Functions of the DRY motif and intracellular loop 2 of human melanocortin 3 receptor

Hui Huang and Ya-Xiong Tao
Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, 212 Greene Hall, Auburn, Alabama 36849, USA

Abstract

The melanocortin 3 receptor (MC3R) regulates several physiological functions, including feed efficiency, nutrient partitioning, fasting response, natriuresis, and immune reactions. Naturally occurring mutations in the MC3R gene have been shown to be associated with increased adiposity and lung diseases such as tuberculosis and cystic fibrosis. The DRY motif at the cytoplasmic end of transmembrane domain 3 (TM3) and the second intracellular loop 2 (ICL2) are known to be important for receptor function in several G protein-coupled receptors (GPCRs). To gain a better understanding of the functions of this domain in MC3R, we performed alanine-scanning mutagenesis on 18 residues. We showed that alanine mutation of 11 residues reduced the maximal binding and maximal cAMP production stimulated by agonists. Mutation of two residues did not change maximal binding but resulted in impaired signaling in the Gs–cAMP pathway. Mutation of five residues impaired signaling in the ERK1/2 pathway. We have also shown that alanine mutants of seven residues that were defective in the cAMP pathway were not defective in the ERK1/2 pathway, demonstrating biased signaling. In summary, we demonstrated that the cytoplasmic end of TM3 and the ICL2 were critical for MC3R function. We also reported for the first time biased signaling in MC3R.

Key Words
- melanocortin 3 receptor
- intracellular loop 2
- DRY motif
- MAP kinase
- biased signaling

Introduction

The melanocortin 3 receptor (MC3R) was identified in 1993 as a receptor for α- and γ-melanocyte-stimulating hormones (MSHs) (Gantz et al. 1993, Roselli-Rehfuss et al. 1993). It is highly expressed in the hypothalamus, especially in the ventromedial nucleus and arcuate nucleus, as well as in several peripheral tissues, such as the heart, liver, lung, kidney, and macrophages (Gantz et al. 1993, Roselli-Rehfuss et al. 1993, Ni et al. 2003, Getting et al. 2006). Co-expressed with proopiomelanocortin (POMC) in the arcuate nucleus, MC3R acts as an inhibitory autoreceptor, providing a short-loop feedback to regulate POMC neuronal activity (Cowley et al. 2001).

MC3R plays vital roles in regulating various physiological functions. It regulates feed efficiency and nutrient partitioning; Mc3r knockout mice have elevated fat mass and reduced lean mass (Butler et al. 2000, Chen et al. 2000). More recently, several studies have shown that MC3R is required for normal nutrient anticipatory activities and fasting responses (Sutton et al. 2008, 2010, Renquist et al. 2012). Deletion of Mc3r results in
Cushing-like phenotypes with increased basal corticosterone levels and decreased bone density (Renquist et al. 2012). Consistent with an important role of MC3R in regulating energy homeostasis, mutations in MC3R have been identified to be associated with human obesity or adiposity (Tao 2010) as well as lung diseases including tuberculosis and cystic fibrosis (Cooke et al. 2008, Wright et al. 2011). In addition to the metabolism-related functions, MC3R is also involved in the regulation of natriuresis (Ni et al. 2003) and immune functions (Getting et al. 2001, 2006).

MC3R is a member of rhodopsin-like family A G protein-coupled receptors (GPCRs). Activation of MC3R results in GDP/GTP exchange in the stimulatory G protein (Gs), which then activates adenyl cyclase to increase the production of cAMP. Although two studies have reported that MC3R activation stimulates ERK1/2 phosphorylation (Chai et al. 2007, Bègriche et al. 2012), an earlier study has reported that while MC4R activation leads to ERK1/2 phosphorylation, MC3R activation does not (Daniels et al. 2003). The existence of biased signaling has been reported in multiple GPCRs with important therapeutic implications (for reviews, see Violin & Lefkowitz (2007), Rajagopal et al. (2010) and Reiter et al. (2012)). In addition to biased ligands, biased signaling may also be induced by mutations in the GPCRs (Rajagopal et al. 2010). We and others have previously identified both biased ligands and receptors in MC4R (Patten et al. 2007, Büch et al. 2009, Huang & Tao 2010, Mo et al. 2012, Mo & Tao 2013; reviewed by Breit et al. (2011) and Tao (2014)). However, until now, no biased signaling of MC3R has been reported. Therefore, it is important to determine whether MC3R mediates ERK1/2 phosphorylation and whether biased signaling also exists in MC3R.

