The Metabolite GnRH-(1-5) Inhibits the Migration of Immortalized GnRH Neurons

Darwin O. Larco, Madelaine Cho-Clark, Shaila K. Mani, and T. John Wu

Program in Molecular and Cellular Biology (D.O.L., T.J.W.) and Department of Obstetrics and Gynecology (T.J.W., M.C.), Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814; and Departments of Molecular and Cellular Biology and Neuroscience (S.K.M.), Baylor College of Medicine, Houston, Texas 77030

The decapeptide GnRH is an important regulator of reproductive behavior and function. In the extracellular matrix, GnRH is metabolized by the endopeptidase EC3.4.24.15 (EP24.15) to generate the pentapeptide GnRH-(1-5). In addition to its expression in the adult hypothalamus, EP24.15 is expressed along the migratory path of GnRH-expressing neurons during development. Although we have previously demonstrated a role for EP24.15 in the generation of the biologically active pentapeptide GnRH-(1-5) in regulating GnRH expression and mediating sexual behavior during adulthood in rodents, the modulatory role of GnRH-(1-5) in the migration of GnRH neurons during development remains unknown. To address this information gap, we examined the effect of GnRH-(1-5) on the cellular migration of a premigratory GnRH-secreting neuronal cell line, the GN11 cell, using a wound-healing assay. Dose- and time-response studies demonstrated that GnRH-(1-5) significantly delayed wound closure. We then sought to identify the mechanism by which GnRH-(1-5) inhibits migration. Because the cognate GnRH receptor is a G protein-coupled receptor, we examined whether GnRH-(1-5) regulates migration by also activating a G protein-coupled receptor. Using a high-throughput β-arrestin recruitment assay, we identified an orphan G protein-coupled receptor (GPR173) that was specifically activated by GnRH-(1-5). Interestingly, small interfering RNA to GPR173 reversed the GnRH-(1-5)-mediated inhibition on migration of GN11 neurons. Furthermore, we also demonstrate that the GnRH-(1-5)-activated GPR173-dependent signal transduction pathway involves the activation of the signal transducer and activator of transcription 3 in GnRH migration. These findings indicate a potential regulatory role for GnRH-(1-5) in GnRH neuronal migration during development. (Endocrinology 154: 783–795, 2013)
associated with KS are not linked to any currently known genetic defect (6).

In addition to the actions of the full-length GnRH, there are also biologically active effects of its cleavage products. We have previously shown that GnRH-(1-5), produced by the zinc metalloendopeptidase EC3.4.24.15 (EP24.15), facilitates lordosis behavior in ovariectomized estrogen-primed rats (7). In addition, GnRH-(1-5) stimulates GnRH mRNA expression in GnRH neurons, whereas treatment with the full-length GnRH inhibits its own expression (8). EP24.15, the enzyme that is responsible for generating GnRH-(1-5), is expressed in the CNS during development along the migratory path of GnRH neurons (9). It is possible that the availability of GnRH-(1-5) during development may contribute to the migration of GnRH neurons before they enter the CNS through the cribriform plate.

The pathway mediated by the signal transducer and activator of transcription 3 (STAT3) plays a critical role in cell survival and differentiation. STAT3 activation is induced by phosphorylation at the residue Y705, leading to subsequent dimerization with another phosphorylated STAT3 (pSTAT3). This event leads to nuclear translocation and initiation of transcription (10). In the CNS, STAT3 signaling has been implicated in neurite outgrowth (11, 12) and in neuronal pathfinding (13). Furthermore, the developmental presence of the cytokine leukemia inhibitory factor (LIF), which induces the phosphorylation of STAT3, has been shown to promote GnRH neuronal migration in vitro (14). Therefore, modulation of STAT3 activity may serve as a mechanism by which the extracellular environment can in part regulate the migration rate of GnRH neurons.

