotide binding and hydrolysis. The GTPase domain consists of a six-stranded β-sheet surrounded by α-helices (Vetter and Wittinghofer, 2001). Most typical Rho proteins consist only of the GTPase domain and short N- and C-terminal
extensions. C-terminal post-translational modifications such as isoprenoid addition facilitate specific subcellular location and association with specific membranes, which is crucial for their function (Wennerberg and Der, 2004). The differences between the GDP- and GTP- bound structural forms of RhoA are confined primarily to two segments, referred to as switch I and switch II, and this feature is probably shared by all Rho GTPases (Ihara et al., 1998, Vetter and Wittinghofer, 2001).

Bearing five glutamine residues in the amino acid sequence (Matos et al., 2000), Rac1 might serve as a suitable substrate of TGases. The transamidation of primary amines to RhoA and Rab4 by TGases renders these small G proteins constitutively active (Walther et al., 2003, Guilluy et al., 2007). Similarly, post-translational modifications, such as transamidation and phosphorylation of Rac1 may affect its ability to interact with regulatory proteins (e.g. GAPs, GEFs and GDIs) or to convert GTP back to GDP, resulting in increased activation of Rac1 and stimulation of its signaling cascade in the cells.

The Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=cdd) provides an interactive tool to identify conserved domains present in protein sequences. The residues, which regulate Rac1 activity after post-translational modifications, are most likely to be located at GTP/Mg\(^{2+}\) binding sites, and GAPs, GEFs and GDIs interaction sites in the Rac1 sequence. Two glutamine residues (Q61, Q74) and three lysine residues (K5, K16, and K116), potential targets of TGase-catalyzed modification, are identified within these
activity-related domains using CDD search of the Rac1 sequence (PSSM-Id: 57957). In line with this finding, it has been reported that site-specific deamidation of a Gln residue in the Rho family of G proteins (Q61 in Rac and Cdc42, Q63 in RhoA) by CNF-1 inhibits both intrinsic and GAPs-stimulated GTP hydrolysis activity, resulting in constitutive activation of these proteins (Flatau et al., 1997, Schmidt et al., 1997). CNF-1 has also been shown to possess in vitro TGase activity. In the presence of primary amines, RhoA is transamidated in vitro at Q63 by CNF-1 and at positions 52, 63 and 136 by guinea pig liver TGase (Schmidt et al., 1998). Similarly, in addition to Q61 which is critical in regulating Rac1 activity, the other four glutamine residues in Rac1 may also be modified by TGase.

**Calmodulin (CaM)**

The other regulator molecule of TGase that will be focused on this dissertation is CaM. It is a 17 kDa, dumbbell shaped, Ca$^{2+}$ binding protein that is ubiquitously expressed in cells (Babu et al., 1985). There are two EF hand motifs at each end of its dumbbell-like structure, separated by a helical linker region. The C-terminal EF hand motifs have three to five fold higher affinity for Ca$^{2+}$ than those in the N-terminal (Chin and Means, 2000). Upon Ca$^{2+}$ binding to the EF hand motifs, both N-terminal and C-terminal domains adopt an open confirmation, exposing hydrophobic surfaces, which then interact with a variety of proteins triggering events such as the release of autoinhibitory domains (for CaM-dependent kinases and calcineurin), active site remodeling (anthrax adenylyl cyclase), and CaM-induced dimerization of membrane
proteins (Bachs et al., 1994, Hoeflich and Ikura, 2002). However, CaM can interact with target proteins in both Ca\(^{2+}\) dependent as well as independent manners. In the absence of Ca\(^{2+}\), the N-terminal domain adopts a closed confirmation whereas the C-terminal domain is in a semi-open state with partial exposure of a hydrophobic patch. Thus the C-terminal domain of CaM could interact with target proteins even in the absence of Ca\(^{2+}\) (Swindells and Ikura, 1996).

The temporal and spatial associations between changes in Ca\(^{2+}\) and the function of CaM have been identified in numerous studies. Changes in intracellular Ca\(^{2+}\) concentration induce dynamic compartmentalization and mobilization of CaM including translocation from the cytosol to the nucleus (Luby-Phelps et al., 1995, Deisseroth et al., 1998, Craske et al., 1999). CaM activation is coupled to elevation in intracellular Ca\(^{2+}\) and also correlates with the spatial pattern of increased Ca\(^{2+}\) (Hahn et al., 1992).

CaM not only acts as a ubiquitous transducer of intracellular Ca\(^{2+}\) signals but also modulates intracellular Ca\(^{2+}\) concentrations. CaM can act as intracellular Ca\(^{2+}\) buffer, regulate the plasma membrane Ca\(^{2+}\) ATPase, ryanodine receptors, IP\(_3\) receptors and cyclic nucleotide gated Ca\(^{2+}\) channels (Baimbridge et al., 1992, Liu et al., 1994, Patel et al., 1997, Balshaw et al., 2001).

Various downstream substrates of CaM have been studied extensively and the studies presented here focus on delineating the role of Ca\(^{2+}\)/CaM in regulation of TGase-catalyzed transamidation activity. CaM increased TGase activity in human
erythrocyte (Billett and Puszkin, 1991), platelets and chicken gizzard (Plank et al., 1983). In the presence of CaM, TGases are activated at lower Ca\(^{2+}\) concentrations than the concentration that is normally needed for activation (Billett and Puszkin, 1991). In cells transfected with TGase2 and mutant htt, CaM co-immunoprecipitates with both TGase2 and mutant htt. A CaM inhibitor can decrease TGases-catalyzed cross-linking of htt (Zainelli et al., 2004). Furthermore, fluorescence confocal microscopy demonstrated that CaM colocalizes with TGase2 and with htt in HD intranuclear inclusions (Zainelli et al., 2004), suggesting that CaM may play a pivotal role in HD.

**Pathological role of TGases in neurodegenerative diseases**

Protein aggregation and inclusion bodies are the common features of several neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease. Selkoe and colleagues were the first to propose the role for TGases in neurodegeneration. They demonstrated that TGases could catalyze the formation of insoluble polymers composed of human neurofilament proteins by forming covalently crosslink (Selkoe et al., 1982). Increasing evidence suggests TGases may be involved in protein aggregation in those neurodegenerative diseases (Ross and Poirier, 2004, Muma, 2007).

**Alzheimer’s disease (AD) and TGase**

Neurofibrillary tangles (NFTs), deposits of tau filaments in the neuronal cell body, are one of the most important pathologic characteristics of AD. Numerous groups have shown that tau is cross-linked by TGases in vitro, forming insoluble
filamentous polymers (Dudek and Johnson, 1993, Appelt and Balin, 1997, Murthy et al., 1998, Norlund et al., 1999). In post-mortem studies, it has been reported that TGase2 colocalizes with paired helical filaments (PHFs), which are composed of tau, in AD brain (Appelt et al., 1996). Our laboratory and others further demonstrated that PHF-tau from AD brain contains TGase-catalyzed gamma-glutamyl-lysine isodipeptide bonds (Norlund et al., 1999), and there is extensive co-localization of TGase-catalyzed isodipeptide bonds and PHF-tau (Citron et al., 2002, Singer et al., 2002), providing circumstantial evidence for the involvement of TGases in AD pathology. Moreover, PHF-tau co-localizes with TGase-catalyzed cross-links in brain regions devoid of NFT in stage II AD, which would be expected to contain them in stage III, suggesting TGase as a contributing factor in NFT formation induces tau cross-links in an early stage of AD (Singer et al., 2002).

Furthermore, TGase protein expression and cross-linking activity are increased in AD brain and cerebrospinal fluid (CSF) as compared to controls (Kim et al., 1999, Bonelli et al., 2002). Interestingly, TGase activity are only increased in the brain regions with abundant neurofibrillary pathology, but not in the cerebellum, a region spared of NFT pathology (Johnson et al., 1997).

**Parkinson's disease (PD) and TGases**

Multiple lines of evidence suggest that TGase-catalyzed bonds are associated with Lewy bodies, the major inclusion body in PD. In vitro and in cell culture, TGase is also capable of crosslinking a-synuclein, a major component of Lewy bodies in PD,
to form intramolecular cross-links and high molecular weight polymers (Jensen et al., 1995, Junn et al., 2003). Moreover, Glu79 and Lys80 in α-synuclein have been identified as the TGase-reactive residues and Lys80 residue is located in a conserved sequence in the synuclein gene family (Jensen et al., 1995). Immunohistochemistry studies demonstrate that TGase-catalyzed cross-links are elevated in dopaminergic neurons in PD substantia nigra, and co-localize with α-synuclein in Lewy bodies (Junn et al., 2003, Andringa et al., 2004). On Western blots, the TGase-catalyzed cross-link bond comigrates with α-synuclein from PD substantia nigra (Citron et al., 2002, Andringa et al., 2004).

**Huntington's disease (HD) and TGase**

HD is an autosomal dominant neurodegenerative disorder characterized by cognitive and behavioral disturbance, involuntary movements (chorea), striatal and cortical neurodegeneration and neuronal inclusions (Gusella and MacDonald, 2000). HD is caused by expansion of CAG repeats in exon1 of HTT gene, which is translated into an expanded polyglutamine (polyQ) stretch at the N-terminus of the huntingtin protein (htt) (Huntington’s Disease Collaborative Research Group, 1993). Consequently, N-terminal proteolytic fragments of mutant htt form intranuclear and cytoplasmic aggregates in neurons, the prominent neuropathologic hallmarks of HD. This CAG “triplet” is normally repeated about 20 times, but an increase in the number of repeats to 40 or more results in the expression of the disease (Duyao et al., 1993, Huntington’s Disease Collaborative Research Group, 1993). Additionally, there is an
inverse correlation between age of onset of HD symptoms and the number of CAG repeats (Duyao et al., 1993).

Several lines of evidence suggest that TGase may be involved in the pathophysiology of HD. It was reported firstly by Cariello and colleagues (Cariello et al., 1996) that TGase activity is above maximum control levels in some HD patients and is correlated with the CAG repeat length. In vitro and in cell culture, htt is an excellent substrate for TGase (Gentile et al., 1998, Kahlem et al., 1998, Karpuj et al., 1999). The rate of the cross-linking reaction increases over an order of magnitude with repeat lengths of over 40 glutamines. Additionally, TGase-catalyzed cross-linking of mutant htt resulted in the formation of htt polymers (Kahlem et al., 1998). TGase mRNA, protein levels, and activity are selectively elevated in HD affected brain regions (Karpuj et al., 1999, Lesort et al., 1999, Lesort et al., 2002, Zainelli et al., 2003).

TGase has become a therapeutic target for HD in both preclinical experiments and clinical trials. Knocking-out TGase2 in an HD transgenic mouse model, R6/1 mice, results in improved motor performance and increases survival (Mastroberardino et al., 2002). R6/2 mice treated with cystamine, a TGase inhibitor, showed extended survival, improved body weight and motor performance and delayed neuropathological sequela (Dedeoglu et al., 2002, Karpuj et al., 2002a). In the other mouse model of HD, the YAC 128 mouse, treatment with cystamine ameliorates striatal volume loss and decreases striatal neuronal atrophy (Van Raamsdonk et al.,
Cysteamine, the dimer of cystamine, is an orphan drug approved for the treatment of nephropathic cystinosis in humans. In 2008, researchers at Raptor Pharmaceuticals Corp began with Phase II clinical trials of cysteamine for HD.

**Distribution and functions htt protein**

As a 350 kDa protein composed of 3,144 amino acids, htt is expressed ubiquitously throughout the body. The highest concentrations are found in the brain and testes, with moderate amounts in the liver, heart, and lungs (Walker, 2007). In the brain, htt has the highest expression in the neocortex, cerebellar cortex, striatum, and hippocampus (Fusco et al., 1999). Although htt is found primarily in the cytoplasm, smaller amounts have also be detected in other cellular compartments, including the nucleus (Hoogeveen et al., 1993, Kegel et al., 2002).

Several studies have revealed the role of wildtype htt in development. Complete knockout of the mouse Htt gene is embryonic lethal between embryonic days 7.5 and 8.5 (Duyao et al., 1995, Nasir et al., 1995, Zeitlin et al., 1995). It was later found that htt contributes to the formation of the nervous system. Mice carrying reduced levels of wild-type htt display profound malformations of the cortex and striatum (White et al., 1997). Several lines of evidence indicate that wild-type htt facilitates neuronal survival as well. For example, overexpression of wildtype htt exhibits neuroprotection against ischemic injury, excitotoxicity, and caspase activation (Rigamonti et al., 2000, Zhang et al., 2003, Leavitt et al., 2006), whereas disruption of the mouse homologue of HTT gene results in a progressive degenerative neuronal
phenotype and sterility (O'Kusky et al., 1999, Dragatsis et al., 2000). Moreover, htt may also participate in the regulation of apoptosis, control of BDNF production, vesicular and mitochondrial transport, neuronal gene transcription, and synaptic transmission (Cattaneo et al., 2005).

Similar to wildtype htt, mutant htt with expanded polyglutamine stretch is found in the cytoplasm as well as the nucleus. In HD, cleavage of the full-length mutant htt is critical for the formation of htt-containing aggregates which are a prominent pathological feature in neurons in the cortex and striatum (DiFiglia et al., 1997, Sapp et al., 1997). Htt has been defined as a caspase substrate with numerous sites for specific caspase activity, such as amino acids 552 (caspase 2), amino acids 513, 552, 530, and 589 (caspase 3), and amino acid 586 (caspase 6) (Wellington et al., 1998, Wellington et al., 2000). In HD and in transgenic mouse models, the activity of caspases 3 and 9 is increased (Wellington et al., 2000, Zeron et al., 2004) and inhibition of caspase 3, 6 or 9 activity decreases toxicity in models of HD (Wellington et al., 2000, Tang et al., 2005).

While mutant htt is primarily cytosolic, the cleavage products of mutant htt, N-terminal fragment with the polyglutamine stretch, can translocate into the nucleus and mediate toxic functions of htt in HD (Hodgson et al., 1999, Graham et al., 2006). Mutant htt can induce neurodegeneration by an apoptotic mechanism in a cell model (Saudou et al., 1998). Proteasomal activity is decreased and the proteasome is unable to process the polyglutamine repeat in htt-transfected cells and HD knock-in mouse
brains (Zhou et al., 2003, Venkatraman et al., 2004), suggesting that mutant htt may disturb normal proteasome function. Also, mutant htt appears to interfere with other cellular mechanisms such as gene transcription. It has been reported that mutant htt causes decreased transcription of the brain-derived neurotrophic factor (BDNF) gene (Zuccato et al., 2001), which is necessary for survival of striatal neurons. cAMP response element (CRE)-mediated transcription, which promotes the expression of several pro-survival genes, are also impaired by mutant htt (Steffan et al., 2000, Wyttenbach et al., 2001).

Although some evidence suggests that htt-associated toxicity is linked to soluble htt and its protein–protein interactions (Petersen et al., 1999), it may also be correlated with the formation of toxic aggregate species, which are found in the neuronal intranuclear inclusions (NIIs) and cytoplasmic aggregates in the cortex and striatum of HD brains (Davies et al., 1997, DiFiglia et al., 1997, Sapp et al., 1997). Interestingly, htt-containing aggregates are predominately nuclear in juvenile HD cases, while they are mainly localized to the neural processes in adult-onset cases (DiFiglia et al., 1997).

**CaM-regulated TGase-modification of htt**

We previously reported that CaM associates with htt and TGase2 as demonstrated by coimmunoprecipitation in transfected cells and fluorescence colocalization in intranuclear inclusions in HD brains (Zainelli et al., 2004). CaM has been shown to increase TGase activity in different cells and tissues (Puszkin and
Raghuraman, 1985, Billett and Puszkin, 1991). Inhibition of CaM results in decreased TGase-catalyzed modifications of htt in cells transfected with mutant htt and TGase2. Using affinity purification, mutant htt with an expanded glutamine repeat binds to CaM with a higher affinity than wild-type htt, suggesting in vivo interaction between CaM and htt as well (Bao et al., 1996). These data, along with the observation that TGase mRNA, protein, and activity are elevated in HD (Karpuj et al., 1999; Lesort et al., 1999; Zainelli et al., 2003), and TGase2 colocalizes with both htt protein and TGase-catalyzed cross-links in HD intranuclear inclusions (Zainelli et al., 2003), implicate CaM and TGase in htt-aggregate formation in HD (Figure 2). Hypothetically, interaction of mutant htt with both CaM and TGase results in an increase in TGase-modified htt and consequent htt stabilization and aggregate formation.
Figure 2. Proposed involvement of TGase and CaM in the formation of htt-containing aggregates. When there are more than 35 glutamines (Gln) repeat in mutant htt, protein conformation is changed. Some proteases such as caspase 3 and calpain can cleave mutant htt and generate a toxic N-terminal htt fragment, which can be transported to nucleus and neurites. We propose that N-terminal htt associated with CaM at a specific binding domain, and htt were modified by TGase. This modification may stabilize htt monomers or polymers and contribute to cytotoxicity and formation of htt-containing aggregates.
CHAPTER TWO

TRANSGLUTAMINASE-CATALYZED TRANSAMIDATION: A NOVEL MECHANISM FOR RAC1 ACTIVATION BY 5-HT2A RECEPTOR STIMULATION

(Published in *J Pharmacol Exp Ther.* 2008; 326(1):153-62)

ABSTRACT

Transglutaminase (TGase)-induced activation of small G proteins via 5-HT$_{2A}$ receptor signaling leads to platelet aggregation (Walther et al., 2003). We hypothesize that stimulation of 5-HT$_{2A}$ receptors in neurons activates TGase, resulting in transamidation of serotonin to a small G protein, Rac1, thereby constitutively activating Rac1. Using immunoprecipitation and immunoblotting, we show that in a rat cortical cell line, A1A1v cells, serotonin increases TGase-catalyzed transamidation of Rac1. This transamidation occurs in both undifferentiated and differentiated cells. Treatment with a 5-HT$_{2A/2C}$ receptor agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), but not the 5-HT$_{1A}$ receptor agonist, 5-hydroxy-2-dipropylamino tetralin (DPAT), increases transamidation of Rac1 by TGase. In A1A1v cells, 5-HT$_{2A}$ receptors mediate the transamidation reaction since expression of 5-HT$_{2C}$ receptors was not detectable and the selective 5-HT$_{2A}$ receptor antagonist blocked transamidation. Time course studies demonstrate that transamidation of Rac1 is significantly elevated after 5 and 15 minutes of serotonin treatment, but returns to
control levels after 30 minutes. The activity of Rac1 is also transiently increased following serotonin stimulation. Inhibition of TGase by cystamine or siRNA reduces TGase-modification of Rac1 and cystamine also prevents Rac1 activation. Serotonin itself is bound to Rac1 by TGase following 5-HT$_{2A}$ receptor stimulation as demonstrated by co-immunoprecipitation experiments and a dose-dependent decrease of serotonin-associated Rac1 by cystamine. These data support the hypothesis that Rac1 activity is transiently increased due to TGase-catalyzed transamidation of serotonin to Rac1 via stimulation of 5-HT$_{2A}$ receptors. Activation of Rac1 via TGase is a novel effector and second messenger of the 5-HT$_{2A}$ receptor signaling cascade in neurons.

INTRODUCTION

5-HT$_{2A}$ receptors are G-protein coupled receptors which are widely expressed in the brain, peripheral vasculature, platelets and skeletal muscle. 5-HT$_{2A}$ receptors are involved in diverse physiological functions, from platelet aggregation to neuroendocrine release (Van de Kar et al., 2001, Walther et al., 2003). Pathophysiologically, 5-HT$_{2A}$ receptor signaling is implicated in a number of disorders including hypertension, atherosclerosis, anxiety and depression (Roth, 1994, Weisstaub et al., 2006). The classical signal transduction pathway of 5-HT$_{2A}$ receptors is $G_{q/11}$-coupled activation of phospholipase C (PLC) (Sanders-Bush et al., 2003), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol
1,4,5-trisphosphate (IP$_3$) and diacylglycerol. IP$_3$ mobilizes calcium from the endoplasmic reticulum and thereby increases intracellular Ca$^{2+}$ (Julius, 1991).

TGases (EC 2.3.2.13) are a family of Ca$^{2+}$-dependent enzymes. Once activated, TGases can catalyze the cross-linking of proteins via the $\gamma$-carboxamide group of peptide-bound glutamine and the $\epsilon$-amino group of peptide-bound lysine, forming an inter- or intramolecular isodipeptide bond (Griffin et al., 2002). TGases can also covalently link biogenic amines and polyamines, such as serotonin or spermine, to a peptide-bound glutamine residue in a transamidation reaction (Folk et al., 1980, Dale et al., 2002). In platelets, activation of 5-HT$_{2A}$ receptors stimulates TGase-catalyzed transamidation of serotonin to small G proteins, such as RhoA and Rab4, rendering them constitutively active (Walther et al., 2003). Furthermore, neuronal differentiation of SH-SY5Y cells induced by retinoic acid increases the expression/activation of TGases, resulting in transamidation of putrescine to RhoA and activation of RhoA (Singh et al., 2003).