The highly conserved DRYxxI motif at the cytoplasmic end of transmembrane domain 3 (TM3) and the intracellular loop 2 (ICL2) are known to be critical for receptor function in several GPCRs. DRY motif forms salt bridges with surrounding residues and with TM6, which forms an ionic lock constraining the receptor in an inactive conformation (Palczewski et al. 2000, Rosenbaum et al. 2009). Upon ligand binding, the ionic lock is broken and DRY forms new interactions with TM5, stabilizing the receptor in an active conformation (Palczewski et al. 2000, Rosenbaum et al. 2009). ICL2 forms a L-shaped structure or a short α-helix parallel to the cell membrane in different GPCRs (Palczewski et al. 2000, Rosenbaum et al. 2009, Chien et al. 2010). ICL2 directly interacts with Gα and β-arrestin and therefore is important for G-protein coupling and receptor desensitization (Han et al. 2001, Rasmussen et al. 2011). Until now, two residues of MC3R in this region, D178 and I183, have been previously reported to be important for MC3R function (Tao & Segaloff 2004, Wang et al. 2008). Hence, our knowledge about the function of the DRYxxI motif and ICL2 of MC3R is still very limited. In this study, we investigated the importance of each residue in the DRYxxI motif and ICL2 in regulating receptor cell surface expression, ligand binding, and their different contributions to the two signaling pathways of MC3R.

**Materials and methods**

**Materials**

([125I]-[Nle⁴,d-Phe⁷]-α-MSH (NDP–MSH) was purchased from the American Radiolabeled Chemicals (St Louis, MO, USA), NDP–MSH from Peptides International (Louisville, KY, USA), and α-MSH from Pi Proteomics (Huntsville, AL, USA). Radiolabeled cAMP was iodinated using the chloramine T method (Tao et al. 2010). The N-terminal 3×HA tagged WT human MC3R (hMC3R) cloned into pcDNA3.1 was obtained from Missouri S&T cDNA Resource Center (http://www.cDNA.org/; Rolla, MO, USA).

**Site-directed mutagenesis**

Mutant hMC3Rs were generated from the WT receptor using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) as described in detail previously (Tao & Segaloff 2003) using primers listed in Table 1. The presence of desired mutations was verified by direct nucleotide sequencing by the DNA Sequencing Facility of University of Chicago Cancer Research Center (Chicago, IL, USA).

**Cell culture and transfection**

Human embryonic kidney 293T (HEK293T) cells and Neuro2a cells were purchased from American Type Culture Collection (Manassas, VA, USA). HEK293T cells were cultured in DMEM supplemented with 10% newborn calf serum, 10 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Neuro2a cells were cultured in the same media except that it was supplemented with 10% fetal bovine serum. Cells were seeded into gelatin-coated six-well clusters and were transfected with 1 µg plasmid/well using the calcium phosphate precipitation method for HEK293T cells as described previously (Tao et al. 2010) or using jetPRIME...
transfection reagent (Polyplus-transfection, New York, NY, USA) for Neuro2a cells (Huang & Tao 2014, Tao & Huang 2014). Approximately 48 h later, cells were used for ligand binding, signaling, or flow cytometry studies. For western blot, HEK293T cells were plated into 100 mm dishes coated with 0.1% gelatin and were transfected using the calcium phosphate precipitation method.