In this study, we investigated whether GnRH-(1-5) regulates GnRH neuronal migration using the GnRH-secreting cell line, the GN11 cell, as a model (15-17). GN11 cells were isolated from a mouse olfactory tumor induced by the SV40 T-antigen driven by the human GnRH promoter (17). These cells are considered an immature GnRH-secreting cell line that has been used extensively to study GnRH neuronal migration in vitro (14, 15, 18). Our results demonstrate that GnRH-(1-5) inhibits GN11 cellular migration by inhibiting STAT3 signaling. Furthermore, we identify the orphan G protein-coupled receptor GPR173 as the signal-transducing element mediating the actions of GnRH-(1-5) on migration in GN11 cells.

Materials and Methods

Cell culture

GN11 cells (generously donated by Dr. S. Radovich, Johns Hopkins University School of Medicine, Baltimore, Maryland) were cultured as described previously (19, 20) with some modification (Novaira, H., personal communication). In brief, cells were grown in DMEM (Mediatech, Herndon, Virginia) without antibiotics supplemented with 7% fetal bovine serum (FBS) (HyClone, Logan, Utah), 3% newborn calf serum (HyClone), 25 mM glucose, and 5 mM L-glutamine. Cells were maintained at 37°C in an atmosphere with 5% CO₂.

Wound closure assay

A wound closure assay was used to determine the effect of GnRH-(1-5) on GN11 cellular migration. GN11 cells were plated on plastic uncoated 6-well plates (Costar, Corning, New York) and allowed to reach 80% confluence. The cells were starved for 24 hours in 10% charcoal-stripped dextran-treated FBS (Atlanta Biologicals, Lawrenceville, Georgia) and tested for migration using an in vitro wound closure assay. The scratch was made through a cell monolayer with a sterile 1000-μL pipette tip. The cells were washed 3 times with PBS to remove detached cells and incubated with 10% stripped FBS containing the indicated treatment condition. Images were taken using a phase-contrast microscope (Olympus IX71; Olympus America Inc, Center Valley, Pennsylvania) of each wound, and its location was noted. Subsequent pictures were taken at 24 or 48 hours after the addition of treatments depending on the experiment. The wound area was measured using Multi Gauge software (Fujifilm, Vallhalla, New York). The percent wound closure was calculated with the following formula: % wound closure = (area_{t=0h} - area_{t=24h})/area_{t=0h} × 100%. Similar protocols have been described previously to measure cellular migration in cancer cells (21, 22) and in GN11 cells (18, 23, 24).

Boyden chamber assay

The QCM Chemotaxis 96-Well Migration Assay (Millipore, Temecula, California) was used as an alternative method to determine the effect of GnRH-(1-5) on GN11 cellular migration. This assay, based on the Boyden chamber assay, has been used previously to characterize GN11 cellular migration (14, 25). GN11 cells were grown in complete medium to 80% confluence and then were starved in serum-free medium for 24 hours. Subsequently, cells were harvested with 2 mM EDTA-PBS, centrifuged for 5 minutes at 1500 rpm, and resuspended in serum-free media. Cells were loaded in the upper compartment of the well separated by an 8-μm porous membrane precoated with 0.2 mg/mL gelatin. The lower compartment contained the specific treatment condition (vehicle [VEH], 1 nM GnRH-(1-5), or 100 nM GnRH-(1-5)) in 10% charcoal-stripped dextran-treated FBS. GN11 cells were allowed to migrate through the pores for 3 hours at 37°C in a cell culture incubator. This time point was established previously to measure GN11 cellular migration (14, 15). After the incubation period, migrating cells attached to the bottom of the membrane were lysed and incubated with the supplied nucleic acid-binding fluorescent dye (CyQuant GR; Molecular Probes, Eugene, Oregon) according to the manufacturer’s instructions (Millipore). Fluorescence was read using a Victor X5 2030 Multilabel Plate Reader (PerkinElmer, Shelton, Connecticut) with a 480/535 filter set. VEH-treated cells were set at 100% for comparison with GnRH-(1-5)-treated cells.
Proliferation assay