Rac1 belongs to the Rho family of small G proteins, a subgroup of the Ras superfamily. Members of the Rho family (e.g. RhoA, Rac1, Cdc42) are associated with a wide array of cellular processes such as cytoskeletal organization, vesicular transport, cell cycle progression, cell adhesion and migration, neuronal differentiation and a variety of enzymatic activities (Etienne-Manneville and Hall, 2002, Burridge and Wennerberg, 2004). Like other small G proteins, Rac1 functions as a molecular switch which cycles between two conformational states: a GTP-bound, active state
and a GDP bound, inactive state. The GTP/GDP cycling is controlled by many regulator molecules such as GTPase-activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and GDP dissociation inhibitors (GDIs) (Hakoshima et al., 2003). Post-translation modification of Rac1 in the regulator-interaction sites or in the GTP-hydrolyzing domain may have an enormous effect on its activation cycle. Bearing five glutamine residues in the amino acid sequence (Matos et al., 2000), Rac1 might serve as a suitable substrate of TGases resulting in the transamidation of primary amines to Rac1 and rendering this small G protein constitutively active.

A1A1v cells derived from embryonic rat cortex (Scalzitti et al., 1998) provide an excellent experimental model to study 5-HT$_{2A}$ receptor signaling. In the present study, we use A1A1v cells to test whether 5-HT$_{2A}$ receptor stimulation increases TGase-catalyzed transamidation and activation of Rac1 since they endogenously express 5-HT$_{2A/1A}$ receptors, Go$_{q/11}$, PLC $\beta$, TGase 2, the serotonin transporter, and small G proteins including Rac1, RhoA and Cdc42.

**MATERIALS AND METHODS**

**Cell Culture**

A1A1v cells, a rat cortical cell line, were grown on 100 mm$^2$ plates coated with poly-L-ornithine (Sigma, St Louis, MO) and maintained in 5% CO$_2$ at 33°C, in Dulbecco’s modified Eagle medium (DMEM) (Fisher Scientific, Pittsburgh, PA) containing 10% fetal bovine serum (FBS) (Fisher Scientific, Pittsburgh, PA). Cells
were induced to differentiate by incubation at 37°C for 4 days. Before each experiment, cells were maintained in DMEM with 10% charcoal-treated FBS for 48 hours. Charcoal adsorption removes most but not all serotonin in the medium. The maximal final concentration of serotonin in the medium is approximately 3 nM (Unsworth and Molinoff, 1992). Cells from passages 8-15 were used for all experiments.

Drugs

The following drugs were used in this study: (-)-1-(2,5-dimethoxy-4-lodophenyl)-2-aminopropane HCl (DOI) and serotonin (Sigma, St Louis, MO). (R)-(+)8-hydroxy-2-dipropylamino tetralin hydrobromide (DPAT) (Tocris, Ellisville, MO). 2-aminoethyl disulfide dihydrochloride (cystamine) (MP biomedicals, Costa Mesa, CA). α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidine methanol (MDL 100907) (a gift from Hoechst Marion Roussel Research Institute, Cincinnati, OH). Serotonin was dissolved in 10 µM HCl and MDL 100907 was dissolved in a minimal volume of Dimethyl sulfoxide (DMSO) then diluted with saline. The remaining drugs were dissolved in purified water. All compounds were further diluted (at least 1:100) in cell culture media before they were applied to the cells and washed away prior to lysing the cells.

Immunoprecipitation of TGase-modified Protein

A1A1v cells were harvested and lysed using lysis buffer A (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 1:1000 protease inhibitor cocktail
(Sigma, St Louis, MO) containing 104 µM AEBSF, 0.08 µM aprotinin, 2 µM leupeptin, 4 µM bestatin, 1.5 µM pepstatin A and 1.4 µM E-64). Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Immunopurification of proteins containing TGase-catalyzed bonds was performed using 81D4 mAb (mouse IgM) prebound to Sepharose beads (Covalab, Lyon, France) using a protocol developed by Covalab and as described previously (Norlund et al., 1999, Zainelli et al., 2005). The 81D4 antibody is well characterized and has been previously shown to be specific for the N epsilon-(gamma-L-glutamy)-L-lysine isopeptide and N epsilon-(gamma-L-glutamy)-L-lysine isopeptide cross-link generated by TGase (Sarvari et al., 2002, Thomas et al., 2004). The 81D4 antibody has been extensively used to demonstrate increases in TGase-catalyzed bonds (Citron et al., 2002, Junn et al., 2003, Andringa et al., 2004). The use of the 81D4 antibody in a competitive ELISA assay was shown to result in isodipeptide cross-link measurements that correlate well to those measured by HPLC analysis but provide more sensitivity than the HPLC approach (Sarvari et al., 2002). Transfection of cells to over-express TGases, including TGase 1, 2 and 3, and a substrate protein such as mutant huntingtin protein results in increased presence of the isodipeptide cross-link in mutant huntingtin protein as demonstrated with immunoprecipitation with the 81D4 antibody (Zainelli et al., 2005).

Briefly, 20µl of sepharose-81D4 beads were washed three times in TBS/0.1% Tween 20 with gentle shaking for 15 minutes, followed by adding 200µg cell lysate (1
μg/μl) to the washed beads and incubating for 2 hours at 37°C. After incubation, the pellets were washed four times in TBS/0.1% Tween 20 for 15 minutes. Then 20μl of loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 5% β-mercaptoethanol) was added to the washed pellets followed by 5 minutes incubation at 90°C. The samples were then centrifuged at 9,000 × g for 2 minutes and the supernatant was transferred and stored at -80°C until immunoblot analysis.

**Immunoprecipitation of Rac1**

200 μg of protein from each sample was brought up to a total volume of 100 μl with IP buffer (50 mM Tris-HCl, pH 7.4, 10 mM EGTA, 100 mM NaCl, 0.5% Triton-X 100 and 0.1% protease inhibitor cocktail), then the samples were precleared using 10μl of recombinant protein G (rProtein G) agarose (Invitrogen, Carlsbad, CA) for 1 hour. The samples were centrifuged at 9,000 × g for 10 minutes and the supernatant was incubated for 1.5 hour at 4°C with either 2 μg of Rac1 antibody (Upstate, Lake Placid, NY) or 2 μg of normal mouse IgG (Santa Cruz, Santa Cruz, CA) as a control for non-specific binding. The immuno-complexes were precipitated using 20 μl of rProtein G agarose at 4°C for 1 hour. The agarose-immuno complexes were washed three times in IP buffer and centrifuged after each wash at 100 × g for 3 minutes. After the last wash, bound proteins were eluted by adding 2× PAGE sample buffer and heating for 5 minutes at 90°C. The samples were centrifuged at 13,000 × g for 5 minutes and the supernatant was transferred and stored at -80°C until immunoblot
analysis.

**Immunoblot**

Immunaoaffinity purified proteins and cell lysates were separated on 12% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. Membranes were then incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween 20, 1× TBS) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibodies on a shaker. Primary antibodies (Upstate Biotechnology, NY: anti-Rac1, mouse IgG, 1:700; anti-Na+/K+ ATPase, mouse IgG, 1:10000; Abcam, Cambridge, MA: anti-serotonin, rabbit IgG, 1:1000; BD Pharmingen, San Jose, CA: anti-5-HT$_{2C}$ receptor, mouse IgG, 1:300; Cell Signaling, Danvers, MA: anti-phosphorylated ERK1/2 at residues Thr202/Tyr204, mouse IgG, 1:1,000; anti-ERK1/2, rabbit IgG, 1:1,000; MP Biomedicals, Aurora, OH: anti-actin, mouse IgG, 1:20,000; CovalAb, Lyon, France: 81D4, mouse IgM, 1: 500; NeoMarkers, Fremont, CA: TG100, mouse IgG, 1:100) were diluted in antibody buffer (1% non-fat dry milk, 0.1% Tween 20, 1× TBS). The next day, membranes were washed with TBS/0.1% Tween 20 and then incubated with goat-anti-mouse or goat-anti-rabbit secondary antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) diluted in antibody buffer. Membranes were washed and signal was detected using enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Using Scion Image for Windows (Scion, Frederick, MD), immunoblots were quantified by calculating the integrated optical density (IOD)
of each protein band on the film.

Expression and Purification of Glutathione S-Transferase (GST)-PAK1

The pGEX-2T plasmid was used to express Rac1 interactive domain of human PAK1 (residues 51–135) fused to GST. The BL21 (DE3) E. coli (Invitrogen life technologies, Carlsbad, CA) were transformed with plasmid expressing GST-PAK1 and grown overnight at 37°C on LB-ampicillin plates. Then a single colony was used to inoculate 25ml LB-ampicillin (100 µg/ml) which was shaken overnight at 37°C. 10 ml of the overnight culture was used to inoculate 500ml LB-ampicillin which was grown for 3 h at 37°C while shaking. After induction with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 h at 30-32°C, bacteria were lysed in lysis buffer B (50 mM Hepes, pH 7.6, 1% Triton X-100, 100 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1 mM PMSF and 0.1% protease inhibitor cocktail). The lysates were sonicated and centrifuged for 15 minutes at 9,000 × g at 4°C. Then the supernatant was added to glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ) and the mixture was incubated at 4°C for 2 hours. The beads were then centrifuged at 4°C at 2,000 × g and were washed four times with lysis buffer B. The beads were resuspended in lysis buffer B and were stored at -80°C for at most two weeks.

Rac1 Activity Assay

A1A1v cells were treated for 5 or 15 minutes with 14 µM serotonin or vehicle (10 µM HCl). The cells were harvested in lysis buffer C (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 0.1% SDS, 1 mM PMSF and 0.1% protease
inhibitor cocktail). Cell lysates were centrifuged at 14,500 × g for 20 minutes at 4°C and 200 µl of the supernatant was incubated with 20 µl of GST-PAK1-Sepharose beads for 40 minutes at 4°C under constant agitation. The beads were washed three times with lysis buffer C, and 2× Laemmli sample buffer was added to the washed beads followed by 5 minutes incubation at 90°C. The samples were then centrifuged at 9,000 × g for 2 minutes and equivalent amounts of proteins in the supernatants were loaded on 12% SDS-PAGE as well as 20 µg cell lysates (in order to detect total Rac1 protein levels), followed by immunoblot analysis as described above.

**Small Interfering RNA**

To induce TGase2 gene silencing, two siRNA duplexes to target the coding sequence of rat TGase2 mRNA were designed and synthesized by QIAGEN (Germantown, MD). The target sequence is 5’-AAGAGCGAGATGATCTGGAAT-3’ for siRNA1 and is 5’- AGAGCCAACCACCTGAACAAA-3’ for siRNA2. The sense strand of each siRNA was labeled at 3’ end with Alexa Fluor 488 to monitor transfection efficiency. At 30–50% confluence, A1A1v cells were transfected with siRNA at a final concentration of 90 nM using Lipofectamine™ 2000 (Qiagen, Germantown, MD) according to the manufacturer’s instructions. 24 hours after transfection, siRNA-lipid complexes were removed by changing medium. Transfection efficiency was assessed based on the percentage of fluorescent cells observed under Nikon Eclipse TE 2000U microscopy. 48 hours after transfection, cells were stimulated with DOI or vehicle for 5 minutes, and then cell lysates were collected to measure TGase2 expression and
TGase-modified Rac1 by immunoblot and immunoprecipitation. Cells incubated with Lipofectamine™ 2000 alone were used as the nontransfected control.

**Statistical Analyses**

All data are presented as group mean ± the standard error of the mean (SEM) and analyzed by one-way or two-way ANOVA. Post hoc tests were conducted using Newman-Keuls Multiple Comparison Test. SYSTAT 11 (Systat Software, Inc., San Jose, CA) and GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) were used for all statistical analyses. A probability level of $p<0.05$ was considered to be statistically significant for all statistical tests.

**RESULTS**

**Serotonin treatment induces Rac1 transamidation in A1A1v cells**

In order to determine whether TGase-catalyzed transamidation of Rac1 is increased after serotonin treatment, we treated A1A1v cells with 14 µM serotonin for 5, 15 or 30 minutes. The transamidation of Rac1 is significantly elevated after 5 or 15 minutes of serotonin treatment by approximately 2~2.5 fold as compared to vehicle (HCl)-treated cells (Fig. 3). However, there is no significant difference in the amount of TGase-modified Rac1 in cells treated with serotonin for 30 minutes as compared to vehicle-treated cells (Fig. 3A, B).

**5-HT$_{2A}$ receptor stimulation increases the transamidation of Rac1**

In order to explore the serotonin receptor specificity for induction of TGase-catalyzed
transamidation, A1A1v cells were stimulated with 14 µM serotonin, 3 µM DOI (5-HT2A/2C receptor agonist), 10 nM DPAT (5-HT1A receptor agonist) or 10 µM HCl (vehicle) for 15 minutes. Treatment with serotonin or DOI significantly ($p<0.05$) increases the amount of TGase-modified Rac1 2-fold as compared to vehicle-treated cells, whereas treatment with DPAT has no effect on the amount of TGase-modified Rac1 compared to vehicle-treated cells (Fig. 4A, B). Expression of 5-HT2A receptors in A1A1v cells has been previously confirmed using anti-sense oligodeoxynucleotide strategies and radioligand binding assays (Scalzitti et al., 1998). Here, 5-HT2C receptor expression in undifferentiated and differentiated A1A1v cells was examined by immunoblot analysis. Choroid plexus tissue from the fourth ventricle of a rat was used as a positive control and HEK 293 cell lysates were used as a negative control. We found that 5-HT2C receptors are expressed in rat choroid plexus, but 5-HT2C receptor expression was not detected in either HEK293 cells or undifferentiated or differentiated A1A1v cells (Fig. 4C). To exclude the possibility that the lack of involvement of the 5-HT1A receptors in Rac1 transamidation is not due to insufficient concentration of DPAT, we treated cells with 14 µM serotonin and increasing concentrations of DPAT (1 nM, 10 nM, and 100 nM) for 5 minutes, and then detected ERK phosphorylation by immunoblot. Treatment with 14 µM serotonin and 10 nM or 100 nM DPAT significantly increased phosphorylation of ERK (Fig. 4D, E), indicating 10 nM DPAT is enough to activate 5-HT1A receptors in A1A1v cells. To further confirm that the effect of DOI on Rac1 transamidation in A1A1v cells is due
to stimulation of the 5-HT\textsubscript{2A} receptors, cells were pretreated with 100 nM MDL 100907 (a selective 5-HT\textsubscript{2A} receptor antagonist) for 15 minutes, and then stimulated with DOI for 5 minutes. We found the pretreatment of MDL 100907 significantly reduced DOI-induced Rac1 transamidation without itself having any effect on Rac1 transamidation (Fig. 4F).

**DOI increases Rac1 transamidation in both undifferentiated and differentiated A1A1v cells**

After differentiation of A1A1v cells, the cytoskeleton undergoes a dramatic reorganization and the cells acquire a neuronal-like cell shape with long processes similar to axons and dendrites compared to undifferentiated cells (Fig. 5A). Rho family GTPases are critical regulators of the actin cytoskeleton organization (Etienne-Manneville and Hall, 2002, Burridge and Wennerberg, 2004). This prompted us to explore the effects of cell differentiation on Rac1 transamidation stimulated by 5-HT\textsubscript{2A} receptor activation. Stimulation of 5-HT\textsubscript{2A} receptors with DOI increased TGase-catalyzed transamidation of Rac1 in both undifferentiated and differentiated cells (Fig. 5B). Treatment with DOI in A1A1v cells significantly increased the amount of TGase-modified Rac1 by approximately 2~2.5 fold as compared to vehicle-treated cells (\(p<0.01\)). In vehicle-treated groups, the amount of TGase-modified Rac1 in differentiated cells was similar to the amount in undifferentiated cells, indicating that cell differentiation has no effect on basal level of Rac1 transamidation. Although the amount of TGase-modified Rac1 in DOI-treated differentiated cells was increased
compared to DOI-treated undifferentiated cells, this increase was not significant, suggesting that cell differentiation also does not have a significant effect on DOI-induced transamidation of Rac1 (Fig. 5C).

**The activity of Rac1 is transiently increased following serotonin stimulation**

Rac1 is GDP-bound in the inactive state and GTP-bound when activated. Activated Rac1 binds to its downstream effectors such as PAK1, therefore a GST-PAK1 fusion protein purified from *E. coli* was used to determine if serotonin stimulation increases the activated form of Rac1. A1A1v cells were treated with serotonin for 5 or 15 minutes, the cells were harvested and cell lysates were incubated with GST-PAK1 prebound to glutathione-Sepharose beads. Then equivalent amounts of the purified proteins were resolved by SDS-PAGE. Immunoblot analysis was performed using an antibody against Rac1. As shown in Fig. 6, the activity of Rac1 is increased by 80 percent after 5 minutes of serotonin treatment, but returns back to baseline after 15 minutes in the presence of serotonin, indicating that Rac1 becomes transiently activated after serotonin treatment in A1A1v cells. In addition, there is no significant change in the total amount of Rac1 in the cell lysate after serotonin treatment (Fig. 6A), suggesting that the activity increase is not due to synthesis of new Rac1.

**TGase inhibition reduces Rac1 transamidation and activity**

The activity of Rac1 is under the direct control of a large set of regulatory proteins. To exam whether the serotonin-stimulated increase of Rac1 activity is due to TGase-catalyzed transamidation, A1A1v cells were treated with increasing
concentrations (0 µM, 100 µM, 500 µM and 1000 µM) of the TGase inhibitor, cystamine, for 1 h, followed by treatment with 14 µM serotonin for 5 minutes. Precipitation of TGase-modified proteins and the activated Rac1 were performed as in previous experiments. Then immunopurified proteins and activated Rac1 were examined on immunoblots using anti-Rac1 and 81D4 antibodies. We found that pretreatment with cystamine significantly decreases Rac1 transamidation (Fig. 7) and activity (Fig. 8) in a dose-dependent manner. Treatment with 100 µM, 500 µM or 1000 µM of cystamine decreases the amount of TGase-modified Rac1 by approximately 50%, 70% or 80%, respectively, as compared to untreated cells (Fig. 7A, C). A similar dose-dependent decrease in TGase-modified Rac1 was also observed when cells were treated with serotonin for 15 minutes following cystamine inhibition (data not shown). Treatment with 500 µM or 1000 µM of cystamine decreases the amount of activated Rac1 by approximately 20% or 40%, respectively, as compared to untreated cells (Fig. 8A, C). These results indicate that TGase-catalyzed transamidation of Rac1 contributes to the increase in Rac1 activity upon serotonin stimulation. The ubiquitously expressed Na⁺/K⁺ ATPase is a well-established plasma membrane marker and its function underlies essentially all of mammalian cell physiology (Kaplan, 2002). In order to verify that the dose-dependent decrease of Rac1 transamidation and activation was not due to cystamine-induced total Rac1 reduction or cellular toxicity, cell lysates from the same experiment were examined on western blots with antibodies for Rac1 or Na⁺/K⁺ ATPase. There is no
significant difference in total Rac1 or Na\(^+\)/K\(^+\) ATPase levels between cystamine-treated and untreated cells, indicating that the concentrations of cystamine were not toxic to the cells (Fig. 7B, 8B).

Next, a second and more specific approach was used to determine the significance of TGase in Rac1 transamidation; we designed two siRNA duplexes to inhibit endogenous TGase expression. Several isoenzymes of TGase are found in the brain, of which TGase2 is the most abundant, therefore siRNAs were developed to silence rat TGase2 gene. At 24 hours post-transfection, transfection efficiency of both siRNA reached about 90%. 48 hours after transfection, siRNA1 and siRNA2 transfection of A1A1v cells resulted in 95% and 65% down-regulation of TGase2 protein expression, respectively (data not shown). Reprobing of membrane with anti-Rac1 antibody revealed that neither siRNA changed Rac1 protein level, indicating no off-target effect on Rac1 (Fig. 9A). DOI induced TGase-modification of Rac1 was significantly reduced by knocking down TGase2 expression with siRNA1 or siRNA2. As compared to DOI-stimulated nontransfected cells, siRNA1 and siRNA2 transfection caused 70% and 30% decreases in TGase-modified Rac1 responding to DOI, respectively (Fig. 9B, C). These results confirm that 5-HT\(_{2A}\) receptor-mediated Rac1 transamidation is dependent on TGase2 expression in A1A1v cells.