**Flow cytometry**

HEK293T and Neuro2a cells were seeded and transfected as described above. On the day of experiment, cells in six-well clusters were placed on ice, washed once with ice-cold PBS for immunohistochemistry (PBS-IH; Tao & Segaloff 2003), detached, and then pelleted by centrifugation at 500 \( g \) for 5 min. To measure the total expression of receptors, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in PBS-IH for 15 min, and then blocked with 5% BSA in PBS-IH for 1 h. To measure the cell surface expression of receptors, cells were directly incubated with the primary antibody after centrifugation. The primary antibody anti-HA.11 (Covance, Princeton, NJ, USA) was added at a dilution of 1:50 in 0.5% BSA in PBS-IH. One hour later, cells were washed once with 0.5% BSA in PBS-IH and then were incubated with the secondary antibody Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) for 1 h. Cells were washed twice with 0.5% BSA in PBS-IH and assayed using a C6 Accuri Cytometer (Accuri Cytometers, Ann Arbor, MI, USA). Cells transfected with the empty vector pcDNA3.1 were used for background staining. Calculations were carried out as described previously (Wang et al. 2008).

**Ligand binding assay**

HEK293T cells were seeded and transfected as described above. On the day of experiment, cells were washed twice with warm Waymouth’s MB752/1 media (Sigma–Aldrich) containing 1 mg/ml BSA (Waymouth/BSA). Then cells were incubated with 100 000 c.p.m. of \( ^{125}\text{I}-\text{NDP–MSH} \) and with or without different concentrations of unlabeled NDP- or \( \alpha \)-MSH at 37 °C. One hour later, cells were washed twice with ice-cold Hank’s balanced salt solution containing 1 mg/ml BSA to terminate the reaction and then were lysed by 100 \( \mu \)l 0.5 M NaOH. Cell lysates were collected by cotton swabs and counted in a gamma counter. GraphPad Prism (San Diego, CA, USA) was used to calculate receptor occupancy (RO, as an estimate of maximal binding; Wang et al. 2008) and the concentration that results in 50% inhibition (IC50).

**cAMP assay**

HEK293T cells were seeded and transfected as described above. On the day of experiment, cells were washed twice with warm Waymouth/BSA. Then cells were incubated with fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma–Aldrich) at 37 °C 15 min before the addition of different concentrations of NDP- or \( \alpha \)-MSH. One hour after incubation with the ligand, cells were lysed with 0.5 M perchloric acid containing 180 \( \mu \)g/ml theophylline (Sigma–Aldrich). The solution was neutralized with 0.72 M KOH/0.6 M KHCO3. Intracellular cAMP levels were measured using RIA as described previously (Tao et al. 2010).

**Protein preparation and western blot**

The method for protein preparation and western blot was described previously in detail (Huang & Tao 2012, Mo et al. 2012). Briefly, HEK293T cells were seeded and transfected as described above. At 24 h after transfection, cells were starved in Waymouth/BSA for 24 h at 37 °C. On the day of experiment, cells were stimulated with or without 10\(^{-6}\) M NDP–MSH for 5 min at 37 °C. Cells were lysed in 0.5% NP-40 lysis buffer and total protein concentrations of cell lysates were measured using the Bradford protein assay. Thirty microgram samples were subjected to 10%
SDS–PAGE and then transferred onto PVDF membranes. Membranes were incubated with 10% non-fat dry milk containing 0.2% Tween 20 for at least 3 h at room temperature, and then immunoblotted with a rabbit phosphorylated ERK1/2 (pERK1/2) antibody (Miyaji et al. 2009) (Cell Signaling, Billerica, MA, USA) at a dilution of 1:1000 and a mouse β-tubulin antibody (Chu & Klymkowsky 1989) (Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA, USA) at a dilution of 1:2000 in 5% BSA in Tris-buffered saline containing Tween 20 (TBST) overnight at 4 °C. After three washes in TBST, membranes were incubated with horseradish peroxidase-linked antibodies, an anti-rabbit antibody (Jackson ImmunoResearch) at a dilution of 1:5000 in 10% non-fat dry milk for 2 h at room temperature. Antibody binding was detected with ECL reagent (Thermo Scientific, Rockford, IL, USA), and staining intensity was determined using the ImageJ Software (NIH, Bethesda, MD, USA).

**Statistical analysis**

All data analyses were performed using the GraphPad Prism 4.0 Software. To compare cell surface expression levels, pERK1/2 levels as well as the binding and signaling parameters between WT and mutant hMC3Rs, Student’s $t$-test was carried out.