Experiments were conducted to determine whether the effect of GnRH-(1-5) on migration was dependent on cell proliferation. The proliferation assay used a colorimetric assay based on measuring the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI). In brief, GN11 cells were plated at 20% confluence on 96-well plates, and on the following day the medium was replaced with 10% charcoal-stripped dextran-treated FBS. After 24 hours, cells were treated with 1 nM VEH or 100 nM GnRH-(1-5) and allowed to grow for either 24 or 48 hours. At the end of each experiment, 20 μL of the CellTiter MTS solution was added to each well and incubated for 30 minutes at 37°C. The absorbance of each plate was read at 490 nm with a Victor X5 2030 Multilabel Plate Reader. The experiments for each assay were performed with at least 8 replicates for each treatment condition and repeated at least 4 times using different cell passages.

Screening for putative GnRH-(1-5) receptors

We narrowed our search for putative GnRH-(1-5) receptors by using the PathHunter Orphan GPCR Biosensor Panel (DiscoveRx, Fremont, California). In this primary screen, 30 μM GnRH-(1-5) (n = 2/cell line) was used to assess ligand activity in an array of identified human orphan G protein-coupled receptors (GPCRs) using standard DiscoveRx protocols (26, 27) (Table 1). Activity was compared with that for the respective VEH (1% dimethyl sulfoxide) after treatment. This technology uses enzyme fragment complementation of the β-galactosidase (β-gal) enzyme, which upon formation of the complete enzyme generates a luminescent signal that can be measured by spectrometry. Specifically, orphan GPCRs were modified as fusion proteins to a portion of the β-gal enzyme. The complementing fragment of β-gal is fused to β-arrestin, which is recruited upon ligand binding to the modified GPCR. In our primary screen, a total of 74 GPCRs were assayed in the presence of GnRH-(1-5) (26, 27). Once we determined that GPR173 was the only receptor expressed in GN11 cells, a secondary screen was implemented using the GPR173-expressing cell line (PathHunter HEK 293 GPR173 Orphan GPCR β-Arrestin High Expression Cell Line, catalog no. 93-0380C1A; DiscoveRx). These cells were grown and maintained in DMEM (catalog no. 12430-054; Invitrogen, Carlsbad, California) containing 10% heat-inactivated FBS (catalog no. 10082-147; Invitrogen), penicillin-streptomycin-glutamine (catalog no. 10378-016; Invitrogen), 200 μg/mL hygromycin (catalog no. 10687-010; Invitrogen), and 800 μg/mL Geneticin (catalog no. 10131-035; Invitrogen). A time-course study was initially conducted to determine the optimal