TGase-mediated transamidation of serotonin to Rac1

TGase-modified Rac1 did not show a significant upward shift on immunoblots
compared to native Rac1 in cell lysates (data not shown), indicating that the molecular weight of Rac1 does not significantly increase after modification by TGase. Therefore, a small amine such as serotonin is most likely incorporated into Rac1 upon stimulation of 5-HT2A receptors. To test this hypothesis, we stimulated A1A1v cells with serotonin or vehicle for 5 minutes then cells were harvested and cell lysates were used to immunoprecipitate Rac1 with an anti-Rac1 antibody, or immunoprecipitate TGase-modified proteins with the 81D4 antibody. The immunopurified proteins were examined on immunoblots using antibodies directed against serotonin. Immunoprecipitation of Rac1 and probing for serotonin reveals an association between Rac1 and serotonin in A1A1v cells (Fig. 10A). However we were unable to detect serotonin in TGase-modified proteins (Fig. 10A). This may be due to a compromise of the serotonin antibody epitope during the immunoprecipitation of TGase-modified proteins, since this procedure uses higher temperatures and longer incubation times compared to the Rac1 immunoprecipitation procedure. Therefore in order to confirm that serotonin is associated with Rac1 by a TGase-catalyzed covalent bond, we treated cells with increasing concentrations (0 μM, 100 μM, 500 μM, 1000 μM) of cystamine for 1 hour followed by stimulating the cells with serotonin for 5 minutes. Then we immunoprecipitated Rac1 and detected associated serotonin by immunoblot, as described above. We found that treatment with cystamine decreases the serotonin-associated Rac1 in a dose-dependent manner (Fig. 10B, D), thus supporting the hypothesis that serotonin is incorporated into Rac1 by a
TGase-catalyzed covalent bond. To compare the effects of different agonists on the incorporated serotonin, cells were exposed to either serotonin or DOI for 5 min. Then Rac1 was immunoprecipitated and the incorporated serotonin was detected by immunoblot. There was no significant difference in serotonin incorporation between serotonin and DOI-treated cells. Pretreatment with cystamine also inhibited DOI-induced serotonin incorporation (Fig. 11).
Fig. 3 Serotonin-induced increase of Rac1 transamidation in A1A1v cells. (A) After 5, 15 or 30 minutes of 14 µM serotonin treatment, TGase-modified proteins were immunoprecipitated with 81D4 antibody coupled to sepharose. TGase-modified Rac1 was then detected on immunoblots with anti-Rac1 antibody. (B) Quantitation of immunoblots for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to vehicle-treated control levels. One-way ANOVA ($F_{(3,8)}=7.31$, $p<0.05$) indicates a significant difference among groups. Newman-Keuls Multiple Comparison Test indicates $p<0.05^*$ as compared to vehicle treatment.
Fig. 4 Serotonin receptor specificity on induction of Rac1 transamidation in A1A1v cells. (A) Cells were stimulated with 14 µM serotonin, 3 µM DOI (5-HT2A/2C receptor agonist), 10 nM DPAT (5-HT1A receptor agonist) or vehicle for 15 minutes. TGase-modified Rac1 was detected by immunoprecipitation and immunoblot analysis. (B) Quantitation of immunoblots for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to vehicle-treated control levels. One-way ANOVA ($F_{(3,8)}=10.05$, $p<0.01$) indicates a significant difference among various treatments. Newman-Keuls Multiple Comparison Test indicates $p<0.05^*$ as compared to vehicle treatment. (C) Immunoblot analysis of 5-HT2C receptor expression in undifferentiated (UD) and differentiated (DI) A1A1v cells. Rat choroid plexus was used as a positive
control and HEK 293 cell was used as a negative control. (D) A1A1v cells were treated with vehicle, 14 µM serotonin or DPAT (1 nM to 100 nM) for 5 minutes. Cell lysates were then resolved by SDS-PAGE, followed by immunoblotting with an anti-phosphorylated ERK antibody. Equal loading was verified by reprobing the same membrane with antibodies to total ERK and actin. (E) Quantitation of immunoblots for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to serotonin-treated control levels. Each phospho-ERK value was normalized using the corresponding total ERK value to control for the possibility of differential loading. One-way ANOVA ($F _ { (4,10)} = 18.12, p < 0.001$) indicates a significant difference among various treatments. Newman-Keuls Multiple Comparison Test *indicates $p < 0.05$ as compared to vehicle treatment and #indicates $p < 0.05$ as compared to serotonin treatment. (F) Upper, cells were stimulated with vehicle, 14 µM serotonin, 3 µM DOI. Some cells were pretreated with 100 nM MDL 100907 prior to treatment with DOI or treated with MDL100907 alone. TGase-modified Rac1 was detected by immunoprecipitation and immunoblot analysis. Lower, quantitation of immunoblots for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to vehicle-treated control levels. One-way ANOVA ($F _ { (4,10)} = 38.96, p < 0.001$) indicates a significant difference among various treatments. Newman-Keuls Multiple Comparison Test indicates $p < 0.05$* as compared to vehicle treatment and $p < 0.05$# as compared to DOI treatment.
Fig. 5 The effects of cell differentiation on Rac1 transamidation. (A) A1A1v cells were incubated at 37°C for 4 days to induce differentiation. Compared to undifferentiated cells (left), differentiated cells (right) develop a neuron-like cell shape with long neurites. (B) Cells were treated with 3 µM DOI for 15 minutes, followed by immunoprecipitation and immunoblot analysis. Stimulation of 5-HT2A receptors by DOI increased the TGase-catalyzed transamidation of Rac1 in both undifferentiated and differentiated cells. (C) Quantitation of immunoblots for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to vehicle-treated control levels in undifferentiated cells. Two-way ANOVA indicates a
significant main effect of DOI treatment ($F_{(1,8)}=49.48, p<0.01$). However, there was no significant main effect of cell differentiation on TGase-modified Rac1 ($F_{(1,8)}=4.07, p=0.08$). The interaction between DOI treatment and cell differentiation was also not significant for TGase-modified Rac1 ($F_{(1,8)}=0.34, p=0.58$). Newman-Keuls Multiple Comparison Test indicates $p<0.01\ast$ as compared to vehicle treatment in undifferentiated cells, $p<0.01\#$ as compared to vehicle treatment in differentiated cells.

Scale bar, 105 µm.
Fig. 6 Rac1 activity was transiently increased after serotonin treatment. (A) Top, activated Rac1 was purified using GST-PAK1 coupled to glutathione-Sepharose beads and detected on immunoblot with an antibody against Rac1. Middle, the total amount of Rac1 in the cell lysates used for pull-down of activated Rac1 was monitored by immunoblot. Bottom, Ponceau staining of GST-PAK1 documents equal amounts of protein loading in each lane. (B) Quantitation of immunoblot for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to vehicle-treated control levels. The two-way ANOVA reveals a significant ($F_{(1,8)}=8.64$, $p<0.05$) main effect for serotonin treatment, and a significant ($F_{(1,8)}=6.32$, $p<0.05$) interaction between serotonin treatment and time course. Newman-Keuls Multiple Comparison Test indicates $p<0.05^*$ as compared to 5 minutes of serotonin treatment.
Fig. 7 Dose-dependent effects of cystamine on Rac1 transamidation. (A) Immunoprecipitation and immunoblot analyses reveal that cystamine causes a dose-dependent (100 to 1000 µM) inhibition of the transamidation of Rac1 as indicated by less intense bands. (B) Cells lysates from the same experiment were examined on immunoblots with antibodies for Rac1 and Na⁺/K⁺ ATPase, which indicate that cystamine has no effect on total Rac1 and cell viability, respectively. (C) Quantitation of effects of cystamine on Rac1 transamidation in 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to untreated control levels. One-way ANOVA ($F_{(3,8)}=19.41$, $p<0.001$) indicates a significant effect of cystamine on Rac1 transamidation. Newman-Keuls Multiple Comparison Test indicates $p<0.05^*$ as compared to untreated cells.
Fig. 8 Rac1 activity is inhibited by cystamine. (A) Top, pull-down and immunoblot analyses reveal that cystamine causes a dose-dependent (100 to 1000 µM) inhibition of the activation of Rac1. Middle, the same membrane was reprobed with antibody 81D4, directed against TGase-catalyzed covalent bonds. Bottom, Ponceau staining of GST-PAK1 (~35 kDa) documents equal amounts of protein loading in each lane. (B) Cell lysates from the same experiment were examined on immunoblots with an antibody for Na⁺/K⁺ ATPase. (C) Quantitation of the effects of cystamine on Rac1 activation in 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to untreated control levels. One-way ANOVA ($F_{(3,8)}=20.94$, $p<0.001$)
indicates a significant effect of cystamine on Rac1 activity. Newman-Keuls Multiple Comparison Test indicates $p<0.05^*$ as compared to untreated cells, $p<0.001^{**}$ as compared to untreated cells.
Fig. 9 Knockdown of TGase2 by siRNAs prevents Rac1 transamidation. (A) Cells were incubated with 90nM of TGase2-specific siRNAs for 24 hours, and then treated with 3 µM of DOI or vehicle for 5 minutes at 48 hours post-transfection. Immunoblot analyses confirmed that transfection of siRNA1 or siRNA2 significantly inhibited TGase2 protein expression compared to nontransfected control (NT). The membranes were stripped and reprobed with anti-Rac1 and anti-actin antibodies. (B) TGase-modified Rac1 was detected by immunoprecipitation and immunoblot analysis. DOI induced TGase-modification of Rac1 were significantly decreased in siRNAs-transfected cells compared to NT cells. (C) Quantitation of effects of siRNAs
on Rac1 transamidation in 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to DOI-stimulated nontransfected cells. Two-way ANOVA indicates a significant main effect of transfection ($F_{(2,12)}=24.68, p<0.0001$), a significant main effect of DOI treatment ($F_{(1,12)}=63.23, p<0.0001$) and a significant interaction between transfection and DOI treatment ($F_{(2,12)}=9.42, p<0.01$) on TGase-modified Rac1. Newman-Keuls Multiple Comparison Test indicates $p<0.01^*$ or $p<0.001^{**}$ as compared to vehicle treatment in nontransfected cells, $p<0.01#$ or $p<0.001^{##}$ as compared to DOI treatment in nontransfected cells.
Fig. 10 Transamidation of serotonin to Rac1 is mediated by TGase. (A) Immunoprecipitation of Rac1, but not TGase-modified proteins, reveals an association between Rac1 and serotonin in A1A1v cells upon 5 minutes of 14 µM serotonin stimulation. (B) Immunoprecipitation and immunoblot analysis reveal that cystamine causes a dose-dependent (100 to 1000 µM) reduction of the serotonin-associated Rac1. Normal mouse IgG is used as a negative control (NC) for immunoprecipitation. (C) Cell lysates from the same experiment were examined on immunoblots with an antibody for Na+/K+ ATPase, which indicates cystamine has no effect on cell viability. (D) Quantitation of the effects of cystamine on
serotonin-induced transamidation of Rac1. Data shown are the mean IOD ± SEM and normalized to untreated control levels. One-way ANOVA ($F_{(3,8)} = 7.15, p<0.05$) indicate a significant difference in serotonin-associated Rac1 among cells treated with different concentration of cystamine. Newman-Keuls Multiple Comparison Test indicates $p<0.05^*$ as compared to untreated cells.
Fig. 11 DOI stimulates transamidation of Rac1 to serotonin to a similar extent as those treated with serotonin. (A) Cells were stimulated with vehicle, 14 µM 5-HT or 3 µM DOI for 5 minutes. Some cells were pretreated with 500 µM cystamine (Cys) for 1 hour prior to stimulation with DOI. Transamidation of serotonin to Rac1 was detected by immunoprecipitation and immunoblot analysis. (B) Quantitation of immunoblot for 3 separate experiments. Data shown are the mean IOD ± SEM and normalized to serotonin-treated levels. One-way ANOVA ($F(3,8)=19.80$, $p<0.001$) indicates a significant difference in Rac1-incorporated serotonin among groups. Newman-Keuls Multiple Comparison Test * indicates $p<0.05$ as compared to vehicle-treated cells and # indicates $p<0.05$ as compared to serotonin-treated cells.
DISCUSSION

In platelets, 5-HT$_2A$ receptor activation causes TGase-catalyzed transamidation of RhoA and Rab4, leading to activation of these proteins and platelet aggregation (Walther et al., 2003). In *aplysia* ganglia, serotonin-treatment induces the activation of Cdc42 and its downstream effector PAK to regulate the actin cytoskeleton (Udo et al., 2005). In the present study, we extend these findings to a rat cortical cell model and find that 5-HT$_2A$ receptor stimulation increased TGase-catalyzed transamidation of Rac1 and Rac1 activity, both of which can be suppressed by TGase inhibition. In elucidating the mechanism further, we identify serotonin as an amine that becomes transamidated to Rac1 by TGase. Activation of Rac1 via TGase is a novel effector and second messenger of the 5-HT$_2A$ receptor signaling pathway in neurons.

To exam the serotonin receptor specificity on induction of Rac1 transamidation, we treated cells with different serotonin receptor agonists and found that the 5-HT$_2A/2C$ receptor agonist DOI, but not the 5-HT$_1A$ receptor agonist DPAT induces a significant increase in TGase-modified Rac1 with a similar magnitude to that observed in serotonin-treated cells. Since there is no 5-HT$_2C$ receptor expression in A1A1v cells and MDL 100907 reversed the effect of DOI on Rac1 transamidation (Fig. 4), serotonin or DOI-induced Rac1 transamidation is selectively mediated by activation of 5-HT$_2A$ receptors in A1A1v cells.

Previous studies demonstrated that TGase-mediated transamidation of the Rho
family of small G proteins blocked the GTP-hydrolyzing activity of these proteins, rendering these small GTPases constitutively active for their respective signaling pathways (Masuda et al., 2000, Walther et al., 2003). Based on these results, we hypothesized that 5-HT$_{2A}$ receptor-stimulated transamidation of Rac1 by TGase will result in prolonged activation of Rac1. Unexpectedly, we found a transient increase in both Rac1 activity and TGase-catalyzed transamidation which returned to baseline levels following continuous exposure to serotonin (Figs. 3 and 6). The transient response may due to selective degradation of transamidated Rac1. Mammalian cells contain multiple proteolytic systems for degradation of various classes of intracellular proteins. Most abnormal proteins (mutant or misfolded) are degraded in the ubiquitin–proteasome pathway. Rac1 is normally a stable protein, but activation by cytotoxic necrotizing factor-1 (CNF-1) in rat bladder carcinoma cells (804G) and human umbilical vein endothelial cells resulted in increased sensitization to ubiquitination and proteasomal degradation (Doye et al., 2002, Munro et al., 2004). Further, dominant-positive forms of Rac1 appeared more susceptible to ubiquitin-mediated proteasomal degradation compared to both dominant-negative and wild-type Rac1 (Doye et al., 2002). Activated and transamidated Rac1 may be targeted for proteasomal degradation, resulting in transient activation of Rac1. However, there is no significant change in total Rac1 protein 15 min after serotonin stimulation (Fig. 4A), suggesting that activated or transamidated Rac1 may only account for small fraction of total Rac1.
In this study, we found differences in the time course for Rac1 transamidation and for its increased activity. After serotonin stimulation for 15 minutes, Rac1 activity is reduced to baseline (Fig. 6) while TGase-catalyzed transamidation of Rac1 still remains at a relatively high level (Fig. 3). These results suggest that transamidation may not only occur at the GTPase activity-related residues, but also at residues which do not have an effect on Rac1 activity. Essentially, transamidation of Rac1 at some sites may induce activation of the small G proteins, while transamidation at other sites may not affect the GTPase activity of Rac1. If activated Rac1 is more sensitive to proteasomal degradation, the activated Rac1 may be degraded earlier than non-activated Rac1 that is transamidated at sites not involved in activation.

Cystamine has been shown to decrease TGase activity and TGase-catalyzed $\varepsilon$-(γ-glutamyl) lysine bonds in cultured cells and animal models of neurodegenerative diseases (Karpuj et al., 2002a, Ientile et al., 2003, Zainelli et al., 2005). As a primary amine, cystamine is a potential substrate for TGase, and acts as a competitive inhibitor for TGase by blocking access to the active site of the enzyme for the glutamine residues in proteins which would otherwise participate in forming $\varepsilon$-(γ-glutamyl) lysine bonds (Lorand et al., 1979). In our study, pretreatment with cystamine reduced both serotonin-stimulated Rac1 transamidation (Fig. 7A, C) and activation (Fig. 8A, C) in a dose-dependent manner. Those observations were further supported by the use of TGase2-specific siRNAs, which significantly suppressed DOI induced TGase-modification of Rac1 (Fig. 9B, C). These results suggest that
cystamine prevented Rac1 transamidation through TGase inhibition, and that transamidation of Rac1 by TGase may contribute to the increase in Rac1 activity. Post-translational modifications, such as transamidation and phosphorylation of Rac1 may affect its ability to interact with regulatory proteins (e.g. GAPs, GEFs and GDIs) or to convert GTP back to GDP, resulting in increased activation of Rac1 and stimulation of its signaling cascade in the cells. The Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=ddd) provides an interactive tool to identify conserved domains present in protein sequences. We searched the CDD for GTP/Mg^{2+} binding sites, and GAPs, GEFs and GDIs interaction sites in the Rac1 sequence (PSSM-Id: 57957) because they will be most likely to modify Rac1 activity after TGase-catalyzed transamidation at these sites. We identified two glutamine residues (Gln61, Gln74) located within these domains. It has been reported that site-specific deamidation of a Gln residue in the Rho family G proteins (Gln61 in Rac and Cdc42, Gln63 in RhoA) by CNF-1 inhibits both intrinsic and GAPs-stimulated GTP hydrolysis activity, resulting in constitutive activation of these proteins (Flatau et al., 1997, Schmidt et al., 1997). CNF-1 has also been shown to possess in vitro TGase activity. In the presence of primary amines, RhoA is transamidated in vitro at Gln63 by CNF-1 and at positions 52, 63 and 136 by guinea pig liver TGase (Schmidt et al., 1998). Similarly, in addition to Gln61 which is critical in regulating Rac1 activity, the other four glutamine residues in Rac1 may also be modified by TGase. It is not surprising, therefore, that the same dosage of cystamine
prevents Rac1 transamidation and its activation to different extents (Fig. 7C, 8C).

Walther et al. found that in platelets TGase covalently cross-links serotonin to RhoA or Rab4 at a position in the phosphate-binding site that is conserved in the sequences of all Ras-related small GTPases (Walther et al., 2003). It has also been shown that polyamines such as putrescine, spermidine and spermine could be incorporated into Rac1 by bacterial TGase in vitro (Masuda et al., 2000). In our study, co-immunoprecipitation assays demonstrated that serotonin is associated with Rac1 in A1A1v cells after serotonin stimulation (Fig. 10A). To explore the association mechanism further, we inhibited TGase by cystamine and found a reduction of serotonin-associated Rac1 with increasing concentrations of cystamine (Fig. 10B, D), indicating that serotonin is incorporated into Rac1 by a TGase-catalyzed bond. Since both serotonin and DOI stimulation can lead to serotonin incorporation to similar levels (Fig. 11), the serotonin bound to 5-HT2A receptors is not likely the source of Rac1-incorporated serotonin; the serotonin bound to Rac1 may originate from endogenously synthesized serotonin or serotonin transported into the cell from serum in the cell culture media.

The first signal transduction mechanism identified for the 5-HT2A receptor was Gq/11 mediated activation of PLC, leading to increased accumulation of IP3 and an increase in intracellular Ca2+ (Boess and Martin, 1994). In addition to activation of PLC, extensive evidence suggests that 5-HT2A receptors couple to other effector pathways, such as phospholipase A2/arachidonic acid cascade (Berg et al., 1994b),
Mitogen-Activated Protein Kinase signaling (Watts, 1998) and phospholipase D/protein kinase C pathway (Mitchell et al., 1998). On the other hand, TGases are subjected to transcriptional regulation by retinoic acid and steroid hormones (Fujimoto et al., 1996, Ou et al., 2000), and require the binding of Ca\(^{2+}\) for their activity (Burgoyne and Weiss, 2001). Evidence indicates that TGase activity can be significantly enhanced in response to increased intracellular Ca\(^{2+}\) through IP\(_3\) generation (Zhang et al., 1998). In *aplysia*, serotonin mediated activation of Cdc42 was dependent on PLC and phosphatidylinositol 3-kinase (PI3K) (Udo et al., 2005). In RA-induced neuronal differentiation of SH-SY5Y cells, TGase-catalyzed transamidation is required for activation of RhoA (Singh et al., 2003), whereas activation of Rac1 is mediated by PI3K in a transamidation-independent manner (Pan et al., 2005). Further studies are needed to elucidate the underlying molecular mechanisms by which the 5-HT\(_{2A}\) receptor signaling regulates TGase-catalyzed activation of Rac1.