**Results**

**Cell surface expression of the mutant hMC3Rs**

To determine the function of each residue at the cytoplasmic end of TM3 and ICL2, we performed alanine-scanning mutagenesis by mutating each residue to alanine or mutating alanine to glycine (Table 1). We generated a total of 18 mutants (Fig. 1).

We first quantified the cell surface expression levels of the mutant hMC3Rs. HEK293T cells and Neuro2a cells transiently transfected with the $3\times$HA-tagged WT or mutant hMC3Rs were used for flow cytometry studies as described in ‘Materials and methods’ section. We found that no mutant had decreased cell surface expression levels in HEK293T cells (Fig. 2A). To confirm the results, we further performed flow cytometry studies using Neuro2a cells because MC3R is widely expressed in the CNS. Consistently, no mutant had significantly decreased total expression levels (data not shown) or cell surface expression levels (Fig. 2B) in Neuro2a cells. Compared

---

**Figure 1**

Schematic of hMC3R. Residues that were found to be important for the receptor function are indicated.
with the WT hMC3R, two mutants (A186G and M193A) had a 40% reduction in cell surface expression levels that did not reach statistical significance in Neuro2a cells (Fig. 2B).

Ligand-binding properties of the mutant hMC3Rs

To study ligand binding and signaling properties of the mutant hMC3Rs, two ligands of MC3R, NDP–MSH, and α-MSH were used. NDP–MSH is a superpotent agonist and α-MSH is an endogenous agonist of the melanocortin receptors except for the MC2R. Both ligands are widely used in MC3R studies.

To perform the competitive binding assay, HEK293T cells transiently transfected with the WT or mutant hMC3Rs were incubated with the radiolabeled 125I-NDP–MSH and different concentrations of unlabeled ligands. NDP–MSH was first studied as the unlabeled ligand. Our data showed that three mutants, D178A, M193A, and T194A, had no detectable binding (Table 2 and Fig. 3). Four mutants, R179A, Y180A, I183A, and I192A, had significantly decreased IC50s compared with the WT hMC3R and therefore increased affinities with the ligand (Table 2 and Fig. 3).

The competitive binding assay was then performed using α-MSH as the ligand. Consistently, three mutants, D178A, M193A, and T194A, had no measurable binding, and one mutant I192A had decreased IC50s (Table 3 and Fig. 4). By contrast, five mutants, R179A, Y180A, T182A, F184A, and L187A, had increased IC50s (Table 3 and Fig. 4).

RO values were also calculated from the binding assays. In addition to the three mutants with no detectable binding, eight mutants, R179A, Y180A, I183A, Y185A, A186G, R188A, Y189A, and I192A, had significantly decreased RO values compared with the WT hMC3R (Table 2). Two mutants, T182A and L187A, had increased RO by ~50% (Table 2).

Signaling properties of the mutant hMC3Rs in the Gs–cAMP signaling pathway

To study whether residues in the DRYxxI motif and ICL2 are required for MC3R signaling in the Gs–cAMP pathway, HEK293T cells transfected with WT or mutant hMC3Rs were stimulated with different concentrations of the ligand and intracellular cAMP accumulation was measured using RIA. We first studied the signaling properties in response to NDP–MSH. We showed that NDP–MSH increased intracellular cAMP production of WT hMC3R with an EC50 of 0.61 nM in a dose-dependent manner. Four mutants, R179A, I183A, M193A, and T194A, had no response to NDP–MSH stimulation (Table 2 and Fig. 5). Additionally, nine mutants, D178A, Y180A, V181A, T182A, Y185A, A186G, L187A, R188A, and I192A, had significantly decreased maximal cAMP production (Rmax) compared with the WT hMC3R (Table 2 and Fig. 5). Although there was no measurable binding, D178A responded to NDP–MSH stimulation with a reduced Rmax, 13% of the WT hMC3R. EC50 values of the mutants were also analyzed compared with the WT hMC3R. We showed that two mutants, T182A and L187A, had increased EC50s (Table 2 and Fig. 5).