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Receptor Accession No.</th>
<th>% Activity Relative to Control (30 μM)</th>
<th>Assay ID</th>
<th>Receptor Accession No.</th>
<th>% Activity Relative to Control (30 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCR1L2 NM_003965.2</td>
<td>ND</td>
<td>31</td>
<td>GPR83 NM_016540.2</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>EBI2 NM_080818.2</td>
<td>ND</td>
<td>32</td>
<td>GPR84 NM_020370.1</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>GHR1b NM_004122</td>
<td>ND</td>
<td>33</td>
<td>GPR85 NM_018970.3</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>GPR1 NM_005279.1</td>
<td>ND</td>
<td>34</td>
<td>GPR88 NM_022049.1</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>GPR3 NM_005281.2</td>
<td>ND</td>
<td>35</td>
<td>GPR97 NM_170776</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>GPR4 NM_005282.1</td>
<td>ND</td>
<td>36</td>
<td>GPR101 NM_054021</td>
<td>–22</td>
</tr>
<tr>
<td>7</td>
<td>GPR6 NM_005284.2</td>
<td>ND</td>
<td>37</td>
<td>GPR107 NM_020960</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>GPR12 NM_005288.1</td>
<td>ND</td>
<td>38</td>
<td>GPR119 NM_178471</td>
<td>–41</td>
</tr>
<tr>
<td>9</td>
<td>GPR15 NM_005290.1</td>
<td>ND</td>
<td>39</td>
<td>GPR120(L) NM_181745</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>GPR17 NM_005291.1</td>
<td>ND</td>
<td>40</td>
<td>GPR123 NM_00108390</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>GPR20 NM_005293.1</td>
<td>ND</td>
<td>41</td>
<td>GPR132 NM_013345.2</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>GPR23 NM_005296.1</td>
<td>ND</td>
<td>42</td>
<td>GPR139 NM_171791</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>GPR25 NM_005298.2</td>
<td>ND</td>
<td>43</td>
<td>GPR141 NM_181791.1</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>GPR26 NM_153442.1</td>
<td>ND</td>
<td>44</td>
<td>GPR143 NM_181790.1</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>GPR27 NM_018971.1</td>
<td>ND</td>
<td>45</td>
<td>GPR143 NM_000273</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>GPR30 NM_001505.2</td>
<td>ND</td>
<td>46</td>
<td>GPR146 NM_138445.1</td>
<td>ND</td>
</tr>
<tr>
<td>17</td>
<td>GPR31 NM_005299.1</td>
<td>ND</td>
<td>47</td>
<td>GPR148 NM_293092</td>
<td>–24</td>
</tr>
<tr>
<td>18</td>
<td>GPR32 NM_001506.1</td>
<td>ND</td>
<td>48</td>
<td>GPR149 NM_293580.1</td>
<td>ND</td>
</tr>
<tr>
<td>19</td>
<td>GPR35 NM_003012.2</td>
<td>ND</td>
<td>49</td>
<td>GPR150 NM_209675</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>GPR37L1 NM_004767.2</td>
<td>ND</td>
<td>50</td>
<td>GPR151 NM_194251</td>
<td>ND</td>
</tr>
<tr>
<td>21</td>
<td>GPR39 NM_001508.1</td>
<td>ND</td>
<td>51</td>
<td>GPR152 NM_352045</td>
<td>ND</td>
</tr>
<tr>
<td>22</td>
<td>GPR45 NM_007227.3</td>
<td>ND</td>
<td>52</td>
<td>GPR157 NM_024980</td>
<td>ND</td>
</tr>
<tr>
<td>23</td>
<td>GPR50 NM_004224</td>
<td>ND</td>
<td>53</td>
<td>GPR162 NM_019858</td>
<td>ND</td>
</tr>
<tr>
<td>24</td>
<td>GPR52 NM_005684.1</td>
<td>ND</td>
<td>54</td>
<td>GPR171 NM_013308.2</td>
<td>ND</td>
</tr>
<tr>
<td>25</td>
<td>GPR61 NM_031936.2</td>
<td>ND</td>
<td>55</td>
<td>GPR173 NM_018969.3</td>
<td>–19</td>
</tr>
<tr>
<td>26</td>
<td>GPR65 NM_003608.2</td>
<td>ND</td>
<td>56</td>
<td>GPR176 NM_007223</td>
<td>ND</td>
</tr>
<tr>
<td>27</td>
<td>GPR75 NM_006794.1</td>
<td>ND</td>
<td>57</td>
<td>HUMNPIY20 NM_022571.5</td>
<td>ND</td>
</tr>
<tr>
<td>28</td>
<td>GPR78 NM_080819.1</td>
<td>ND</td>
<td>58</td>
<td>MRGPRE NM_171536.3</td>
<td>ND</td>
</tr>
<tr>
<td>29</td>
<td>GPR79 NM_001272</td>
<td>ND</td>
<td>59</td>
<td>OXGR1 NM_080818</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>GPR83 NM_016540.2</td>
<td>ND</td>
<td>60</td>
<td>SUCNR1 NM_033050.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviation: ND, no difference.

a A panel of orphan GPCRs was screened for GnRH-(1-5) binding by measuring β-arrestin recruitment activity. The screen was run in duplicate, and cells were treated with 30 μM GnRH-(1-5).
time course for this cell line, ranging from 15 to 90 minutes. We found that 15 minutes yielded the optimal results (data not shown) and subsequently conducted a dose-response curve ranging from 0.01 to 1000 nM GnRH-(1-5). β-Gal activity was determined using the PathHunter Detection Kit (catalog no. 93-0001; DiscoveRx) and measured on a Victor X5 2030 Multilabel Plate Reader. The EC50 was calculated by applying a 4-parameter logistic curve using SigmaPlot (Systat Software, Inc, San Jose, California).