The present study provides the first evidence in neurons that stimulation of 5-HT\(_{2A}\) receptors induces an increase in TGase-catalyzed transamidation of serotonin into Rac1 and constitutive activation of Rac1. Further studies using alternative approaches to measure Rac1 transamidation, such as mass spectroscopy, are needed to confirm this hypothesis. Rac1 activation via 5-HT\(_{2A}\) receptor stimulation may have an important functional impact given the plethora of pathways that utilize this small G protein. For example, Rac1 is best known as a regulator for the assembly of the actin
cytoskeleton, thereby playing a role in neurite outgrowth and neuronal differentiation. We demonstrate that stimulation of 5-HT$_{2A}$ receptor can increase the transamidation of Rac1 in both undifferentiated and differentiated A1A1v cells, suggesting that transamidation and constitutive activation of this signal transducer are not altered by neuronal differentiation of cells. However, the effect of 5-HT$_{2A}$ receptor-mediated activation of Rac1 on cytoskeleton organization, cell cycle progression, transcriptional activation or other crucial cellular functions in neurons has yet to be explored.
CHAPTER THREE:

PHOSPHOLIPASE C, CALCIUM AND CALMODULIN SIGNALING ARE REQUIRED FOR 5-HT$_{2A}$ RECEPTOR-MEDIATED TRANSMIDATION OF RAC1 BY TRANSGLUTAMINASE

ABSTRACT

Increasing evidence indicates that small G proteins undergo transglutaminase (TGase)-catalyzed transamidation and activation in neurons, platelets, and smooth muscle cells. We have previously reported that 5-HT$_{2A}$ receptor stimulation increased TGase-catalyzed transamidation and activation of Rac1 small G protein in A1A1v cells, a rat embryonic cortical cell line. In this study, we explore the signaling pathways involved in 5-HT$_{2A}$ receptor-mediated Rac1 transamidation. In cells pretreated with phospholipase C (PLC) inhibitor U73122, the 5-HT$_{2A}$ receptor specific agonist, DOI-stimulated increase in intracellular Ca$^{2+}$ concentration and TGase-modified Rac1 were significantly attenuated as compared to those pretreated with U73343, an inactive analog. Addition of the membrane-permeant Ca$^{2+}$ chelator, BAPTA-AM strongly reduced TGase-catalyzed Rac1 transamidation upon DOI stimulation. Conversely, when cells were treated with Ca$^{2+}$ ionophore, ionomycin at a concentration that produced an elevation of cytosolic Ca$^{2+}$ to a level comparable to cells treated with DOI, ionomycin mediated an increase in TGase-modified Rac1.
without 5-HT<sub>2A</sub> receptor activation. Moreover, calmodulin (CaM) inhibitor W-7, significantly decreased Rac1 transamindation in a dose-dependent manner in DOI-treated cells. To identify potential TGase-targeting residues that could render Rac1 constitutively active upon transamidation, a search in NCBI Conserved Domain Database was conducted, which revealed that two glutamine residues (Q61, Q74) and three lysine residues (K5, K16, and K116) are located within activity-related domains. Taken together, these results indicated that 5-HT<sub>2A</sub> receptor-coupled PLC activation and subsequent Ca<sup>2+</sup>/CaM signaling are necessary in TGase-catalyzed Rac1 transamidation, and an increase in intracellular Ca<sup>2+</sup> is sufficient to induce Rac1 transamination in A1A1 cells.

INTRODUCTION

Rac1 (Ras-related C3 botulinum toxin substrate 1), one of the most extensively characterized members in the Rho family of small G proteins, was initially discovered as a substrate for ADP-ribosylation induced by the C3 component of botulinum toxin (Didsbury et al., 1989). Subsequently, Rac1 and its downstream effectors were identified as key signaling molecules in various cell functions, such as cytoskeleton reorganization, cell transformation, axonal guidance, and cell migration (Etienne-Manneville and Hall, 2002, Bosco et al., 2009).

Like all members of the Rho superfamily, Rac1 functions as a molecular switch, cycling between an inactive GDP-bound state and an active GTP-bound state. Rho
small G proteins can be activated by different signal transduction pathways initiated by extracellular factors such as platelet-derived growth factor, insulin or epidermal growth factor (Bishop and Hall, 2000). Serotonin has also been shown to induce activation via transglutaminase (TGase)-catalyzed transamidation of small G proteins in neurons, platelets, and aortic smooth muscle cells (Walther et al., 2003, Guilluy et al., 2007, Dai et al., 2008). TGases catalyze the transamidation of protein-bound glutamines and primary amines such as serotonin in a Ca\(^{2+}\)-dependent manner (Ahvazi et al., 2002). We previously reported that the effect of serotonin on Rac1 transamidation in A1A1v cells, a rat cortical cell line, is due to stimulation of the 5-HT\(_{2A}\) receptors (Dai et al., 2008). However, the underlying molecular mechanisms by which the 5-HT\(_{2A}\) receptor signaling regulates TGase-catalyzed transamidation and activation of Rac1 are still unclear.

5-HT\(_{2A}\) receptor activation can increase IP\(_3\) via G\(_{q/11}\)-mediated activation of phospholipase C (PLC) (Conn and Sanders-Bush, 1984, de Chaffoy de Courcelles et al., 1985). PLC plays an important role in intracellular signal transduction by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2), a membrane phospholipid, and thereby generating two second messengers: inositol trisphosphate (IP\(_3\)) which diffuses through the cytosol and releases Ca\(^{2+}\) from intracellular endoplasmic reticulum stores and diacylglycerol (DAG) (Boess and Martin, 1994). It has been reported that TGase activity can be significantly enhanced in response to increased intracellular Ca\(^{2+}\) through inositol 1,4,5-trisphosphate (IP\(_3\)) generation (Zhang et al.,
Moreover, serotonin-mediated activation of small G protein cdc42 was dependent on PLC signaling pathways (Udo et al., 2005).

Calmodulin (CaM) has been shown to increase TGase enzymatic activity. In the presence of CaM, a membrane-associated erythrocyte TGase is activated at physiological and lower than physiological Ca$^{2+}$ concentrations (Billett and Puszkin, 1991). Moreover, in the presence of Ca$^{2+}$, CaM enhanced TGase activity 3-fold in human platelets and the chicken gizzard (Puszkin and Raghuraman, 1985). A CaM inhibitor prevented TGase-catalyzed cross-linking of huntingtin in cells co-transfected with mutant huntingtin and TGase (Zainelli et al., 2004). Taken together, we hypothesize that TGase-catalyzed Rac1 modification upon 5-HT$_{2A}$ receptor stimulation is dependent on PLC-mediated increases in intracellular Ca$^{2+}$ and is regulated by CaM. To test this hypothesis, we examined the effects of PLC inhibition, CaM inhibition, and manipulation of intracellular Ca$^{2+}$ by means of a Ca$^{2+}$ ionophore or a chelating agent on TGase-modified Rac1 in response to 5-HT$_{2A}$ receptor activation in A1ATv cells.

MATERIALS AND METHODS

Cell Culture

A1ATv cells, a rat cortical cell line, were grown on 100 mm$^{2}$ plates coated with poly-L-ornithine (Sigma, St Louis, MO) and maintained in 5% CO$_2$ at 33°C, in Dulbecco’s modified Eagle medium (DMEM) (Fisher Scientific, Pittsburgh, PA)
containing 10% fetal bovine serum (FBS) (Fisher Scientific, Pittsburgh, PA). Before each experiment, cells were maintained in DMEM with 10% charcoal-treated FBS for 48 hours. Charcoal adsorption removes most but not all serotonin in the medium. The maximal final concentration of serotonin in the medium is approximately 3 nM (Unsworth and Molinoff, 1992). Cells from passages 8-15 were used for all experiments.

**Drugs**

The following drugs were used in this study: (-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI) (Sigma, St Louis, MO), U73122 (Tocris Bioscience Ellisville, MO), U73343 (Sigma, St Louis, MO), BAPTA AM (Invitrogen, Carlsbad, CA), ionomycin (Invitrogen, Carlsbad, CA), and W-7 hydrochloride (Tocris Bioscience Ellisville, MO).

**Measurement of intracellular Ca²⁺ concentration**

Cells were grown to 90% confluence in black-sided 96-well plates (Fisher Scientific, Pittsburgh, PA). Cells were washed twice with modified Kreb's medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 11.5 mM D-glucose, 11.6 mM Hepes, pH 7.3) and then incubated in the same medium with 5 µM Fura-2 AM (Molecular Probes, Carlsbad, CA), 0.1% bovine serum albumin, and 0.02% Pluronic F127 detergent (Poenie et al., 1986) for 60 min at 33°C incubator in the dark. After loading, the cells were washed twice and incubated in the dark in modified Kreb's
medium or pretreated with drugs for 30 min. Following 1 min of equilibration, the cells were stimulated with a single injection of 3 µM DOI, and the response was recorded until a plateau was reached (about 4 min). Fura-2 fluorescence (at 510 nm) was measured with a BioTek fluorescence plate reader. After background fluorescence was subtracted, the ratio of fluorescence at 340 nm excitation to that at 380 nm excitation was calculated and used as an index of the intracellular Ca\(^{2+}\) concentration (the 340/380 nm fluorescence ratio is positively correlated with the absolute values of intracellular Ca\(^{2+}\) concentration).

**Immunoprecipitation of TGase-modified Protein**

A1A1v cells were harvested and lysed using lysis buffer A (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 1:1000 protease inhibitor cocktail (Sigma, St Louis, MO) containing 104 µM AEBSF, 0.08 µM aprotinin, 2 µM leupeptin, 4 µM bestatin, 1.5 µM pepstatin A and 1.4 µM E-64). Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Immunopurification of proteins containing TGase-catalyzed bonds was performed using 81D4 mAb (mouse IgM) prebound to Sepharose beads (Covalab, Lyon, France) using a protocol developed by Covalab and as described previously (Norlund et al., 1999, Zainelli et al., 2005). The 81D4 antibody has been extensively used to demonstrate increases in TGase-catalyzed bonds (Citron et al., 2002, Junn et al., 2003, Andringa et al., 2004). The use of the 81D4 antibody in a competitive ELISA assay was shown to result in isodipeptide cross-link measurements that correlate well with
those measured by HPLC analysis but provide more sensitivity than the HPLC approach (Sarvari et al., 2002). Transfection of cells to over-express TGases, including TGase 1, 2 and 3, and a substrate protein such as mutant huntingtin protein results in increased presence of the isodipeptide cross-link in mutant huntingtin protein as demonstrated with immunoprecipitation with the 81D4 antibody (Zainelli et al., 2005).

Briefly, 20µl of sepharose-81D4 beads were washed three times in TBS/0.1% Tween 20 with gentle shaking for 15 minutes, followed by adding 200µg cell lysate (1 µg/µl) to the washed beads and incubating for 2 hours at 37°C. After incubation, the pellets were washed four times in TBS/0.1% Tween 20 for 15 minutes. Then 20µl of loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 5% β-mercaptoethanol) was added to the washed pellets followed by 5 minutes incubation at 90°C. The samples were then centrifuged at 9,000 × g for 2 minutes and the supernatant was transferred and stored at -80°C until immunoblot analysis.

**Immunoblot**

Immunoaffinity purified proteins and cell lysates were separated on 12% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. Membranes were then incubated in blocking buffer (5% non-fat dry milk, 0.1% Tween 20, 1× TBS) for 1 hour at room temperature. Membranes were incubated overnight at 4°C with primary antibodies on a shaker. Primary antibodies (Upstate
Biotechnology, NY: anti-Rac1, mouse IgG, 1:700; anti-Na+/K+ ATPase, mouse IgG, 1:10000) were diluted in antibody buffer (1% non-fat dry milk, 0.1% Tween 20, 1× TBS). The next day, membranes were washed with TBS/0.1% Tween 20 and then incubated with goat-anti-mouse or goat-anti-rabbit secondary antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) diluted in antibody buffer. Membranes were washed and signal was detected using enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). Using Scion Image for Windows (Scion, Frederick, MD), immunoblots were quantified by calculating the integrated optical density (IOD) of each protein band on the film.

Statistical Analyses

Data are presented as mean ± the standard error of the mean (SEM) and analyzed by one- or two-way ANOVA. Post hoc tests were conducted using Bonferroni’s multiple comparison tests. GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses. A probability level of p < 0.05 was considered to be statistically significant for all statistical tests.

RESULTS

PLC inhibitor decreases the response of intracellular Ca²⁺ and TGase-modified Rac1 to 5-HT₂A receptor activation

We previously reported that 5-HT₂A receptor stimulation induced
TGase-catalyzed Rac1 transamidation and activation in A1A1v cells (Dai et al., 2008).

To test the dependence of the intracellular Ca\(^{2+}\) response and Rac1 transamidation on PLC activation, cells were pretreated with 5 µM of the PLC inhibitor, U73122, or an inactive analog, U73343, for 30 min prior to the stimulation of 5-HT\(_{2A}\) receptors by 3 µM DOI. U73122 inhibits both PLC-mediated hydrolysis of PIP2 to IP3 and the coupling of G protein-PLC activation (Bleasdale et al., 1990). Changes in intracellular Ca\(^{2+}\) were monitored by the 340/380 nm fluorescence ratio of the Fura-2 loaded cells. DOI-stimulation induced a robust increase (by at least 200%) in the intracellular Ca\(^{2+}\) concentration within cells pretreated with U73343, whereas PLC inhibition by U73122 reduced the intracellular Ca\(^{2+}\) response to DOI (Fig. 12A). TGase-modified Rac1 was evaluated by immunoprecipitation using the 81D4 antibody directed against the TGase-catalyzed covalent bond and immunoblots in parallel experiments. There were significant increases in TGase-modified Rac1 within DOI-stimulated cells, but pretreatment with U73122 significantly attenuated DOI-induced Rac1 modification by TGase (Fig. 12B, C). These results suggest a specific role of PLC upstream of Rac1 transamidation in A1A1v cells. Two-way ANOVA indicates a significant main effect of DOI [F(1,8) =108.3; p<0.001] and U73122 [F(1,8) =29.99; p<0.001] on TGase-modified Rac1. There was also a significant interaction between DOI and U73122 [F(1,8) =17.40; p<0.01]. The ubiquitously expressed Na\(^{+}/K\(^{+}\) ATPase is a well-established plasma membrane marker, and its function underlies essentially all of mammalian cell physiology (Kaplan, 2002). To verify that the PLC inhibitor-mediated
the decrease in Rac1-transamidation by TGase was not due to U73122-induced cellular toxicity, cell lysates from the same experiment were examined on immunoblot with antibodies for Na+/K+ ATPase. There are no significant differences in Na+/K+ ATPase levels among cells with different treatments (Fig. 12B). Similar measurements were used in all of the following experiments.

**Ca^{2+} chelator inhibits DOI-stimulated modification of Rac1 by TGase**

In order to examine whether TGase-modified Rac1 specifically depends on an increase in intracellular Ca^{2+} upon 5-HT_{2A} receptor activation, A1A1v cells were pretreated with increasing concentrations of the membrane-permeant Ca^{2+} chelator, BAPTA-AM (0, 5, 10, 20 µM) for 30 min, then the cells were challenged by DOI. Dose-dependent reductions in the DOI-mediated Ca^{2+} response were observed by measuring Fura-2 fluorescence (Fig. 13A). Since 20 µM BAPTA caused the most significant inhibition in Ca^{2+} increase, it was used in the parallel experiments that evaluated TGase-modified Rac1. DOI-stimulation in cells without BAPTA-pretreatment significantly increases the amount of TGase-modified Rac1 about 2-fold compared with vehicle-stimulated cells, whereas pretreatment with BAPTA has a notable inhibitory effect on DOI-induced Rac1 modification by TGase (Fig. 13B, C), suggesting that an increase in intracellular Ca^{2+} is required for DOI-mediated Rac1 transamidation. Two-way ANOVA indicates a significant main effect of DOI [F(1,8) =37.09; p<0.001] and BAPTA [F(1,8) =9.85; p<0.05] on TGase-modified Rac1. There was also a significant interaction between DOI and
BAPTA [F(1,8) =9.50; p<0.05].

**Ca^{2+} ionophore induces TGase-modified Rac1 without 5-HT_{2A} receptor activation**

To determine if an increase in intracellular Ca^{2+} is sufficient to enhance Rac1 modification by TGase, a Ca^{2+} ionophore, ionomycin was used to produce an elevation of cytosolic Ca^{2+} concentration without 5-HT_{2A} receptor activation. Ionomycin is an effective and specific Ca^{2+} carrier, so it has been used in studies of Ca^{2+} flux across plasma and ER membranes. To determine the concentration of ionomycin that raises the levels of cytosolic Ca^{2+} to levels comparable to 3µM DOI (the concentration of DOI used to induce TGase-modified Rac1), serial dilutions of ionomycin (from 1000 nM to 12.5 nM) were tested for their ability to induce increases in intracellular Ca^{2+}. As shown in Fig. 14A, 25 nM ionomycin was able to produce a comparable increase in cytosolic Ca^{2+} in comparison to 3 µM DOI, so this concentration was used in the parallel experiments to measure the effects on TGase-modified Rac1. After cells were treated with either 3 µM DOI or 25 nM ionomycin for 15 min, ionomycin mediated an increase in TGase-modified Rac1 to levels similar to those induced by DOI. One-way ANOVA indicated there is no significant difference in TGase-modified Rac1 between DOI- and ionomycin-treated cells. Taken together, these data suggest an increase in intracellular Ca^{2+} via an ionophore can mimic 5-HT_{2A} receptor-induced cytosolic Ca^{2+} increases and is sufficient to induce TGase-catalyzed Rac1 transamidation.
**TGase-modified Rac1 is reduced by CaM inhibition in a dose-depend manner**

TGase is not only a Ca\(^{2+}\)-dependent enzyme, but its activity is also positively regulated by CaM. Moreover, Ca\(^{2+}\) signaling in cells is mainly mediated by CaM, a potent Ca\(^{2+}\)-binding protein. The CaM inhibitor, W-7, binds with high affinity to CaM in the presence of Ca\(^{2+}\) and thereby inhibits the activation of CaM-dependent enzymes (Asano and Hidaka, 1984). To test whether CaM modulates TGase-catalyzed transamidation of Rac1, cells were pre-incubated for 1 h with 0, 0.5 or 5 µM W-7 hydrochloride, and then stimulated with 3 µM DOI or vehicle for 15 min. We found that in DOI-stimulated cells, the levels of TGase-modified Rac1 were significantly higher than in vehicle-stimulated cells, whereas pretreatment with W-7 decreased DOI-stimulated Rac1 modification by TGase in a dose-dependent manner (Fig. 15). Two-way ANOVA indicates a significant main effect of DOI [F(1,12) =144.6; p<0.001] and W-7 [F(2,12) =8.152; p<0.01] on TGase-modified Rac1. There was also a significant interaction between DOI and W-7 [F(2,12) =5.01; p<0.05].