The cAMP assay was also carried out using α-MSH as the ligand. The EC50 of α-MSH was 3.3 nM for the WT hMC3R. Three mutants, I183A, M193A, and T194A, did not respond to α-MSH stimulation. Additionally, nine mutants, D178A, R179A, Y180A, Y185A, A186G, L187A, R188A, Y189A, and I192A, had significantly reduced Rmax.
showed that one mutant, V181A, had significantly altered basal signaling, intracellular cAMP concentrations with the WT hMC3R. Three mutants, Y180A, F184A, and L187A, had significantly reduced basal cAMP levels (Table 2). The other mutants had similar basal cAMP levels as the WT hMC3R (Table 2).

(Table 3 and Fig. 6). The results were consistent with the results using NDP–MSH with three exceptions, R179A, Y189A, and S191A. Although they did not respond to NDP–MSH stimulation, R179A partially responded to α-MSH stimulation with an $R_{\text{max}}$ 16% of the WT hMC3R. Y189A and S191A had similar $R_{\text{max}}$ values with NDP–MSH, but a reduced $R_{\text{max}}$ value with α-MSH. When EC_{50} of the mutants were analyzed, six mutants, D178A, R179A, Y189A, and T182A, had a dramatic increase in $R_{\text{max}}$ (by more than 40%) and significantly increased EC_{50} (Table 3 and Fig. 6).

The basal signaling of MC3R is low compared with another neural melanocortin receptor, the MC4R (Tao 2007, Tao et al. 2010). To study whether the mutants had altered basal signaling, intracellular cAMP concentrations were measured in the absence of any ligand. Our data showed that one mutant, V181A, had significantly increased basal cAMP levels, increased by ~50% compared with the WT hMC3R. Three mutants, Y180A, F184A, and L187A, had significantly reduced basal cAMP levels (Table 2). The other mutants had similar basal cAMP levels as the WT hMC3R (Table 2).

Figure 3
Ligand-binding properties of the WT and mutant hMC3Rs with NDP–MSH as the ligand. Intact cell surface binding was measured by competitive inhibition of $^{125}$I-NDP–MSH with different concentrations of unlabeled NDP–MSH. Results are expressed as percentage of WT maximal binding. Each point is expressed as mean ± S.E.M. of at least three independent experiments. All curves are representative of at least three independent experiments.
Table 3 The ligand binding and signaling properties of WT and mutant hMC3Rs in response to α-MSH stimulation. Values are expressed as mean ± S.E.M. of at least three independent experiments. The Rmax of WT hMC3R was 2851.50 ± 466.51 pmol/10⁶ cells with α-MSH stimulation.

<table>
<thead>
<tr>
<th>hMC3R construct</th>
<th>α-MSH binding (IC₅₀ (nM))</th>
<th>α-MSH-stimulated cAMP (EC₅₀ (nM))</th>
<th>α-MSH-stimulated cAMP (Rmax (% WT))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>66.88 ± 4.57</td>
<td>3.30 ± 1.04</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>D178A</td>
<td>42.08 ± 13.49</td>
<td>16.5 ± 2.35</td>
<td>9.0 ± 4.16</td>
</tr>
<tr>
<td>R179A</td>
<td>63.04 ± 10.17</td>
<td>6.71 ± 0.24</td>
<td>10.1 ± 3.06</td>
</tr>
<tr>
<td>Y180A</td>
<td>62.38 ± 9.81</td>
<td>6.94 ± 0.36</td>
<td>8.93 ± 6.80</td>
</tr>
<tr>
<td>V181A</td>
<td>2.54 ± 0.85</td>
<td>17.44 ± 6.39</td>
<td>62.89 ± 14.48</td>
</tr>
<tr>
<td>T182A</td>
<td>1.47 ± 0.79</td>
<td>3.47 ± 2.17</td>
<td>ND</td>
</tr>
<tr>
<td>I183A</td>
<td>4.37 ± 2.94</td>
<td>7.12 ± 2.94</td>
<td>111.96 ± 16.62</td>
</tr>
<tr>
<td>F184A</td>
<td>64.98 ± 17.28</td>
<td>65.96 ± 11.55</td>
<td>44.80 ± 10.91</td>
</tr>
<tr>
<td>Y185A</td>
<td>11.96 ± 15.73</td>
<td>30.62 ± 0.79</td>
<td>36.40 ± 1.58</td>
</tr>
<tr>
<td>A186G</td>
<td>13.83 ± 5.47</td>
<td>5.43 ± 0.63</td>
<td>10.79 ± 1.18</td>
</tr>
<tr>
<td>L187A</td>
<td>11.96 ± 11.55</td>
<td>6.80 ± 0.36</td>
<td>30.67 ± 7.67</td>
</tr>
<tr>
<td>R188A</td>
<td>3.30 ± 1.28</td>
<td>46.11 ± 7.63</td>
<td>46.11 ± 7.63</td>
</tr>
<tr>
<td>Y189A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
<tr>
<td>H190A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
<tr>
<td>S191A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
<tr>
<td>I192A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
<tr>
<td>M193A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
<tr>
<td>T194A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
<tr>
<td>V195A</td>
<td>113.66 ± 15.73</td>
<td>ND</td>
<td>82.82 ± 7.00</td>
</tr>
</tbody>
</table>