**Searching potential TGase-targeting residues at the activity-related domains in Rac1**

We have previously reported that TGase-catalyzed transamidation of Rac1 was able to transiently increase Rac1 activity (Dai et al., 2008). Like other small G proteins, Rac1 acts as a molecular switch and cycles between the GDP-bound inactive and GTP-bound active forms. There are at least three types of regulators for small G proteins: GTPase activating protein (GAP) which accelerates GTP hydrolysis,
resulting in inactivation of small G proteins; guanine nucleotide exchange factors (GEF) which stimulates conversion from the GDP-bound to the GTP-bound form; and GDP dissociation inhibitor (GDI) which inhibits this reaction. Post-translational modifications, such as TGase-catalyzed transamidation, of Rac1 at the conserved domains that interact with these regulators may affect its ability to convert GTP to GDP, resulting in increased activation. The Conserved Domain Database (available on http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) provides an interactive tool to identify conserved domains present in protein sequences. We searched the Conserved Domain Database for GTP/Mg2+ binding sites, and GAP, GEF, and GDI interaction sites in the Rac1 sequence (accession numbers: NP_008839) since TGase-catalyzed modifications at these sites would more likely lead to functional changes that would impact on Rac1 activity (Fig. 16A). TGase-catalyzed transamidation may not only incorporate an amine into the glutamine residue of the acceptor protein, but also form intra-molecular cross-links between glutamine and lysine residues within the protein (Hand et al., 1993). Two glutamine residues (Q61, Q74) and three lysine residues (K5, K16, and K116) are located at the activity-related domains and are highlighted in Rac1 structure (Fig. 16B).
Figure 12. Effect of PLC inhibition on DOI-induced Ca\textsuperscript{2+} signals and TGase-modified Rac1 in A1A1v cells. (A) After pretreatment with 5 µM U73122 or U73343 (negative control) for 30 min, and stimulation with either 3 µM DOI or vehicle (Ve), changes in the fluorescence ratio (340/380 nm) were recorded from cells loaded with Fura-2. A representative example from three independent experiments conducted in triplicate is shown. Arrows indicate the time of drug application. (B) Upper, immunoprecipitation (IP) and immunoblot (IB) analyses revealed that U73122
pretreatment inhibited the increase in TGase-modified Rac1 upon DOI stimulation. Lower, cells lysates from the same experiment were examined on IB with Na+/K+ ATPase antibody, which indicated that U73122 had no effect of on cell viability. (C) Quantitation of effects of U73122 and DOI on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD±S.E.M., and they were normalized to U73343- and Ve- treated cells. Two-way ANOVA followed by Bonferroni test: * and *** indicated p<0.05 and 0.001 as compared to U73343- and Ve- treated cells, respectively; # indicated p<0.001 as compared to U73343- and DOI- treated cells.
Figure 13. Pre-incubation with a Ca\(^{2+}\) chelator attenuated the DOI-mediated elevation of cytosolic Ca\(^{2+}\) and TGase-modified Rac1. (A) Dynamic changes in fluorescence ratio (340/380 nm) in Fura-2 loaded A1A1v cells. The Ca\(^{2+}\) response to exposure to 3 µM DOI was gradually reduced with the increasing concentrations of BAPTA. A representative example of three independent experiments conducted in triplicate is shown. (B) Upper, cells were pretreated with 20 µM BAPTA or the vehicle for BAPTA (Ve1) for 30 mins, and then stimulated with 3 µM DOI or the vehicle for DOI (Ve2) for 15 mins. TGase-modified Rac1 was detected by IP and IB analysis. BAPTA inhibited the increase in TGase-modified Rac1 in DOI-stimulated cells. Lower, cells
lysates from the same experiment were examined on IB with Na+/K+ ATPase antibody. There was no significant difference among various treatments. (C) Quantitation of effects of BAPTA and DOI on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD±S.E.M., and they were normalized to Ve1- and Ve2- treated cells. Two-way ANOVA followed by Bonferroni test: * indicated p<0.01 as compared to Ve1- and Ve2- treated cells; # and ## indicated p<0.05 and p<0.01 as compared to Ve1- and DOI- treated cells, respectively.
Figure 14. The Ca\textsuperscript{2+} ionophore mimiced the DOI-induced increase in cytosolic Ca\textsuperscript{2+} and TGase-catalyzed transamidation of Rac1. (A) Fura-2 loaded cells were stimulated with either 3µM DOI or different concentrations of ionomycin. The changes in the fluorescence ratio (340/380 nm) were recorded. A representative example from three independent experiments conducted in triplicate is shown. (B) Upper, cells were treated for 15 mins with 3µM DOI, 25 nM ionomycin or vehicles (Ve). Ionomycin caused an increase in TGase-modified Rac1 comparable to that induced by DOI. Lower, cell viability, as indicated by Na\textsuperscript{+}/K\textsuperscript{+} ATPase levels was not significantly different.
among the various treatments. (C) Quantitation of effects of ionomycin and DOI on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD±S.E.M., and were normalized to Ve-1-treated cells. One-way ANOVA followed by Bonferroni test: * indicates p<0.01 as compared to Ve-1-treated cells; # indicates p<0.01 as compared DOI-treated cells.
Figure 15. The CaM inhibitor W-7 caused a dose-dependent reduction in DOI-stimulated TGase-modified Rac1. (A) Cells were pretreated with increasing concentrations of W-7 for 1 h and then stimulated with either 3 μM DOI or vehicle (Ve). TGase-modified Rac1 and Na⁺/K⁺ ATPase were measured by IP and/or IB. W-7 decreased the levels of TGase-modified Rac1 upon DOI stimulation, but did not significantly affect Na⁺/K⁺ ATPase at the concentrations applied. (B) Quantitation of effects of W-7 on TGase-modified Rac1 in three separate experiments. Data shown were the mean IOD±S.E.M., and were normalized to Ve-stimulated cells without W-7 pretreatment. Two-way ANOVA followed by Bonferroni test: * and *** indicate
p<0.05 and p<0.001 as compared to Ve-stimulated cells without W-7 pretreatment, respectively; # and ## indicate p<0.01 and p<0.001 as compared to DOI-stimulated cells without W-7 pretreatment, respectively.
Figure 16. Rac1 activity-related domains and potential TGase-targeted amino acid residues. (A) Amino acid sequence alignment of human Rac1. Conserved activity-related sites were identified by searching NCBI Conserved Domain Database (CDD). # indicates a GTP/Mg2+ binding site; * indicates GAP interaction sites; + indicates GEF interaction sites; & indicated GDI interaction sites. Two glutamine residues (Q61, Q74) and three lysine residues (K5, K16, and K116), highlighted in gray, are directly localized in these potential activity-related domains. (B) Rac1 structure adapted from http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=56843. The potential TGase-targets, glutamine and lysine residues at Rac1
activity-related domains are highlighted in yellow.
Figure 17. Schematic representation showing 5-HT$_{2A}$ receptor signaling-mediated transamidation of Rac1 by TGase. DOI stimulates Gq/11 proteins-coupled 5-HT$_{2A}$ receptors, leading to the activation of PLC, which in turn hydrolyses PIP2 into IP$_3$. IP$_3$ releases Ca$^{2+}$ from ER and results in the activation of Ca$^{2+}$-dependent proteins, such as CaM and TGase. Positively regulated by both Ca$^{2+}$ and CaM, TGase catalyzes transamidation of Rac1 to bioamines, such as serotonin transported into the cytoplasm by SERT. Abbreviations: 5-HT$_{2A}$R, 5-HT$_{2A}$ receptor; CaM, calmodulin; ER, endoplasmic reticulum; PLC, phospholipase C; PIP2, phophatidylinositol-1,4-bisphosphate; IP$_3$, inositol-1,4,5-triphosphate; SERT, serotonin transporter; TGase, transglutaminase; $\alpha$, $\beta$, $\gamma$, G protein $\alpha$, $\beta$, $\gamma$ subunits
DISCUSSION

Several lines of evidence indicate G_q/11-protein-coupled receptors, such as bradykinin or endothelin-1 receptor, are also able to stimulate the activation of Rac1 (van Leeuwen et al., 1999, Clerk et al., 2001), but the mechanisms by which Rac1 becomes activated through these receptors remains poorly characterized. Nevertheless, it is worth noting that the activation of Rac1 is highly regulated by physiological stimuli, and aberrant activation occurs under many pathological conditions such as Salmonella invasion, tumor cell migration, and retinal degeneration (Bourguignon et al., 2000, Belmonte et al., 2006, Brown et al., 2007). Therefore, further studies that delineate signaling pathways involved in Rac1 activation may uncover new potential therapeutic targets for these diseases. We have previously demonstrated that the 5-HT_2A receptor, another G_q/11-protein-coupled receptor, is responsible for TGase-catalyzed transamidation and activation of Rac1 (Dai et al., 2008). In the present study, we investigated the mechanisms involved in Rac1 transamidation in response to 5-HT_2A receptor stimulation. Our experimental results identified that PLC-mediated increase in intracellular Ca^{2+} and subsequent CaM activation are required for 5-HT_2A receptor-mediated Rac1 transamidation. 5-HT_2A receptor stimulation activates PLC which catalyzes the hydrolysis of PIP2 into IP_3. IP_3 mobilizes Ca^{2+} from ER stores and thereby increases the intracellular Ca^{2+} and leads to the activation of CaM. Our data suggest that both Ca^{2+} and CaM activate TGase, which catalyzes transamidation of Rac1 to bioamines, such as serotonin (Fig. 17).
Except for the 5-HT\textsubscript{3} receptor, which is a ligand-gated ion channel, the other 5-HT receptors belong to the G protein-coupled receptor (GPCR) superfamily. G proteins such as Ga\textsubscript{12} and Ga\textsubscript{13}, have been shown to transduce signals from GPCR to activate Rho small G proteins (Suzuki et al., 2003). One of the GEFs for Rho, p115RhoGEF, has served as a direct link between Ga\textsubscript{12}/Ga\textsubscript{13} and Rho (Hart et al., 1998, Kozasa et al., 1998). However, the signaling pathways between 5-HT\textsubscript{2A} receptors and Rac1 transamidation and activation are not well understood. 5-HT\textsubscript{2A} receptor is primarily coupled to G\textsubscript{q/11} and activates various isoforms of PLC (Conn and Sanders-Bush, 1984, de Chaffoy de Courcelles et al., 1985). It also induces the activation of phospholipase A2 (PLA2) and the release of arachidonic acid (Felder et al., 1990, Kurrasch-Orbaugh et al., 2003). Moreover, through interaction with ADP-ribosylation factor 1 (ARF1) via its C-terminal domain, 5-HT\textsubscript{2A} receptor is able to signal through the phospholipase D (PLD) pathway (Mitchell et al., 1998, Robertson et al., 2003). Our results suggest that 5-HT\textsubscript{2A} receptor activation triggers TGase-catalyzed Rac1 transamidation at least predominantly through a pathway involving PLC (Fig.12).

Although it has been reported that PLC activates Rac in a manner dependent on Iba1, a Ca\textsuperscript{2+}-binding protein specifically expressed in macrophage/microglia (Kanazawa et al., 2002), Rac1 transamidation and activation in A1A1v cells, a neuronal cell line, could be mediated by other signaling pathways. Cleavage of PIP2 by PLC yields two important second messengers, IP\textsubscript{3} and DAG, to initiate a variety of
cellular functions. Since Ca\textsuperscript{2+} chelation reduces DOI-stimulated Rac1 transamidation to basal levels (Fig.13), IP\textsubscript{3}-induced Ca\textsuperscript{2+} release from ER rather than DAG signaling plays a pivotal role in TGase-catalyzed Rac1 transamidation. These results are in agreement with an earlier study suggesting that in thrombin- and collagen-stimulated human blood platelets, PLC activity and an increase in intracellular Ca\textsuperscript{2+} concentration were required for Rac activation, whereas PKC inhibition had no effect (Soulet et al., 2001). Increases in intracellular Ca\textsuperscript{2+} concentration upon 5-HT\textsubscript{2A} receptor stimulation is achieved by releasing intracellular Ca\textsuperscript{2+} stores and/or by activating Ca\textsuperscript{2+} channels, depending on the cell of interest. 5-HT\textsubscript{2A} receptor-induced contraction of rat aorta may require the concerted action of voltage-gated Ca\textsuperscript{2+} channels and phosphoinositide turnover (Nakaki et al., 1985, Roth et al., 1986, Watts, 1998). More interestingly, stimulation of 5-HT\textsubscript{2A} receptors on astrocytes caused Ca\textsuperscript{2+} influx through voltage independent Ca\textsuperscript{2+} channels which were dependent of the IP\textsubscript{3} production and subsequent Ca\textsuperscript{2+} release from internal stores (Eberle-Wang et al., 1994, Hagberg et al., 1998). Therefore, both Ca\textsuperscript{2+} mobilization from internal stores and influx of extracellular Ca\textsuperscript{2+} could contribute to the PLC/IP\textsubscript{3}-mediated intracellular Ca\textsuperscript{2+} increase.

CaM and TGase are Ca\textsuperscript{2+} dependent proteins. CaM activation is coupled to intracellular Ca\textsuperscript{2+} elevation and also correlates with the spatial pattern of increased Ca\textsuperscript{2+} (Hahn et al., 1992). Upon Ca\textsuperscript{2+} binding to the EF hand motifs of CaM, both N- and C-terminal domains adopt an open confirmation, exposing hydrophobic surfaces,
which then interact with a variety of target proteins including TGase (Puszkin and Raghuraman, 1985, Zainelli et al., 2004). TGase contains a cysteine in their active site that is unmasked only in the presence of Ca\(^{2+}\) (Hand et al., 1993) and Ca\(^{2+}\) activation of TGase is further regulated by other signal modulators such as GTP (Monsonego et al., 1998) and CaM (Puszkin and Raghuraman, 1985, Zainelli et al., 2004). In order for TGase to become receptive to the glutamine substrate, Ca\(^{2+}\) first must bind to TGase, inducing its conformational change which permits the glutamine substrate binding to the catalytic center (Folk, 1983, Greenberg et al., 1991). A rise of Ca\(^{2+}\) concentration increases TGase activity in a dose dependent manner in vitro and in cells (Siefring et al., 1978, Shin et al., 2004, Li et al., 2009). Consistent with these results, the elevation of intracellular Ca\(^{2+}\) induced via a Ca\(^{2+}\) ionophore lead to increased TGase-catalyzed Rac1 transamidation (Fig. 14).

In addition to Ca\(^{2+}\), CaM also interacts with TGase and regulates its cross-linking activity. CaM has been shown to coimmunoprecipitate with TGase2 in transfected cells (Zainelli et al., 2004). CaM increased TGase activity in human erythrocyte (Billett and Puszkin, 1991), platelets, chicken gizzard (Puszkin and Raghuraman, 1985) and rat liver (Hand et al., 1985). Interestingly, exogenous CaM with a binding site on the α-subunits of the hexadecameric phosphorylase kinase molecule (αβγδ)\(_4\), stimulates TGase-catalyzed incorporation of putrescine into the β- and γ-subunits, but inhibits incorporation of amines into the α-subunit (Nadeau and Carlson, 1994). Some of the CaM antagonists, such as dansylcadaverine, inhibit TGase
(Lorand et al., 1979) and its protein cross-linking ability. We have previously reported that a CaM antagonist or inhibitory CaM fragments, attenuated TGase-modified mutant huntingtin in cell and animal models of Huntington's disease (Zainelli et al., 2004, Dai et al., 2009, Dudek et al., 2009). CaM antagonists and Ca\(^{2+}\)-chelators also have been shown to depress the motile progression of sperm cells possibly mediated by TGase-catalyzed cross-linking of the contractile proteins (Cariello and Nelson, 1985).

Our present results revealed that CaM inhibitor, W-7, reduced TGase-modified-Rac1 in dose-dependent fashion (Fig. 15), indicating that TGase-catalyzed Rac1 transamidation, at least in part, was positively regulated by CaM. Moreover, in the presence of 5-HT\(_{2A}\) receptor agonist, CaM has been shown to interact with 5-HT\(_{2A}\) receptor at the sites that are important for G protein coupling, suggesting a putative role for CaM in regulating 5-HT\(_{2A}\) receptor function (Turner and Raymond, 2005). Thus, one could not exclude the possibility that the effect of CaM inhibition is mediated by alterations in 5-HT\(_{2A}\) receptor signaling.

The significance of TGase in Rac1 transamidation was confirmed by TGase siRNA and inhibitor in our previous studies (Dai et al., 2008). In addition to small G proteins such as Rac1 and RhoA (Walther et al., 2003, Dai et al., 2008) that could undergo TGase-catalyzed transamidation to serotonin (serotonylation), more recently, arterial delete cytoskeletal proteins including actin, myosin heavy chain and filamin A have been identified as serotonylated proteins (Watts et al., 2009). However, serotonin may not be the only amine that could be incorporated in these proteins. Neuronal
differentiation of SH-SY5Y cells induced by retinoic acid increases the expression/activation of TGases, resulting in transamidation of putrescine to RhoA and activation of RhoA (Singh et al., 2003). Moreover, spermidine and putrescine are suitable substrates for transglutamination of RhoA in vitro (Schmidt et al., 2001). In addition to amine incorporation, another important TGase-catalyzed modification of small G proteins is deamidation of glutamine residue. The site-specific deamidation of Gln53 and Gln61 of RhoA and Ras, respectively, by the bacterial toxin TGases inhibits the GTPase activity and renders small G proteins constitutively active (Lorand and Graham, 2003).

In addition to TGase-catalyzed reactions, other Ca\textsuperscript{2+/CaM dependent pathways may also contribute to Rac1 activation. For example, platelet-derived growth factor (PDGF) stimulates phosphorylation of Tiam1, the Rac1-specific GEF, and this phosphorylation was significantly reduced by Ca\textsuperscript{2+} chelation, inhibition of Ca\textsuperscript{2+}/CaM dependent kinase II (CaMKII), or disruption of PLC-\gamma expression in Swiss 3T3 fibroblasts (Fleming et al., 1998). Targeted CaMKII knockdown using siRNA resulted in the inhibition of wound-induced Rac activation and vascular smooth muscle cell migration (Mercure et al., 2008).

In conclusion, our study provides important new insights into the signaling mechanisms underlying 5-HT\textsubscript{2A} receptor-induced Rac1 transamidation and activation by TGase in A1A1v cells, and highlights the necessary role of PLC-mediated Ca\textsuperscript{2+} mobilization and CaM in TGase-catalyzed Rac1 transamidation. As an activating
signal for both TGase and CaM, an elevation of intracellular Ca$^{2+}$ is sufficient to induce Rac1 transamidation. Accumulating evidence suggests that Rho family of small G proteins is subject to TGase-mediated modification and activation. The essential role of Rac1 in cytoskeletal organization is well characterized, while the biological functions of Rac1 transamidation by TGase of in neurons have yet to be explored.
CHAPTER FOUR

STRIATAL EXPRESSION OF A CALMODULIN FRAGMENT IMPROVED MOTOR FUNCTION, WEIGHT LOSS AND NEUROPATHOLOGY IN THE R6/2 MOUSE MODEL OF HUNTINGTON’S DISEASE

(Published in J Neurosci. 2009; 29(37):11550-9)

ABSTRACT

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder, caused by a polyglutamine expansion in the huntingtin protein (htt). Increasing evidence suggests that transglutaminase (TGase) plays a critical role in the pathophysiology of HD possibly by stabilizing monomeric, polymeric and aggregated htt. We previously reported that in HEK293 and SH-SY5Y cells expression of a calmodulin (CaM)-fragment, consisting of amino acids 76-121 of CaM, decreased binding of CaM to mutant htt, TGase-modified htt and cytotoxicity associated with mutant htt and normalized intracellular calcium release. In this study, an adeno-associated virus (AAV) that expresses the CaM-fragment was injected into the striatum of HD transgenic R6/2 mice. The CaM-fragment significantly reduced body weight loss and improved motor function as indicated by improved rotarod performance, longer stride length, lower stride frequency, fewer low mobility bouts and longer travel distance than HD controls. A small but insignificant increase in survival was observed in R6/2 mice with CaM-fragment expression.
Immunoprecipitation studies show that expression of the CaM-fragment reduced TGase-modified htt in the striatum of R6/2 mice. The percentage of htt-positive nuclei and the size of intranuclear htt aggregates were reduced by the CaM-fragment without striatal volume changes. The effects of CaM-fragment appear to be selective, as activity of another CaM-dependent enzyme, CaM-dependent kinase II, was not altered. Moreover, inhibition of TGase-modified htt was substrate-specific since overall TGase activity in the striatum was not altered by treatment with the CaM-fragment. Taken together, these results suggest that disrupting CaM-htt interaction may provide a new therapeutic strategy for HD.

INTRODUCTION

Patients with Huntington’s disease (HD) present symptoms such as chorea, irregular gait, reduced motor coordination and weight loss (Harper, 1991). HD is a progressive and fatal neurological disorder caused by expansion of CAG repeats in HTT gene, conferring a toxic gain of function to the huntingtin (htt) protein (Huntington's Disease Collaborative Research Group, 1993). It is characterized neuropathologically by intranuclear inclusions and cytoplasmic aggregates composed of htt with an expanded polyglutamine domain (DiFiglia et al., 1997, Ross et al., 1998). Currently, there are no effective treatments to prevent or slow the progression of the disease.

Increasing evidence indicates that TGases may contribute to the
pathophysiology of HD (Cooper et al., 1999, Karpuj et al., 2002b). TGases, a family of Ca\(^{2+}\)-dependent enzymes, catalyze a covalent bond between peptide-bound glutamine residues and either lysine-bound peptide residues or mono- or polyamines (Folk et al., 1980, Griffin et al., 2002). In cell culture, mutant htt is an excellent substrate for TGases (Gentile et al., 1998, Kahlem et al., 1998, Zainelli et al., 2005). The mRNA, protein and enzymatic activity of TGases are elevated in HD brain (Karpuj et al., 1999, Lesort et al., 1999, Zainelli et al., 2003). TGase 2 ablation in HD transgenic mice resulted in a drastic reduction in \(\epsilon-(\gamma\text{-glutamyl})\) lysine bond levels and neuronal death in the cortex and striatum (Mastroberardino et al., 2002). Administration of the TGase inhibitor, cystamine, extended survival and improved motor performance in HD transgenic mice (Karpuj et al., 2002a).

Calmodulin (CaM) increased TGase activity in human erythrocyte (Billett and Puszkin, 1991), platelets and chicken gizzard (Puszkin and Raghuraman, 1985). CaM activates a host of enzymes upon Ca\(^{2+}\) binding (Cheung, 1982) and associates with mutant htt as demonstrated by affinity purification (Bao et al., 1996) and immunoprecipitation (Zainelli et al., 2004). Moreover, CaM colocalizes with htt and TGase 2 in HD intranuclear inclusions (Zainelli et al., 2004). Since a CaM inhibitor decreased TGase-catalyzed cross-linking of htt in cells (Zainelli et al., 2004), we tested the ability of fragments of CaM to interrupt the interaction of CaM and mutant htt and decrease the deleterious effects of TGase in HEK-293 cells (Dudek et al., 2008). A CaM-fragment, containing amino acids 76-121, reduced binding of CaM to
mutant htt \textit{in vitro} and in HEK-293 cells. AAV-mediated expression of the CaM-fragment significantly decreased TGase-modified htt, and mutant htt-associated cytotoxicity in differentiated SH-SY5Y cells, a neuroblastoma cell line, which stably express mutant htt. Importantly, CaM-fragment did not alter the total activity of TGase or another CaM-dependent enzyme, CaM kinase II, suggesting that CaM-fragment has specific effects on the CaM-htt interaction (Dudek et al., 2009). In the present study, we assessed the therapeutic potential of CaM-fragment in the R6/2 mouse model of HD. R6/2 transgenic mice express exon 1 of the human HD gene with an increased CAG repeat. Consequently, they develop a progressive neurological phenotype and pathological changes that resemble many features of HD (Mangiarini et al., 1996, Li et al., 2000). Survival, body weight, motor performance and neuropathological features were monitored to determine whether AAV-mediated expression of the CaM-fragment has beneficial effects on the course of HD in R6/2 mouse model.

\textbf{MATERIALS AND METHODS}

\textit{Animals.} Male R6/2 transgenic mice (with 100-115 CAG repeats in the transgene) and wild-type littermate mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 weeks of age. The mice had free access to food and water in an environment controlled for temperature and humidity and a 12 h light/dark cycle. The behavioral tests were conducted in the light part of the cycle. All procedures were
conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee.

Adenoassociated Virus (AAV) Injections. Recombinant AAV serotype 2 vectors were generated as described in our previous studies (Dudek et al., 2009). Three resultant vectors expressed each of the following: GFP and the CaM-fragment (containing amino acids 76-121 of calmodulin), GFP and a scrambled peptide (containing the same amino acids of the CaM-fragment but in a randomly scrambled sequence), and GFP alone. The titers of final AAV products were ranged from 2~5 × 10^{14} vector genomes per ml. Bilateral striatal injections of GFP-expressing AAV were performed in 7-week-old mice (1 µl per each side with 200 nl/min infusion rate at coordinates of anterior/posterior 0.5 mm, lateral/medial ±2 mm and dorsal/ventral -3 mm relative to bregma (Franklin et al., 1997)). Mice were randomly divided into five groups (11~14 mice for each group) with a defined genotype and treatment: CaM-HD (R6/2 mice injected with AAV expressing the CaM-fragment), Vec-HD (R6/2 mice injected with empty vector AAV expressing GFP alone), Scr-HD (R6/2 mice injected with AAV expressing a scrambled version of the CaM-fragment), CaM-WT (wild-type mice injected with AAV expressing CaM-fragment) and Vec-WT (wild-type mice injected with empty vector AAV).

Body weight and survival. Body weights were measured twice a week. Survival was checked twice daily at 8 h intervals. Brains were removed after death
and snap frozen in 2-methylbutane/dry ice bath and stored at -80°C.

*Behavioral tests.* Three behavioral tests were performed between 10 and 14 weeks of age at weekly intervals. Rotarod performance, gait dynamics and locomotion were examined every Wednesday, Friday and Sunday, respectively.

*Gait dynamics.* The DigiGait imaging system (Mouse Specifics, Inc., Boston, MA) was utilized for gait analyses as previously described (Hampton et al., 2004). Briefly, mice were placed on a motorized transparent treadmill belt moving at a speed of 14 cm/s. Digital images of paw placement were recorded at 150 Hz through a video camera mounted below the animal. The proprietary software analyzed the resulting digital images to generate a set of periodic waveforms that described the paw area and movement of each limb relative to the treadmill belt through consecutive strides. Gait data were pooled from all four paws and used to determine numerous gait dynamic measures including stride length and frequency, variability of stance width and paw area at peak stance.

*Locomotion.* To accurately measure locomotor behaviors including the total distance traveled and the number of low mobility bouts (defined as remaining continuously in a virtual circle of 15 mm radius for 10 sec), a force-plate actometer (constructed in Dr. Fowler’s laboratory, University of Kansas) was used as described previously (Fowler et al., 2001). Mice were placed in a 28 cm × 28 cm force-plate actometer for a 30-min recording once a week. When the animal moves on the plate, its movements are sensed by four supporting force transducers positioned at the
corners of the plate. The signals are processed by specialized computer algorithms written in house to yield measurements of travel distance and bouts of low mobility.

**Rotarod performance.** A rotarod apparatus (MED-Associates, St. Albans, VT) was used to measure fore- and hindlimb motor coordination and balance. The mice were given a training session at 9 weeks of age (four trials per day for 3 consecutive days) to acclimate them to the rotarod apparatus. During the test period, each mouse was placed on the rotarod with increasing speed, from 4 rpm to 40 rpm in 300 seconds. The latency to fall off the rotarod within this time period was recorded. Mice were tested on the rotarod once a week. Each mouse received two consecutive trials and the mean latency to fall was used in the analysis.

**Immunoprecipitation (IP).** Mouse brains were bisected mid-saggitally. One half was used in IP, CaM Kinase II Activity and TGase assays. The other half was used for immunohistochemistry assays. To prepare striatal homogenates for IP, a half brain was cut into 300-µm coronal sections with a cryostat (Leica, Nussloch, Germany). Striatum was punched out from five consecutive sections and homogenized in lysate buffer (10 mM Tris-HCl, pH 7.5, 0.14 M NaCl, 1 mM EDTA, and 1:1000 protease inhibitor mixture) in a Tissue Tek homogenizer. Protein concentration was determined using the BCA Protein Assay kit (Pierce Chemical, Rockford, IL) and tissue homogenates containing 150 µg of protein were centrifuge at 12,000 × g for 5 min at 4°C to separate the insoluble fraction. After removal of the supernatant, the insoluble fraction was resuspended in 60 µl of 95% formic acid and
incubated in a shaking water bath at 37°C for 40 min. The formic acid was then evaporated using a CentriVap Speed Vacuum (Labconco, Kansas City, MO) for 1.5 h at 45°C. The pellets were resuspended in 150 µl of IP wash buffer (10 mM Tris-HCl, pH 7.5, 0.14 M NaCl, and 0.1% Tween 20) and sonicated on ice at 10 x 5 sec pulse. Immunopurification of proteins containing TGase-catalyzed bonds was performed using 81D4 monoclonal antibody prebound to Sepharose beads (Covalab, Lyon, France) using a protocol developed by Covalab and as described previously (Norlund et al., 1999, Zainelli et al., 2005).

**Immunoblot.** Immunoaffinity-purified proteins were separated on 10% SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. Membranes were then incubated in blocking buffer (5% nonfat dry milk, 0.1% Tween 20, and 1× TBS) for 1 h at room temperature. Membranes were incubated overnight at 4°C with primary antibody on a shaker. Primary antibody (Chemicon International, Temecula, CA: anti-Huntingtin amino acids 1-82, mouse IgG, 1:500) was diluted in antibody buffer (1% nonfat dry milk, 0.1% Tween 20, and 1× TBS). The next day, membranes were washed with TBS/0.1% Tween 20, and then they were incubated with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted in antibody buffer. Membranes were washed, and signal was detected using enhanced chemiluminescence Western blotting detection reagents (GE Healthcare, Chalfont, St.Giles, UK). Using Scion Image for Windows (Scion Corporation,
Frederick, MD), immunoblots were quantified by calculating the integrated optical density (IOD) of each protein band on the film.

**Evaluation of htt aggregates by immunofluorescence.** Sections of frozen mouse brain tissue (20 µm thick) were mounted on glass slides, and then fixed in 4% paraformaldehyde. After fixation, the sections were washed in PBS (pH 7.4), and then nonspecific binding was blocked with 5% normal goat serum (NGS). Sections were incubated overnight with primary antibody: MAB5374 (mouse IgG, 1:100, directed against human huntingtin amino acids 1~256) in 1% NGS + 1X PBS. Next, sections were washed and incubated for 1h with secondary antibody: goat anti-mouse IgG, Fcγ fragment specific, conjugated to DyLight 649 (1:400) (Jackson ImmunoResearch, West Grove, PA). Next, coverslips were mounted on tissue sections with Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). As a control for nonspecific labeling with secondary antibody, omission of the primary antibody was also performed on a slide from each case. Htt-aggregate positive nuclei and size of htt-aggregates were measured in each of ten 215 × 215 µm microscope fields by Olympus IX-81 microscope (Olympus, Japan), in each of five rostrocaudally spaced sections in the striatum of 5 mice from the three groups of R6/2 mice (wild-type littermates did not show htt immunoreactivity). At least 450 nuclei per mouse were obtained and the percentage of nuclei containing htt-aggregates and area of htt-aggregates were calculated by CellProfiler software (Whitehead Institute, Cambridge, MA) and a mean value was obtained for each group.
**Measurement of striatal volume by Nissl staining.** Cryostat sections (20 µm thick) were postfixed in 4% paraformaldehyde, then dehydrated in graded alcohols. After delipidated in 1:1 alcohol/chloroform for 1.5 h, sections were rehydrated through graded alcohols and stained with 0.2% aqueous solution of cresyl violet (Sigma, St. Louis, MO) for 5 min, followed by a brief rinse with water and dehydration in graded alcohols. Sections were cleared in xylene (two changes, 5 min each) and cover-slipped with Permount (Fisher Scientific, Fair Lawn, NJ). The volume of the striatum was measured according to the principle of Cavalieri (Cyr et al., 2005) (volume=\(s_1d_1+s_2d_2+...+s_n d_n\), where \(s\)=surface area and \(d\)=distance between two sections). We considered 15 coronal levels of the striatal sections (from Bregma 1.7 mm, with an interval of 200 µm between the sections) for the volumetric measurement study. Image capturing was performed by using an inverted microscope (Olympus IX-81) coupled to a digital camera (Hamamatsu EMCCD, Japan). The montage image of a coronal brain section was constructed by Slidebook (Intelligent Imaging Innovations, Denver, CO). The surface area of striatum in each section was measured using NIH Image J [http://rsbweb.nih.gov/ij/]. Data were expressed as the average Cavalieri volume ±SEM (mm\(^3\)) of 4~5 mice per group.

**CaM Kinase II Activity Assay.** CaM kinaseII enzyme activity was analyzed using the SignaTECT Calcium/Calmodulin-Dependent Protein Kinase Assay System (Promega, Madison, WI), according to the manufacturer's protocol. Mouse striatum was homogenized in the extraction buffer (20mM Tris-HCl pH 8.0, 2mM EDTA,
2 mM EGTA, 2 mM DTT, 1 mM PMSF and 1:1000 Protease Inhibitor Cocktail (Sigma, St Louis, MO)). The homogenate was centrifuged at 350 × g for 5 min at 4°C and the supernatant was mixed with [γ-32P]ATP (at 3000 Ci/mmol, 10 mCi/ml), 50 µM biotinylated CaM kinase II substrate, reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 0.5 mM DTT, with or without control buffer containing 1 mM EGTA. After incubation at 30°C for 2 min, the reaction was terminated by adding 7.5 M guanidine hydrochloride and spotted to a streptavidin-impregnated membrane. Membranes were washed and the retained radioactive substrate of CaM kinase II was quantitated by liquid scintillation counting (Beckman, Fullerton, CA). Radioactive counts were used as an index of endogenous CaM kinase II activity in the sample.

**TGase II Activity Assay.** TGase activity was measured by detecting the incorporation of a biotinylated TGase amine substrate, 5-(biotinamido)pentyamine (Pierce, Rockford, IL) into N,N’-dimethylcasein (Calbiochem, San Diego, CA) as previously described (Dudek et al., 2009). 96-well Immulon 4 HBX plates (Dynatech, Franklin, MA) were coated with 100 µl of N,N’-dimethylcasein (20 mg/ml) in 0.05 M sodium bicarbonate, and stored at 4°C overnight. After the unbound N,N’-dimethylcasein was discarded, the plate was washed with PBS and blocked with nonfat dry milk (2% milk in PBS) for 1 hour at 37°C, followed by 3 washes with PBS. Striata from the various mice were homogenized in 0.1 M Tris-HCl pH 8.3, 1 mM EDTA, 1 mM PMSF, and protease inhibitor cocktail. Striatal homogenates (15 µg of protein/sample) were added to the plate with 0.1 M Tris-HCl pH 8.5, 0.15 M NaCl, 5
mM DTT, 0.5 mM biotin-labeled pentylamine, and 5 mM CaCl₂. The mixture was incubated for 1 hour at 37°C. The plate was washed with TBS and 0.001% Tween followed by incubation with streptavidin conjugated to HRP (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature. After five washes, 1 x TMB substrate solution (eBioscience, SanDiego, CA) was added and color was allowed to develop for 3 min. Then the reaction was stopped with 3N sulfuric acid and the absorbance was read at 450nm. For GTP-inhibited TGase enzymatic activity, the assay was performed in the presence of 500 µM GTPγS (Sigma-Aldrich, St Louis, MO). Negative controls were run in the absence of pentylamine or dimethylcasein. Each assay also included a standard curve of varying amounts of guinea pig TGase (Sigma, St. Louis, MO). Each sample was measured in triplicate. TGase activity was converted to a percentage control based on the mean value for the Vec-WT mice.

Statistics. All data are presented as mean ± S.E.M. and analyzed by one- or two-way ANOVA with repeated measures. Post hoc tests were conducted using Bonferroni's multiple comparison tests. There were one or two mice dead after week 13 in each HD group, so the last observed value was used for the subsequent missing values. Survival time was demonstrated by Kaplan-Meier survival curves and analyzed by a log-rank test. GB-STAT 10.0 (Dynamic Microsystems, Inc., Silver Spring, MD) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) were used for all statistical analyses. A probability level of p < 0.05 was considered to be statistically significant for all statistical tests.
RESULTS

**AAV-mediated delivery of CaM-fragment in R6/2 mice attenuated body weight loss**

Body weight was monitored from 7 weeks of age onwards. All groups of mice were of similar initial body weight (22.9±0.3 g, n=10~14 mice in each group). Changes in body weight were expressed as a percentage of body weight measured at 7 weeks of age (Fig. 18a). Body weight in all groups slowly increased or remained unchanged until week 10. Thereafter, mice in the two wild-type (WT) groups continued gaining body weight. The Vec-HD (R6/2 mice injected with empty vector AAV expressing GFP alone) and Scr-HD mice (R6/2 mice injected with AAV expressing a scrambled version of the CaM-fragment) had profound weight loss over the duration of the observation period, whereas CaM-HD mice (R6/2 mice injected with AAV expressing the CaM-fragment) maintained their body weight, or slightly increased their body weight (Fig. 18a). One-way ANOVA \([F(4,50)=82.48; \ p<0.0001]\) followed by Bonferroni's multiple comparison test indicated that mice in CaM-HD group had less change in body weight than those in the Vec-HD group at 12-16 weeks of age (p < 0.05), and there was no significant difference in body weight change between Scr-HD and Vec-HD at each time point. Similarly, body weight change in the CaM-WT (WT mice injected with AAV expressing the CaM-fragment) was not significantly different from the Vec-WT (WT mice injected with empty vector AAV expressing GFP alone).

**CaM-fragment expression did not significantly increase R6/2 mice survival**
Although the first death took place on day 88, 78 and 67 and mean survival time was 112, 102.9, 99.7 days in CaM-HD, Vec-HD and Scr-HD mice, respectively, suggesting a small (about 10%) increase in life span of CaM-HD mice, Kaplan-Meier survival curves demonstrated no statistically significant effect of CaM-fragment expression on the mortality in R6/2 mice (Fig. 18b). By log-rank comparison, the three groups of HD mice did not significantly differ from each other (p > 0.05). None of the mice in WT groups died during the observation period (from 6 to 27 weeks of age).

**CaM-fragment expression increases stride length and lowers stride frequency in R6/2 mice**

Gait dynamics were characterized using the DigiGait Imaging System. Generally, there is a clear difference between WT mice and Vec-HD or Scr-HD mice starting from week 11 in all the parameters measured, whereas the difference between WT mice and CaM-HD mice is not significant in stride length and frequency. At a speed of 14 cm/s, WT mice maintained a regular alternating gait at a consistent stride length of 5.8±0.3 cm and stride frequency of 2.5±0.1 steps/sec, whereas Vec-HD and Scr-HD mice walked at a gradually reduced stride length and higher stride frequency. For stride length (Fig. 19a), the two-way ANOVA with repeated measures indicated a significant main effect of group \[F(4,126)= 35.77; p<0.0001\] and week \[F(3,126)= 12.12; p<0.0001\]. There was also a significant interaction between group and week \[F(12,126)= 5.71; p<0.0001\]. The post hoc Bonferroni test indicated that stride length of CaM-HD
mice is indistinguishable from the two HD control groups at 10 weeks of age, but CaM-HD mice displayed a longer stride length as compared to Vec-HD or Scr-HD mice at week 11~13. Stride length of CaM-HD mice was not significantly shorter than WT mice until at week 13. For stride frequency (Fig. 19b), two-way ANOVA indicated significant differences among groups \( [F(4,126)=11.99; p<0.0001] \), but there is no significant effect of week \( [F(3,126)=0.202; p=0.895] \) or interaction between group and week \( [F(12,126)=1.368; P=0.190] \). The Bonferroni test indicated that CaM-HD mice had a significantly lower stride frequency than Vec-HD at week 11-13. Although Vec-HD and Scr-HD were statistically indistinguishable at any time point, there was a statistically significant difference between CaM-HD and Scr-HD at week 12. There was no significant difference between CaM-HD and WT mice in stride frequency. Analysis of some other gait dynamics revealed differences between genotypes. For example, the measure of stance width variation between steps was greater (Fig. 19c) and paw area at peak stance was smaller (Fig. 19d) in HD mice than in WT mice, but the CaM-fragment had no beneficial effects on these gait indices. For stance width variation, two-way ANOVA followed by Bonferroni test indicated significant differences between HD and WT groups \( [F(4,126)=13.61; p<0.0001] \), but no significant differences among HD groups or WT groups (\( P>0.05 \)). There is no significant effect of week \( [F(3,126)=1.21; p=0.31] \) although interaction between group and week is significant \( [F(12,126)=1.97; P=0.03] \). Similar results were obtained in the analysis of paw area at peak stance [Group factor: \( F(4,126)=10.42; p<0.0001 \)], whereas week effect
is significant \[ F(3,126) = 13.68; p<0.0001 \] and interaction between group and week is not \[ F(12,126) = 1.55; P=0.11 \].

**CaM-HD mice had fewer low mobility bouts and longer travel distance than Scr-HD or Vec-HD mice**

Force-plate actometers were used to measure the locomotor activity of R6/2 and WT mice. The number of low mobility bouts (LMB, defined as remaining continuously in a virtual circle of 15 mm radius for 10 sec) is presented in Fig. 20a. Two-way ANOVA with repeated measures indicated a significant effect of group \[ F(4,144) =27.75, p < 0.001 \] and a significant effect of week \[ F(3, 144) = 3.179, p < 0.05 \], but the interaction between group and week was not significant. The number of LMB gradually increased in HD mice without CaM-fragment expression, whereas CaM-HD mice, similar to WT mice, maintained LMB at a relatively lower level. At week 12 and 13, the Bonferroni post hoc test detected significantly fewer LMB in CaM-HD mice as compared to either Scr-HD or Vec-HD mice. Total travel distance in a 30 min session provided another index of locomotor activity of HD mice (Fig. 20b). Two-way ANOVA with repeated measures indicated a significant effect of group \[ F(4,138) = 3.40, p = 0.02 \], but the effect of week \[ F(3,138) = 0.17, p=0.92 \] and interaction between group and week \[ F(12,138) =1.12, p=0.35 \] were not significant. Consistent with reduced LMB in HD mice treated with CaM-fragment, CaM-fragment expression also increased the locomotor activity in HD mice as indicated by a significantly greater travel distance in CaM-HD mice than in either Scr-HD (at week 12,13) or Vec-HD.
mice (at week 12). There was no significant difference between CaM-HD and WT mice in distance traveled in 30 min.

**CaM-fragment improved performance on the rotarod in R6/2 mice**

Rotarod was used to measure fore- and hindlimb motor coordination and balance starting at 10 weeks of age (i.e., three weeks after the AAV injections). At ten weeks of age, the two groups of WT mice were able to stay on the rod during the 300 sec time period measured, whereas none of the three groups of HD mice could finish the test without falling in 300 sec. Even at this early time point after injection of the AAV, the mean latency to fall was significantly differed among the HD groups, 245.9±32.0, 150.2±29.5, 98.1±19.8 sec in CaM-HD, Vec-HD and Scr-HD mice, respectively. After 10 weeks of age, there was a progressive decline in performance of all HD mice, but the CaM-HD mice still achieved better performance than the other two HD groups (Fig 21). Using analysis using two-way ANOVA with repeated measurements, we found a significant main effect of group ($F_{(4,188)} = 49.35$, $p < 0.0001$), a significant main effect of week ($F_{(4,188)} = 18.86$, $p < 0.0001$) and a significant interaction between group and week ($F_{(16,188)} = 4.714$, $p < 0.0001$) on latency to fall off the rotarod. Bonferroni post hoc test indicated that CaM-HD mice had longer latency to fall as compared to Scr-HD mice at 10-14 weeks of age and Vec-HD mice at 10-11 weeks of age ($p < 0.05$).

**CaM-fragment expression reduced TGase-modified htt in R6/2 mice striatum**

Cell culture studies indicated that AAV-mediated CaM-fragment expression can
significantly inhibit the formation of TGase-modified htt (Dudek et al., 2009). Here we used striatal homogenates from HD or WT mice to detect TGase-catalyzed cross-linking of htt. Immunopurification of proteins containing ε-(γ-glutamyl) lysine bonds was performed using 81D4 mAb prebound to Sepharose beads. TGase-modified htt was then detected on immunoblots as shown in the Fig. 22a. TGase-modified htt was only detected in HD but not WT mice. One-way ANOVA \( F(2,15)=18.44, p<0.0001 \) followed by Bonferroni’s comparison test found there was a significant reduction (by approximate 50%) in TGase-modified htt in the striatum of CaM-HD mice as compared to Scr-HD or Vec-HD mice (Fig. 22b).

**CaM-fragment expression decreases the percentage of htt-positive nuclei and the size of htt aggregates in the striatum of R6/2 mice**

Striatal intranuclear and neuropil inclusions containing mutant htt are prominent neuropathological hallmarks of HD and may play an important role in disease progression. In R6/2 mice, intranuclear aggregates are much larger than neuropil aggregates and amenable to quantification, so we examined the effect of CaM-fragment on intranuclear htt aggregates in the striatum of R6/2 mice. In CaM-HD mice, the percentage of htt-positive nuclei was lower and the size of intranuclear aggregates appeared smaller than in the control virus-treated HD mice (Fig. 23a). These observations were confirmed by quantification of nuclear aggregates (Table 1). We found a significant reduction in the percentage of striatal nuclei containing htt-aggregates (approximately 18%) and the nuclear aggregate size
(approximately 35%) in CaM-HD mice as compared with either Vec- or Scr-HD mice. GFP fluorescence was observed in coronal brain sections from AAV-injected mice, indicating a widespread infection and expression of CaM-fragment in the striatum (Fig. 23a). Both human HD and R6/2 mouse brains are characterized by atrophy of the striatum. To exam whether the CaM-fragment delivery decreases gross striatal atrophy, we used Nissl staining and the Cavalieri principle to estimate striatal volumes. There was no statistically significant difference in mean striatal volumes between CaM-HD and the two groups of control HD mice. However, all three groups of HD mice had significantly smaller striatal volumes as compared with WT littermates (Fig. 23b, Table 1). As expected, striatal neuronal atrophy was observed in HD mice in high-power images.