ND, not detected.

*Significantly different from WT hMC3R, P < 0.05.

**Significantly different from WT hMC3R, P < 0.01.

***Significantly different from WT hMC3R, P < 0.001.

Signaling properties of the mutant hMC3Rs in the ERK1/2 signaling pathway

To study the ERK1/2 signaling pathway of MC3R, HEK293T cells starved for 24 h were stimulated with or without 10⁻⁶ M NDP–MSH for 5 min. We showed that stimulation of WT hMC3R with NDP–MSH in turn stimulated ERK1/2 phosphorylation (Fig. 7). Compared with the basal pERK1/2 level, the pERK1/2 level of WT hMC3R was increased by more than twofold in the presence of NDP–MSH. When the 18 mutants were studied, our data indicated that five mutants (Y185A, A186G, R188A, M193A, and T194A) did not respond to NDP–MSH stimulation (Table 2 and Fig. 7). NDP–MSH stimulation increased ERK1/2 phosphorylation in two mutants, F184A and S191A, although not statistically significant, probably due to larger variation and increased basal ERK1/2 activation. The other 11 mutants had significantly increased ERK1/2 phosphorylation when stimulated by NDP–MSH (Table 2 and Fig. 7).

Discussion

In this study, we systematically studied the function of 18 residues in the DRYxxl motif and the ICL2 of hMC3R.

We showed that i) 11 residues, D178, R179, Y180, I183, Y185, A186, R188, Y189, I192, M193, and T194, were critical for ligand binding and signaling; ii) two residues, T182 and L187, were critical for signaling in the Gα–cAMP pathway.
pathway; iii) five residues, Y185, A186, R188, M193, and T194, were critical for signaling in the ERK1/2 pathway; and iv) seven residues, D178, R179, Y180, T182, I183, T194, and I192, were critical for conferring biased signaling in MC3R.

Alanine or glycine mutations of 11 residues, D178, R179, Y180, I183, Y185, A186, R188, Y189, I192, M193, and T194, did not interfere with the receptor cell surface expression but resulted in decreased RO (Fig. 2, Tables 2 and 3). These mutations might interfere with the ligand binding with a consequent defect in signaling. We have reported previously that D178E and D178Q are expressed well on the cell surface but have either no detectable (D178Q) or reduced RO (D178E) (Wang et al. 2008). These 11 residues are located in the cytoplasmic side of MC3R and, therefore, are not expected to directly interact with the ligand. Instead, these residues may indirectly modulate the ligand–receptor interaction through a conformational change. Although it is not a common feature of residues that are important for ligand binding located remotely from the binding sites, observations have been reported previously in MC3R (Wang et al. 2008) as well as other GPCRs, such as gonadotropin-releasing hormone receptor (Lu et al. 2005) and V2 vasopressin receptor (Erlenbach et al. 2001).