**CaM Kinase II activity was not affected by CaM-fragment expression**

To test if expression of the CaM-fragment would interfere with other CaM-dependent enzymes, we measured the activity of calmodulin-dependent protein kinase II (CaM kinase II) in mouse striatal homogenates. We found no significant differences in CaM kinase II activity among the various HD or WT mouse groups in either the presence or absence of EGTA, a calcium chelator (Fig. 24a). However, EGTA caused a general decrease in CaM kinase II activity in all mouse groups. Two-way ANOVA indicated a significant main effect of EGTA $[F_{(1,50)}=72.12; p<0.0001]$, but there was no significant effect of mouse group $[F_{(4,50)}=0.543; p=0.705]$ or an interaction between EGTA and group $[F_{(4,50)}=0.966; p=0.435]$. 

CaM-fragment did not change total TGase activity

In order to explore whether the reduced TGase-modified htt in CaM-HD mice is due to a substrate-dependent inhibition or a general decrease in TGase enzymatic activity, we compared total TGase activity in the striatum among the different groups of mice. There is a significant increase in TGase activity in the three groups of HD mice as compared to CaM-WT or Vec-WT mice (p<0.01). Expression of the CaM-fragment did not have an effect on TGase activity in either HD or WT mice. In the presence of GTPγS, which specifically inhibits TGase activity, TGase activity is dramatically reduced (at least 60%) in all groups (Fig. 24b). Two-way ANOVA indicated a significant main effect of GTPγS \( [F(1,50)=428.149; \ p<0.0001] \) and mouse group \( [F(4,50)= 5.535; \ p<0.01] \), and there was a significant interaction between GTPγS and mouse group \( [F(4,50)= 5.032; \ p<0.01] \). The activity of guinea pig liver TGase was measured in each assay in the range from 0.01 to 0.5 milliunits/ml and resulted in a linear correlation with an R-square value from 0.95 to 0.99. The activity of TGase in the striatal samples fell within this linear range for guinea pig liver TGase.
Fig. 18 Effect of CaM-fragment on body weight and survival. (a) Changes in body weight were expressed as a percentage of body weight measured at 7 week of age. One-way ANOVA and Bonferroni's test found that the change in body weight was significantly smaller in CaM-HD than in Vec-HD (p < 0.05) starting from week 12, and there is no significant difference between Scr-HD and Vec-HD. * indicates p < 0.05 (CaM-HD vs. Vec-HD). (b) Kaplan-Meier survival curves showed the first death was at day 88, 78 and 67 in CaM-HD, Vec-HD and Scr-HD mice, respectively. By log-rank comparison, three groups of HD mice did not differ from each other (p > 0.05). (n = 9~14 in each group)
Fig. 19 Video-based gait analysis on the treadmill. Mice were placed on a treadmill belt moving at a speed of 14 cm/s. Gait data were pooled from all four paws. CaM-HD exhibited a significantly greater stride length (a), and a lower stride frequency (b). There is no significant difference between CaM-HD and Vec-HD or Scr-HD in stance width variability (c) and paw area at peak stance (d). Two-way ANOVA with repeated measures followed by Bonferroni test. * indicates $p<0.05$ (CaM-HD vs. Vec-HD), # indicates $p<0.05$ (CaM-HD vs. Scr-HD), n=6-14 in each group.
Fig. 20 Expression of CaM-fragment enhanced the locomotor activity in R6/2 mice. Spontaneous locomotor activities of R6/2 and WT control mice were recorded in a force-plate actometer apparatus for 30 min. (a) CaM-HD mice displayed a significantly fewer number of low mobility bouts than the two HD control groups at week 12-13. (b) Distance traveled by CaM-HD mice was significantly longer than Scr-HD at week 12-13 and Vec-HD at week 12. Two-way ANOVA with repeated measures followed by Bonferroni test. * indicates $p<0.05$ (CaM-HD vs. Vec-HD), # indicates $p<0.05$ (CaM-HD vs. Scr-HD), n=9-14 in each group.
Fig. 21 CaM-fragment expression delayed the onset of the rotarod defects in R6/2 mice. Mice were placed on a rotating rod with increasing speed, from 4 rpm to 40 rpm in 300 seconds. The latency to fall off the rotarod within this time period was recorded. Two-way ANOVA with repeated measures followed by Bonferroni test found that CaM-HD mice had significantly longer latency to fall than Scr-HD starting from week 10, and than Vec-HD at week 10-11. * indicates $p<0.05$ (CaM-HD vs. Vec-HD), # indicates $p<0.05$ (CaM-HD vs. Scr-HD), n=9-14 in each group.
Fig.22 TGase-modified htt in R6/2 mice striatum was reduced by CaM-fragment expression. (a) The insoluble fraction from mouse striatal homogenates was dissolved with formic acid. Immunopurification (IP) of proteins containing ε-(γ-glutamyl) lysine bonds was performed using 81D4 mAb prebound to Sepharose beads. Immunopurified proteins were then examine on immunoblots (IB) using an antibody against htt. (b) Quantitation of immunoblots. Data shown are the mean IOD± S.E.M., and they are normalized to CaM-HD mice. * indicates $p<0.05$ as compared to CaM-HD (n=5 in each group at 13-14 weeks of age).
Fig. 23 Histological evaluation of neuropathology (a) Top and middle, immunofluorescent labeling of striatum in R6/2 mice at 14 weeks of age. Htt-aggregates were labeled with htt antibody MAB5374 (red), the nuclei were labeled with DAPI (blue). The composite images show that the percentage of htt-positive nuclei and the size of nuclear htt-aggregates were decreased in CaM-HD as compared to the control-HD mice. Scale bar=30µm. Bottom, photomicrographs of
GFP protein distribution in the brain of representative AAV-injected animals indicated that the same vector-derived CaM-fragment was expressed in the striatum. Scale bar=1 mm. (b) Top, montage images of Nissl-stained brain coronal sections from CaM-HD, Vec-HD and CaM-WT mice at the level when the corpus callosum starts to merge in the middle. Scale bar=1 mm. Bottom, high magnification of micrograph of the dorsomedial aspect of the striatum from the sections above. There is marked neuronal atrophy with small angulated neurons in Vec-HD mouse, with relative preservation of neuronal size in CaM-HD mouse. Scale bar=50 µm.
Fig. 24 Expression of CaM-fragment in mouse striatum did not significantly affect the activity CaM kinase II or TGase. (a) Mouse striatal homogenates were used to measure CaM kinase II activity. Two-way ANOVA indicates a significant main effect of EGTA. However, there was no significant main effect of mouse group. The interaction between EGTA and group was also not significant. Bonferroni posttest indicates no significant differences in CaM kinase II activity among the various groups in either the presence or absence of EGTA. (b) The presence of the
CaM-fragment did not significantly change the levels of TGase activity in either HD or WT mice. However, a significant elevation of TGase activity in three groups of HD mice was observed as compared with WT mice. When 500 µM GTPγS was added, there is a dramatic reduction in all mouse groups. Two-way ANOVA indicates a significant main effect of GTPγS and mouse group, and there is a significant interaction between GTPγS and group. Bonferroni posttest *indicates p<0.01 as compared to Vec-WT; # indicates p<0.01 as compared to CaM-HD in the absence of GTPγS. Each column represents the mean ± SEM. (n=5 in each group at 13-14 weeks of age).
<table>
<thead>
<tr>
<th></th>
<th>CaM-HD</th>
<th>Vec-HD</th>
<th>Scr-HD</th>
<th>CaM-WT</th>
<th>Vec-WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of nuclei containing htt aggregates (%)</td>
<td>71.7 ± 5.6</td>
<td>85.1 ± 2.1*</td>
<td>89.5 ± 0.8**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size of intranuclear htt aggregates (µm²)</td>
<td>248.4 ± 27.1</td>
<td>378.1 ± 31.5*</td>
<td>391.0 ± 28.2* b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatal volume (mm³)</td>
<td>14.5 ± 0.3</td>
<td>13.8 ± 0.7</td>
<td>13.6 ± 0.3</td>
<td>17.0 ± 0.8*</td>
<td>17.2 ± 0.4* c</td>
</tr>
</tbody>
</table>

* P<0.05 as compared to CaM-HD (One-way ANOVA, n=5 in each group at 13-14 weeks of age); # P<0.05 as compared to CaM-HD (One-way ANOVA, n=4-5 in each group at 14-20 weeks of age for HD mice, at 27-28 week of age for WT mice); a: F_{(2,12)} = 6.83, b: F_{(2,12)} = 7.38, c: F_{(4,17)} = 11.77
DISCUSSION

Although there have been enormous strides in understanding the molecular and mechanistic pathways that mediate the progression of HD, an effective treatment to slow or prevent the disease-associated physical and mental decline has yet to be discovered. A number of reports in cultured cells have shown that interfering peptides can selectively inhibit pathological interactions between mutant htt and other proteins, and therefore are a potential therapeutic strategy for HD (Nagai et al., 2000, Dudek et al., 2008, 2009). However, only a few studies have been performed to verify the efficacy of these peptides in vivo (Kazantsev et al., 2002, Tang et al., 2009). We previously found that plasmid or AAV-mediated delivery of CaM-fragment significantly attenuated TGase-modified htt, htt-associated cytotoxicity and intracellular Ca^{2+} disturbances without interfering with the activity of other CaM-dependent enzymes in HD cell models (Dudek et al., 2008, 2009). We now report that expression of the CaM-fragment in the striatum ameliorated body weight loss, improved motor performance of R6/2 mice, reduced TGase-modified htt and decreased the number and size of nuclear htt-aggregates in the striatum.

Striatal delivery of CaM-fragment significantly delayed the onset of movement abnormalities and improved motor function as measured by analysis of gait, rotarod performance and locomotor behavior. It has been reported that gait deficiencies in R6/2 mice became apparent from 8 weeks of age. As visualized in the footprint analysis, R6/2 mice displayed a significantly shorter stride length, a
staggering movement and a gait that lacked a uniform step pattern compared to WT littermates (Carter et al., 1999). Striatal injection of AAV-CaM-fragment conferred significant improvements in stride length and frequency measurements, and a mild, insignificant increase in paw area at peak stance in R6/2 mice. Decreased motor function on the rotarod is the most commonly reported measure among animal models of HD (Schilling et al., 1999, von Horsten et al., 2003). Three weeks after CaM-fragment expression, R6/2 mice started to show significantly improved rotarod performance and this beneficial effect was maintained from 10 weeks of age onward. Although motor abnormalities such as bradykinesia (abnormally slow movements) and pronounced hypoactivity were frequently observed in HD animal models (Mangiarini et al., 1996, Keene et al., 2002), there have been relatively few quantitative studies on spontaneous locomotor activity of R6/2 mice. In the present experiments using a force-plate actometer, progressive locomotor deterioration (assessed by number of low mobility bouts and travel distance) was observed in R6/2 mice injected with control AAV, whereas delivery of AAV expressing CaM-fragment to R6/2 mice striatum significantly improved their locomotor deficits.

In comparison to the TGase inhibitor cystamine, which prolonged the life span of R6/2 mice by 12%–19% (Dedeoglu et al., 2002, Karpuj et al., 2002a), there was a non-significant increase in the mean survival time by approximately 10% in the CaM-HD mice. Differences in other mechanisms of action could possibly account for the different outcomes. In addition to inhibiting TGase, cystamine increased
transcription of neuroprotective genes, increased glutathione levels, and ameliorated apoptosis (Dedeoglu et al., 2002, Karpuj et al., 2002a). In contrast, that CaM-fragment decreased TGase-modified htt, normalized intracellular calcium release, and reduced mutant htt-associated cytotoxicity but had no effect on TGase activity (Dudek et al., 2008, 2009). Secondly, the different outcomes could be due to the different brain regions treated. The CaM-fragment was expressed in the striatum only while the cystamine-treatment was systemically delivered and could affect other brain regions that display neuropathology in HD such as the frontal cortex. Rather than unspecific inhibition of TGase by cystamine, disrupting the CaM-htt interaction, as with the CaM-fragment, is a promising new approach for the treatment HD.

As a result of its expanded N-terminal polyglutamine region, mutant htt is processed and deposited as a component of insoluble protein aggregates that persist in neuronal nuclei, perikarya, and processes (DiFiglia et al., 1997). Although some evidence suggests that htt-associated toxicity is linked to soluble htt and its protein–protein interactions (Petersen et al., 1999), htt aggregates act as a phenotypic readout that can reflect pathologically relevant processes such as htt cleavage, misfolding, and sequestration (Chopra et al., 2007). A number of compounds which are neuroprotective in R6/2 mice, such as the TGase inhibitor cystamine (Dedeoglu et al., 2002), the antioxidant coenzyme Q10 (Ferrante et al., 2002) and the energy buffer creatine (Ferrante et al., 2000), significantly suppress htt aggregates. Our present results revealed that CaM-fragment significantly reduced TGase-modified htt in the
insoluble fraction, the percentage of htt-positive nuclei and intranuclear htt-aggregate size in the striatum, which may contribute to the benefits of CaM-fragment. These findings support the hypothesis that CaM-regulated TGase modification of htt contributes to the formation and stabilization of htt-aggregates and may play a role in the pathogenesis of HD. However, it is difficult to speculate whether there would be an associated increase in soluble or oligomeric htt in the R6/2 mouse striatum with CaM-fragment expression. There is evidence that TGase modifications stabilize proteins (Tucholski et al., 1999), so inhibiting TGase modifications to htt may increase clearance of the htt protein.

R6/2 mice developed significant striatal atrophy as compared to WT littermates, a pathological characteristic associated with the gradual neurodegeneration occurring in the mice. Although there is no significant difference in striatal volume between CaM-HD and control-HD mice by light microscopy, we could not exclude the possibility that expression of CaM-fragment would reduce the striatal atrophy if a more sensitive technology (MR scanning) was applied. Moreover, if striatal injections of AAV were performed at an earlier age, there might be more notable beneficial results including increases in survival and striatal volume.

Similar to increases in TGase protein level and enzymatic activity in human HD brain (Karpuj et al., 1999, Lesort et al., 1999), our results show that TGase activity is elevated in R6/2 mice compared with WT littermates. Interestingly, striatal delivery of CaM-fragment did not change the overall activity of TGase in either R6/2
mice or WT mice. These results are consistent with our previous studies in cell culture (Dudek et al., 2009), suggesting that CaM-fragment may specifically affect the CaM-mutant htt interaction and selectively alter TGase-catalyzed modifications of mutant htt without interfering with the activity of TGase on other substrate proteins. Furthermore, CaM-fragment expression did not affect CaM kinase II activity, indicating that CaM-fragment had no effect on the activity of other CaM-dependent enzymes. Most importantly, expression of CaM-fragment in WT mice had no effect on any of the behavioral tests nor striatal histopathology, suggesting there is minimal, if any, deleterious effect of long-term expression of CaM-fragment.

A prominent hypothesis on the pathogenesis of HD is that the aberrant interactions of the extended polyglutamine repeats (polyQs) of mutant htt, either with other proteins or with each other result in htt-aggregate formation (Wanker, 2000). Glutamine repeats may function as polar zippers and expansion of polyQs may increase the affinity of htt for its protein binding partners (Perutz et al., 1994). The principal proteins that are known to interact with htt are: htt associated protein 1 (Li et al., 1995), htt interacting protein (Kalchman et al., 1997), type 1 inositol 1,4,5-trisphosphate receptor (InsP3R1) (Tang et al., 2003) and calmodulin (Bao et al., 1996, Zainelli et al., 2004). As revealed by our previous in vitro assay (Dudek et al., 2009), the neuroprotective effects of CaM-fragment may depend on its ability to competitively bind to mutant htt thereby disrupting the CaM-mutant htt interaction. First, this disruption may result in the CaM and mutant htt no longer in close
proximity to each other, thus the TGase activity in htt modification and aggregate formation may be reduced. Second, the association between mutant htt and CaM-fragment may prevent the further interaction of mutant htt with other proteins, such as InsP3R1, an intracellular Ca\textsuperscript{2+} release channel. Since mutant htt selectively associates with and activates InsP3R1 (Tang et al., 2003), disrupting their interaction should stabilize neuronal Ca\textsuperscript{2+} homeostasis. This hypothesis is supported by in vitro experiments that indicated the CaM-fragment (Dudek et al., 2008) or InsP3R-fragment (Tang et al., 2009) attenuates mutant htt-associated intracellular Ca\textsuperscript{2+} disturbances.

Although it will be advantageous to confirm the results from the current study using a different HD animal model, special care should be taken to choose a different strain other than R6/2 mice. Instead of body weight loss, YAC128 mice have significantly (27%) increased body weight at 12 months of age (Van Raamsdonk et al., 2006, Van Raamsdonk et al., 2007), which in turn can cause bias in most behavioral tests. Obvious or diffuse nuclear htt accumulation is not detectable in the striatum or cortices of BAC HD mice even in 18-month-old mice (Gray et al., 2008). Intergenerational instability and somatic instability of the HD CAG repeat have been observed in Hdh\textsuperscript{Q111} knock-in mice in all three background strains: C57BL/6, FVB/N and 129Sv (Lloret et al., 2006). The phenotype of N171-82Q mice is more variable than that of R6/2 mice, and therefore a much larger number of mice are necessary to provide adequate power (Hersch and Ferrante, 2004).
Many therapeutic targets for HD today are based on the pathogenesis hypotheses, from mitochondrial dysfunction to transcriptional perturbation to TGase hyperactivity (Beal and Ferrante, 2004). However, since clinical trials of mechanism-based therapies for HD have been limited by insufficient statistical power and insignificant improvement (Handley et al., 2006), current clinical treatments are symptomatic. The major neurological symptoms associated with HD include disordered voluntary movements: uncoordinated, arrhythmic, and slow fine motor movements; rigidity; and gait disturbances (Harper, 1991). Our in vivo results demonstrated that viral delivery of CaM-fragment to the striatum of R6/2 mice significantly improved motor coordination and balance, enhance locomotor activity and alleviated gait disturbances, suggesting the possibility of a better quality of life for HD patients if this new therapeutic strategy is successfully translated to the clinical trials.

The present studies provide, for the first time, in vivo evidence that CaM-fragment has significant efficacy in improving the behavioral deficits and neuropathological phenotype in the HD animal model. The positive effects of CaM-fragment in R6/2 mice provide further evidence that CaM-regulated TGase modification of htt may contribute to HD pathogenesis. More importantly, these studies have identified a novel therapeutic strategy for treating HD patients.
CHAPTER FIVE
GENERAL CONCLUSION

REVIEW OF RESULTS AND SIGNIFICANCE

In chapter two, we identified a novel effector mechanism attributed to 5-HT$_{2A}$ receptor signaling. This chapter described the effect of 5-HT$_{2A}$ agonists (5-HT or DOI) on transamidation and activation of Rac1 in a cell line derived from rat embryonic cortex. The transamidation is mediated by TGase, as suggested by a TGase inhibitor, cystamine or knockdown of TGase with siRNA. The transamidation of Rac1 contributed to its transient activation, since TGase inhibition significantly reduced both transamidation and activation. Moreover, serotonin was identified as an amine that becomes transamidated to Rac1 by TGase.

TGase-catalyzed transamidation of small G proteins not only occurred in platelets, vascular smooth muscle cells and cells derived from cervical cancer (Singh et al., 2001, Walther et al., 2003, Guilluy et al., 2007), but also occurred in neuronal cell lines, such as SH-SY5Y neuroblastoma cell line (Singh et al., 2003) as well as A1A1v cell line derived from rat embryonic cortex (Dai et al., 2008). One of the consequences of small G protein transamidation was constitutive activation. Members of the Rho family, particularly Rac1, are associated with a wide array of cellular
processes such as cytoskeletal organization, vesicular transport, cell cycle progression, cell adhesion and migration (Etienne-Manneville and Hall, 2002, Burridge and Wennenberg, 2004) and pathological conditions such as Salmonella invasion, tumor cell migration, and retinal degeneration (Bourguignon et al., 2000, Belmonte et al., 2006, Brown et al., 2007). The physiological functions of TGase-catalyzed transamidation and activation of small G proteins in the nervous system remains poorly understood. Recent studies have demonstrated that activation of TGase and transamidation of RhoA to putrescine induced by retinoic acid in SH-SY5Y cells, results in activation of ERK1/2, JNK1, and p38 MAP kinases, which may stimulate transcription factors and induce gene expression for neuronal differentiation, as indicated by neurite outgrowth and neuronal marker expression (Singh et al., 2003). In addition to small G proteins, cytoskeletal proteins such as intermediate filaments in neurons, have also been shown to undergo TGase-catalyzed transamidation in vitro (Selkoe et al., 1982, Miller and Anderton, 1986) and in human cerebral cortex (Norlund et al., 1999). We demonstrate that stimulation of 5-HT2A receptors can increase the transamidation of Rac1 in both undifferentiated and differentiated A1A1v cells, suggesting that transamidation and constitutive activation of this signal transducer are not altered by neuronal differentiation of cells. The functional consequences of transamidation of Rac1 by TGase especially in neurons needs to be further explored.

The signaling pathways involved in Rac1 transamidation have been
investigated in chapter three. The different components in the potential signaling pathways have been targeted by the antagonists and/or agonists. We found inhibition of PLC decreased intracellular Ca\(^{2+}\) concentration as well as TGase-modified Rac1 upon 5-HT\(_{2A}\) receptor activation. The crucial role of Ca\(^{2+}\) signaling in Rac1 transamidation was confirmed by manipulation of intracellular Ca\(^{2+}\) by means of a Ca\(^{2+}\) chelating agent or an ionophore. When cells were pretreated with a Ca\(^{2+}\) chelator, TGase-catalyzed Rac1 transamidation was significantly reduced. Conversely, treatment with a Ca\(^{2+}\) ionophore was sufficient to induce an increase in Rac1 transamidation by TGase. Moreover, the transamidation reaction was also regulated by CaM, a Ca\(^{2+}\) binding protein, as demonstrated by a CaM antagonist which depresses the stimulatory effect of 5-HT\(_{2A}\) receptor activation on Rac1 transamidation. Searching the Conserved Domain Database revealed that two glutamine residues (Q61, Q74) and three lysine residues (K5, K16, and K116) are located at the activity-related domains. These results provided new insights into the signaling mechanisms underlying 5-HT\(_{2A}\) receptor-induced Rac1 transamidation and activation by TGase in A1A1\(_{v}\) cells. The present study indicated that 5-HT\(_{2A}\) receptor-coupled PLC activation and subsequent Ca\(^{2+}\)/CaM signaling are necessary in TGase-catalyzed Rac1 transamidation, and identified potential TGase-targeting residues that could render Rac1 constitutively active upon transamidation.

In the studies presented in chapter four, we shifted our focus from Rac1 to mutant htt as another substrate of TGase-catalyzed transamidation reaction. Our
previous studies in the cell models of HD screened various CaM fragments based on their ability to decrease TGase-modified htt and cytotoxicity, as well as normalize intracellular Ca$^{2+}$ release. In this study, an AAV that expresses a CaM fragment was delivered into the striatum of HD transgenic R6/2 mice. Ameliorated body weight loss and improved motor performance were observed in those mice as compared to control virus-injected HD mice. CaM-fragment expression also specifically reduced TGase-modified htt, the percentage of htt-positive nuclei and the size of intranuclear htt aggregates in the striatum.

Although the life span of CaM-fragment treated HD mice was not significantly prolonged (there was a non-significant increase in the mean survival time by ~10%), our results demonstrated that CaM-fragment expression in the striatum of HD mice significantly improved motor coordination and balance, enhance locomotor activity and alleviated gait disturbances. The gene mutation that causes HD was identified in 1993 (Huntington’s Disease Collaborative Research Group, 1993), but there is currently no effective therapy and the disease inevitably leads to death within 10 to 15 years of symptom onset (Bates, 2003). Medications are available to help manage the signs and symptoms associated with HD, but will not stop the progressive physical and mental decline. AAV-mediated gene therapy for HD by preventing mutant htt expression or interrupting its interaction with other proteins has emerged as an attractive new strategy. The AAV-mediated knock-down of htt via bilateral injections into the striatum of HD transgenic mice resulted in a reduction in
neuronal intranuclear inclusions, and improvements on behavioral phenotypes and histological deficits (Harper et al., 2005, Rodriguez-Lebron et al., 2005). Disruption interaction mutant htt with either CaM (Dai et al., 2009) or type 1 IP$_3$ receptor (Tang et al., 2009) by AAV expressing competitive fragments alleviated motor deficits and improved neuropathological abnormalities. Our results demonstrate the importance of CaM-htt association for HD pathogenesis and suggested that CaM fragment is a potential therapeutic agent for HD.

According to our in vivo studies, wild-type mice injected with the AAV expressing CaM-fragment did not differ from those injected with AAV vector in terms of body weight, any of the behavioral tests, striatal histopathology, nor CaM KII or TGase enzyme activity up to 29 weeks old, suggesting there are minimal, if any, deleterious effects of long term expression of CaM-fragment. The CaM-fragment might not be able to disrupt the binding of CaM to other proteins to inhibit other biologic functions. The CaM target database (http://calcium.uhnres.utoronto.ca/ctdb/flash.htm) provides a program that searches for possible CaM-binding motifs in a protein sequence (Yap et al., 2000). The database currently includes the CaM-binding sites identified in over 300 proteins. No putative binding sites were located in the sequence of CaM-fragment suggesting that the interruption of binding of CaM to mutant huntingtin protein is selective.

**LIMITATIONS OF THE PRESENT STUDIES**

There are certain limitations that should be noted when interpreting the data of
present studies.

We did not have direct evidence to establish a causal relationship between Rac1 transamidation and activation. Based on the facts that inhibition of TGase caused decreases in both Rac1 transamidation and activation, and that two glutamine residues (Gln61, Gln74) located in Rac1 activity-related domain are potential targets of transglutaminase, we are only able to conclude that 5-HT$_{2A}$ receptor stimulation increased TGase-catalyzed transamidation of Rac1, which may contribute to the increase in Rac1 activity. Although retinoic acid promoted activation of RhoA by TGase-catalyzed transamidation in neuroblastoma SH-SY5Y cells (Singh et al., 2003), transamidation-independent activation of Rac1 was observed in SH-SY5Y cells stimulated with retinoic acid (Pan et al., 2005). Further experiments could be carried out to explore if other mechanisms are also involved in 5-HT$_{2A}$ receptor-mediated Rac1 activation in A1A1v cells.

Although the TGase inhibitor cystamine decreased the serotonin-associated Rac1 in co-immunoprecipitation experiments, we could not confirm a direct association between Rac1 and serotonin via a TGase-catalyzed covalent bond. Another common binding partner could link serotonin to Rac1. However, this possibility is undermined by the fact that serotonin-associated Rac1 did not show a significant upward shift on immunoblots compared with native Rac1 in cell lysates, indicating that the molecular weight of serotonin-associated Rac1 does not significantly increase. Therefore, the binding partner, if it exists, should also be a
small molecule likely an amine.

The attempt to replicate 5-HT$_{2A}$ receptor-mediated Rac1 transamidation in rat cortex failed. In Sprague Dawley rats that were given subcutaneously injections of DOI (2.5 mg/kg) and sacrificed 5 or 15 minutes later, there is no significant increase in TGase-modified Rac1 in the frontal cortex compared to saline-treated rats. The discrepancy between cell-based and in vivo studies could be attributed to the following possibilities. The DOI dosage and route of administration in rats may not be able to mimic the circumstances under which 5-HT$_{2A}$ receptor stimulation induces Rac1 transamidation in A1A1v cells. Moreover, the time course of reactions for DOI-mediated Rac1 transamidation may be different between cultured cells and whole animals. Lastly, the cultured cells grow in an environment that lack intercellular communication or neurotransmitter cross-talk, which differs from the normal surrounding milieu presenting in the brain.

In our studies that evaluated protective effects of CaM-fragment in R6/2 mice, immunofluorescence staining demonstrated that the percentage of htt-positive nuclei and the size of intranuclear htt aggregates in the striatum were reduced by CaM-fragment expression. Although TGase-modified htt in the striatal homogenates was decreased in CaM-expressing HD mice as compared to the other two groups of control HD mice (as indicated in immunoprecipitation and immunoblotting experiments), we were unable to confirm that the reduction of htt aggregates was due to decreased TGase-modification of htt. Our original experimental design that aimed
to measure the colocalization of htt aggregates with TGase-modified covalent bonds could help to test this hypothesis. However, the only commercially available antibody directed against TGase-modified covalent bonds (81D4) is raised in mouse. High levels of background staining were observed with the 81D4 antibody in R6/2 mouse tissues, likely due to the binding of secondary anti-mouse antibody to endogenous mouse tissue Igs and other components. This technical difficulty compromised our ability to explore the role of TGase-modified covalent bonds in the formation of htt aggregation in HD mice.

Our previous in vitro experiment indicated that CaM-fragment can directly inhibit the binding of mutant htt and CaM (Dudek et al., 2009). In this in vivo study, since the striatum of age matched HD mice were used in immunofluorescence staining, Nissil staining, immunoprecipitation, TGase and CaM activity assay, we did not have enough striatal homogenates to confirm the hypothesis that striatal CaM-fragment expression disrupted mutant htt-CaM association in vivo. The other experiment that could support the hypothesis that the CaM-fragment can directly disrupt the interaction between mutant htt and CaM is co-immunoprecipitation to demonstrate the binding of CaM-fragment to mutant htt. However, we have not been able to find a specific antibody directed against this CaM-fragment. Moreover, due to the small size of the CaM-fragment (consisting of 46 amino acids and weighing 5,158.6 daltons), detection of the proteins on immunoblots was difficult.

As for the timing of the delivery of AAV expressing CaM-fragment, our
original plan was to inject AAV to R6/2 mice at a much younger age, but the youngest mice we could purchase from Jackson Laboratories were at 6 weeks of age due to the genotyping process. Therefore, we chose the new R6/2 strain (B6CBA-Tg(HD exon1)62Gpb/3J) which has fewer CAG repeats in exon 1 (100-115 CAG repeats) over the previous strain (B6CBA-Tg(HD exon1)62Gpb/1J) with 154-159 CAG repeats. The new strain exhibited a concomitant decrease in severity and delayed onset in the expected neurological phenotype (Jackson Laboratories, http://jaxmice.jax.org/strain/002810.html), which actually gave CaM fragment more time to perform its function. However, if striatal injection of AAV was performed at a younger age, we might have more beneficial results including significant increases in survival and striatal volume.

FUTURE STUDIES: DISRUPTING CAM-HTT INTERACTION AS A POTENTIAL THERAPEUTIC STRATEGY IN HUMANS

Currently there is no cure for HD. On August 15, 2008 the U.S. Food and Drug Administration (FDA) approved the use of tetrabenazine to treat Huntington’s chorea (the involuntary writhing movements), making it the first drug approved for use in the United States to treat the disease. Tetrabenazine works mainly as a Vesicular Monoamine Transporter (VMAT)-inhibitor (Zheng et al., 2006) and thereby increases the early metabolic degradation of dopamine. Other agents targeting dopamine system have also been used to ameliorate chorea and treat irritability, hallucinations and delusions. Those agents include dopamine antagonists such as
haloperidol, fluphenazine and pimozide (Caine and Shoulson, 1983, Girotti et al., 1984, Barr et al., 1988).

Our in vivo studies demonstrated that AAV-mediated striatal expression of CaM-fragment improved motor function, weight loss, and neuropathology in HD mice. In the long run, to extend the application of CaM-fragment to HD patients, a less invasive method has to be developed since AAV-mediated delivery of CaM-fragment by stereotaxic surgery could cause brain injury. The less invasive approaches include intravenous administration or systemic injection. The delivery of CaM-fragment to the brain by the intravenous route could be efficient and widespread to all parts of the brain once the blood-brain barrier (BBB) is traversed. Certain endogenous large-molecule neuropeptides such as insulin, transferrin, or leptin are transported into the brain from blood via receptor mediated transport (RMT) across the BBB (Pardridge, 2003). These transporters are regulated by ligand-specific receptors such as insulin receptors and transferring receptors, which are highly expressed on the capillary endothelium of brain (Rip et al., 2009). Molecular Trojan horses (MTH) are certain monoclonal antibodies (MAbs) or peptide ligands that target RMT systems (e.g., receptor-binding sequences of insulin) that bind to BBB receptors and induce receptor mediated transcytosis through the BBB (Pardridge, 2002). Therefore, the construction of fusion proteins between the CaM-fragment and the MTH may enable the CaM-fragment to cross the BBB in vivo. It has been reported that a fusion protein between the insulin receptor mAb and brain-derived neurotrophic
factor (BDNF) enables rapid brain penetration of the neurotrophin in animal models, including nonhuman primates and is intended for treatment of stoke and neurodegenerative diseases (Boado et al., 2007, Boado et al., 2008, Boado and Pardridge, 2009).

On the other hand, the identification of the sites on mutant htt that binds to CaM may also provide an alternative approach to disrupt CaM-htt interaction. A yeast two-hybrid assay or affinity purification could be used to rapidly screen the sequential deletions of htt to identify binding domain, and then test their ability to disrupt the binding of CaM to mutant huntingtin. Next, similar to CaM-fragment (Dudek et al., 2008, 2009), we could test the ability of these fragments from mutant htt for their ability to inhibit TGase-catalyzed modifications to mutant htt and for their ability to increase cell viability in HD cell models. Once the beneficial effects were observed, we could deliver these fragments to the animal models of HD via different approaches depending on their molecular size and charge character. The binding domain in mutant htt could be located at the amino acid sequences upstream or downstream of the expanded polyglutamine tract or in the middle of the polyglutamine tract. If the polyglutamine domain is responsible for binding to CaM, the therapeutic strategy that targets CaM-htt interaction could be applied to the other eight polyglutamine repeat diseases as well.

CONCLUSIONS

TGase-catalyzed transamidation and activation of the small G protein Rac1 in
A1A1v cells is regulated by 5-HT$_{2A}$ receptor signaling and Ca$^{2+}$/CaM (Figure 25). Active Rac1 has various physiological functions in neuronal cells, including neurite outgrowth and neuronal differentiation, via modulating the activity of diverse effector proteins (Leeuwen et al., 1997, Pan et al., 2005). Pathologically, TGase-modification of htt is regulated by the association of htt with CaM, which may be involved in the formation and stabilization of htt-aggregates and play a role in the pathogenesis of HD. Direct inhibition of TGase or CaM may be able to suppress TGase- and htt-mediated neuropathological perturbations, but also will interrupt the normal physiological functions of TGase and CaM because both enzymes regulate a variety of effectors including kinases and cytoskeletal proteins. Alternatively, specific disruption of the interaction of CaM with mutant htt, by a competitive CaM-fragment, is a useful tool to determine the pathogenic mechanisms involved in HD and can be used to develop new therapeutic strategies for HD (Figure 25). Our in vivo study demonstrated that striatal expression of the CaM-fragment significantly improved motor function and neuropathological perturbations in HD mice. Moreover, TGase and CaM activity were not altered by the CaM-fragment in either HD or WT mice, suggesting that disrupting CaM-htt interaction is an important new therapeutic strategy for HD.
Figure 25. Regulation of TGase by 5-HT$_{2A}$ receptor signaling and CaM. In A1A1v cells, upon stimulation of Gq/11 protein-coupled 5-HT$_{2A}$ receptor, the phosphatidyl inositol pathway was activated, resulting in PLC-mediated hydrolysis of PIP2 to IP$_3$, which in turn releases Ca$^{2+}$ from ER stores. A rise of cytoplasmic Ca$^{2+}$ is sufficient to activate TGase, which catalyzed the transamidation of Rac1 to bioamines, such as serotonin transported into the cytoplasm by SERT. CaM, another Ca$^{2+}$-dependent enzyme, also contributed to Rac1 transamidation by positive regulation of TGase. In HD cells, mutant htt associated with CaM at a specific binding domain, and htt were modified by TGase. This modification may stabilize htt monomers or polymers and contribute to cytotoxicity and formation of htt-containing aggregates. CaM-fragment may disrupt CaM-htt interaction by competing with endogenous CaM, resulting in
decreased TGase-modified htt and aggregates. Abbreviations: 5-HT2AR, 5-HT2A receptor; CaM, calmodulin; ER, endoplasmic reticulum; PLC, phospholipase C; PIP2, phosphatidyl inositol-1,4-bisphosphate; IP3, inositol-1,4,5-triphosphate; SERT, serotonin transporter; TGase, transglutaminase; αq/11, Gq/11 protein α subunits
REFERENCES LIST


Aghajanian GK, Marek GJ (Serotonin, via 5-HT2A receptors, increases EPSCs in layer V pyramidal cells of prefrontal cortex by an asynchronous mode of glutamate release. Brain Res 825:161-171.1999).


Belmonte MA, Santos MF, Kihara AH, Yan CY, Hamassaki DE (Light-Induced photoreceptor degeneration in the mouse involves activation of the small GTPase Rac1. Invest Ophthalmol Vis Sci 47:1193-1200.2006).


Bergamini CM, Signorini M, Poltronieri L (Inhibition of erythrocyte transglutaminase by GTP. Biochim Biophys Acta 916:149-151.1987).

Bernassola F, Federici M, Corazzari M, Terrinoni A, Hriba ML, De Laurenzi V, Ranalli M, Massa O,


Boado RJ, Pardridge WM (Comparison of Blood-Brain Barrier Transport of GDNF and an IgG-GDNF Fusion Protein in the Rhesus Monkey. Drug Metab Dispos.2009).


De Laurenzi V, Melino G (Gene disruption of tissue transglutaminase. Mol Cell Biol

Dean B, Hayes W (Decreased frontal cortical serotonin2A receptors in schizophrenia. Schizophr Res 21:133-139.1996).


Gray M, Shirasaki DI, Cepeda C, Andre VM, Wilburn B, Lu XH, Tao J, Yamazaki I, Li SH, Sun YE, Li XJ, Levine MS, Yang XW (Full-length human mutant huntingtin with a stable polyglutamine repeat can elicit progressive and selective neuropathogenesis in BACHD mice. J Neurosci


Hand D, Bungay PJ, Elliott BM, Griffin M (Activation of transglutaminase at calcium levels consistent with a role for this enzyme as a calcium receptor protein. Biosci Rep 5:1079-1086.1985).


Hemrick-Luecke SK, Evans DC (Comparison of the potency of MDL 100,907 and SB 242084 in blocking the serotonin (5-HT)(2) receptor agonist-induced increases in rat serum corticosterone concentrations: evidence for 5-HT(2A) receptor mediation of the HPA axis. Neuropharmacology 42:162-169.2002).


Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, Humphrey PP (International Union of Pharmacology classification of receptors for 5-hydroxytryptamine


Li X, Wei XL, Meng LL, Chi MG, Yan JQ, Ma XY, Jia YS, Liang L, Yan HT, Zheng JQ (Involvement of tissue transglutaminase in endothelin 1-induced hypertrophy in cultured neonatal rat cardiomyocytes. Hypertension 54:839-844.2009).


Muma NA (Transglutaminase is linked to neurodegenerative diseases. J Neuropathol Exp Neurol 66:258-263.2007).


Murga C, Zohar M, Teramoto H, Gutkind JS (Rac1 and RhoG promote cell survival by the activation of PI3K and Akt, independently of their ability to stimulate JNK and NF-kappaB. Oncogene 21:207-216.2002).


Nakaki T, Roth BL, Chuang DM, Costa E (Phasic and tonic components in 5-HT2 receptor-mediated rat aorta contraction: participation of Ca++ channels and phospholipase C. J Pharmacol Exp Ther 234:442-446.1985).


Singh RK, Dai Y, Staudinger JL, Muma NA (Activation of the JAK-STAT pathway is necessary for


Venkatraman P, Wetzel R, Tanaka M, Nukina N, Goldberg AL (Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing


Willins DL, Deutch AY, Roth BL (Serotonin 5-HT2A receptors are expressed on pyramidal cells and interneurons in the rat cortex. Synapse 27:79-82.1997).


Zemskov EA, Janiak A, Hang J, Waghray A, Belkin AM (The role of tissue transglutaminase in


Ying Dai was born on July 3, 1978 in Hunan, China to Yiquan Dai and Zhiyang Song. He attended Nanhua University, China, where he earned his Bachelor of Medicine degree, with highest distinction in 2001. After graduation, Ying entered the Master’s Program in the Department of Anatomy and Histology at Jinan University, China, and joined the laboratory of Dr. Yunquan Liu. His project focused on cerebral ischemia and reperfusion injury. He completed his Master of Medicine in July, 2004.

In August of 2004, Ying began to pursue a doctorate of philosophy in Neuroscience Program at Loyola University Chicago. In 2005, Ying joined the laboratory of Dr. Nancy A. Muma in the Department of Pharmacology and Experimental Therapeutics, to study the regulation of transglutaminases by 5-HT$_{2A}$ receptor signaling and calmodulin. In January of 2007, Ying moved to University of Kansas with his advisor Dr. Muma, and continued his research at the Department of Pharmacology and Toxicology.

Following the completion of the doctoral program in Neuroscience at Loyola University Chicago, Ying will continue to pursue his interest in development of new therapeutic strategies for neurodegenerative diseases.
DISSERTATION APPROVAL SHEET

The dissertation submitted by Ying Dai has been read and approved by the following committee:

Nancy A. Muma, Ph.D., Director
Professor and Chair
Department of Pharmacology and Toxicology, University of Kansas

George Battaglia, Ph.D.
Professor of Pharmacology
Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago

Thackery Gray, Ph.D.
Professor of Anatomy and Associate Dean
School of Medicine, American University of the Caribbean

Edward J. Neafsey, Ph.D.
Professor and Program Director,
Neuroscience Program, Loyola University Chicago

Karie Scrogin
Associate Professor of Pharmacology
Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

__________________      ____________________________
Date                Director’s Signatur