It has been suggested that D3.49 forms hydrogen bonds with R3.50, which is critical for the conformational switch between inactive and active states of GPCRs (the superscript numbers refer to the Ballesteros & Weinstein (1995) numbering scheme; Palczewski et al. 2000, Rosenbaum et al. 2007, Wacker et al. 2013). In addition to be involved in multiple intramolecular interactions constraining the receptor in an inactive status (Flanagan 2005), R3.50 has also been indicated to directly contact Gz stabilizing the active status of the receptor-G protein complex upon ligand binding (Scheer et al. 2008, Rasmussen et al. 2011). Compared with D3.49 and R3.50, Y3.51 is the least conserved in the DRY motif. The side chain of Y3.51 has been indicated to direct toward the inside of the receptor and form hydrophobic interaction with P3.57 in rhodopsin (Okada et al. 2001). Consistent with these well-known findings, we observed that the DRY motif of MC3R also played a vital role in MC3R binding and signaling (Fig. 2, Tables 2 and 3).

Alanine mutant of I1833.54 (I1833.54A) with partially reduced RO did not respond to NDP- or α-MSH stimulation (Tables 2 and 3). We reported that mutations of I1833.54 to polar residues result in a profound loss of function, whereas valine mutation (I183 3.54 V) does not alter receptor activity (Tao & Segaloff 2004), implying that I1833.54 forms hydrophobic interactions required for MC3R activation. Phosphorylation of T1573.65 has been suggested to regulate the forward trafficking of MC1R (Sanchez-Laorden et al. 2009). However, in MC3R,
Figure 7
ERK1/2 signaling properties of the WT and mutant hMC3Rs. In (A), HEK293T cells were transiently transfected with WT or mutant hMC3Rs. Cells were starved for 24 h and then stimulated with or without 10^{-6} M NDP–MSH for 5 min. Lysates were analyzed by western blot using antibodies against pERK1/2 or antibodies against β-tubulin as a control. In (B), results are expressed as mean ± S.E.M. of at least four independent experiments. *Significantly different from the basal signaling of WT or mutant hMC3Rs, P < 0.05. Images had not been modified and are representative of at least four independent experiments. The β-tubulin and pERK1/2 bands were developed from the same blot.
T1943.65 did not affect MC3R trafficking but abolished MC3R binding and signaling.

Although T1823.53A and L1873.58A bound similarly with the ligands as the WT hMC3R, they had impaired cAMP production upon NDP- or α-MSH stimulation (Tables 2 and 3). These two positions are highly conserved in rhodopsin-like GPCRs, with 79% being A/S/T at position 3.53 and 75%/L/I/V at position 3.58 respectively (Marion et al. 2006). The position 3.58 was reported to be critical for G protein coupling, and mutations result in impaired receptor activation in β2-AR (F1393.58A; Rasmussen et al. 2011) and GPR54 (L1483.58S; Wacker et al. 2008). Consistently, our data indicated that L1873.58 was also a key residue in MC3R activation.

The basal activity of MC3R is relatively low compared with the closely related MC4R. Until now, there is only one artificial mutation of MC3R, F3477.64A, that has been reported to be constitutively active (Tao et al. 2010). In this study, we found that only one mutant (T1823.53A) had a less than twofold increase in basal activity. It has been suggested that the hydrogen bonding interaction between D3.49 and Y3.60 in β1-AR and A2A-adenosine receptor accounts for their low basal activities, whereas the interaction between D3.49 and S3.62 in β2-AR accounts for its high basal activity (Jaakola et al. 2008, Rosenbaum et al. 2009). In the MC4R, mutation of D1463.49N identified from obese patients pronouncedly increases the basal activity (Wang & Tao 2011). However, in this study, alanine mutation of D1783.49A and Y1893.60A had no effect on the basal activity of MC3R, implying that such hydrogen bonding interactions do not exist or do not affect the basal activity of MC3R. There is usually a correlation between the basal activity of the WT receptor and the propensity of mutations to be constitutively active (Tao et al. 2002, Tao 2008). As MC3R basal activity is low, the propensity of MC3R mutations to be constitutively active is probably low.

To delineate the ERK1/2 signaling pathway of MC3R, we also studied the pERK1/2 levels of WT and mutant hMC3Rs stimulated with NDP–MSH. We showed that activation of MC3R by NDP–MSH activated ERK1/2. Five mutants, Y185A, A186G, R188A, M193A, and T194A, did not activate ERK1/2 when stimulated with NDP–MSH; these mutants also had decreased maximal cAMP production. Interestingly, some of the mutants with decreased RO, especially D178A that had almost no detectable binding, still functioned well in ERK1/2 signaling. It is possible that the mutation D178A increased the disassociation rate of the ligand from MC3R, resulting in decreased RO; however, as ERK1/2 activation is rapid and transient, ERK1/2 was still activated. ERK1/2 are known to be involved in various processes, such as cell proliferation and cell migration (Roskoski 2012). However, the physiological role of ERK1/2 mediated by MC3R is still not clear. Further studies need to be conducted to determine and differentiate the different functions of the cAMP and ERK1/2 pathways of MC3R.

Seven mutants (D178A, R179A, Y180A, T182A, I183A, L187A, and I192A) that had very low or even no cAMP production had similar ERK1/2 signaling as the WT MC3R, suggesting that these seven mutants had ERK1/2 biased signaling (Table 2 and Fig. 7). A previous study had reported that MC3R induces ERK1/2 phosphorylation not through cAMP–PKA but through phospho-inositol-3 kinase (Chai et al. 2007). To the best of our knowledge, this is the first report describing biased signaling in MC3R. Biased signaling induced either by mutation or by biased ligand has been reported in the MC4R (Patten et al. 2007, Büch et al. 2009, Huang & Tao 2012, Mo et al. 2012, Mo & Tao 2013; reviewed by Breit et al. (2011) and Tao (2014)) and several other GPCRs (reviewed by Violin & Lefkowitz (2007) and Lefkowitz (2013)). It has been suggested that biased ligands interact with different residues of the receptor and, therefore, induce different conformational changes in β1-AR (Warne et al. 2012) and serotonin receptors (Wacker et al. 2013). The biased mutants of MC3R might also have different structures from the WT receptor and, consequently, have different signaling capacities when stimulated by NDP–MSH.

In summary, our present data demonstrated that the cytoplasmic end of TM3 and ICL2 were critical for MC3R function. We identified residues that were critical for ligand binding, signaling in the Gs–cAMP pathway, and signaling in the ERK1/2 pathway. We also reported biased signaling of MC3R that advances our understanding of the complexity of the MC3R signaling cascades and also might have therapeutic potential.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This study was supported by grants from the National Institutes of Health R15DK077213 and Animal Health and Disease Research Program of Auburn University College of Veterinary Medicine.

Acknowledgements
The authors thank Dr Shu-Xiu Wang for generating the mutant constructs and performing some of the preliminary experiments. The E7 β-tubulin...
antibody developed by M Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

References


Daniels D, Patten CS, Roth JD, Yue DK & Hluhtry SJ 2003 Melanocortin receptor signaling through mitogen-activated protein kinase in vitro and in rat hypothalamus. Brain Research 986 1–11. (doi:10.1016/S0006-8993(03)01627-2)


Flanagan CA 2005 A GPCR that is not “DRY”. Molecular Pharmacology 68 1–3. (doi:10.1124/mol.105.014183)


Ni XP, Pearce D, Butler AA, Cone RD & Humphreys MH 2003 Genetic disruption of the /-melanocyte-stimulating hormone signaling leads to salt-sensitive hypertension in the mouse. Journal of Clinical Investigation 111 1251–1258. (doi:10.1172/JCI200316993)


Tao YX & Hansen MA, Lefkowitz RJ 2014 Ipsen 5i is a novel potent pharmacopereone for intracellularly retained melanocortin-4 receptor mutants. Frontiers in Endocrinology **5** 131. (doi:10.3389/fendo.2014.00131)


Tao YX, Miczack D & Segaloff DL 2002 Chimeras of the rat and human FSH receptors (FSHRs) identify residues that permit or suppress transmembrane 6 mutation-induced constitutive activation of the FSHR via rearrangements of hydrophobic interactions between helices 6 and 7. Molecular Endocrinology **16** 1881–1892. (doi:10.1210/me.2001.0199)


Wang SX, Fan ZC & Tao YX 2008 Functions of acidic transmembrane residues in human melanocortin-3 receptor binding and activation. Biochemical Pharmacology **76** 520–530. (doi:10.1016/j.bcp.2008.05.026)


Received in final form 12 September 2014
Accepted 15 September 2014
Accepted Preprint published online 16 September 2014