Reagents

GnRH-(1-5) was synthesized on-site (Biomedical Instrumentation Center, Uniformed Services University, Bethesda, Maryland) as described previously (28). In brief, the peptide was synthesized by 9-fluorenylmethoxycarbonyl chemistry using a 433A Peptide Synthesizer (Applied Biosystems, Foster City, California). Synthesized GnRH-(1-5) was divided into 10- to 20-mg aliquots and stored at −80°C with a desiccant. Before use, GnRH-(1-5) was dissolved in water to 1 mM and subsequently serially diluted in culture medium to its final concentration. n-Ser GnRH was purchased from Bachem (Torrance, California; catalog no. H-6395). The STAT3 inhibitor WP1066 was purchased from EMD Millipore (Darmstadt, Germany; catalog no. 573097) and dissolved in 100% ethanol to 100 mM. Control experiments included the appropriate solvent (water or ethanol) not exceeding more than 0.01% indicated as VEH.

RT-PCR

To determine the expression of target GPCRs in the GN11 cell line, total RNA was extracted with TRIzol reagent (Invitrogen), and 2 μg of RNA was reverse transcribed (Maxima First Strand cDNA Synthesis Kit; Fermentas, Glen Burnie, Maryland) for PCR analysis. In parallel, RNA samples in the absence of reverse transcriptase were used as a negative control (−RT) to exclude genomic contamination during cDNA synthesis. PCRs used cDNA templates equivalent to 50 ng of RNA using PCR Master Mix (Promega) and 200 nM concentrations of each primer. The PCR conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 30 seconds), and extension (72°C, 30 seconds). There was a final extension period of 5 minutes at 72°C. Amplicons were visualized by agarose gel electrophoresis (1%) using GelStar Nucleic Acid Gel Stain (Lonza, Rockland, Maine) and verified by sequencing. Primers specific to each target gene were generated with the National Center for Biotechnology Information Primer-BLAST Web site (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with the exception of GPR119 (29) and GnRH (20), which were described previously. All primers were made to span at least 1 intron on the corresponding gene (see Table 2 for more details). Mouse hypothalamic tissue was used as a positive control for each primer pair. The putative mRNA structure for GPR173 was obtained from the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

GPCR silencing by small interfering RNA (siRNA)

GN11 cells were plated at 20% confluence and transfected with a cocktail of siRNA (25 nM) specific to GPR173 (SMART-pool, catalog no. L-059639-00-0005; Dharmacon, Lafayette, Colorado). Control cells were transfected with a nontargeting siRNA mixture (25 nM) (SMARTpool, catalog no. D-001810-10-05; Dharmacon). Dharmafect4 (Dharmacon) was used as the transfection reagent. All treatments were performed in 10% charcoal/dextran–stripped FBS (Atlanta Biologicals, Lawrenceville, Georgia). Cells were incubated with the siRNA treatments for 48 hours, and then a scratch was made as described above in our wound closure assay protocol. Cells were washed 3 times, and the medium was replaced either with VEH or 100 nM GnRH-(1-5). Images were taken immediately after the addition of GnRH-(1-5) treatment and 24 hours later with a phase-contrast microscope (Olympus IX71).

Quantitative RT-PCR (qPCR)

The efficiency of GPR173 silencing by siRNA was measured in GN11 cells by qPCR. Total RNA was harvested 48 hours after transfection with siRNA as described earlier. Then 2 μg of RNA was reverse transcribed using the Maxima First Strand cDNA Synthesis Kit, and the cDNA was analyzed by qPCR using Fast SYBR Green Master Mix (catalog no. 330609; Qiagen, Valencia, California) with a 200 nM concentration of the appropriate primer pair. All samples were assayed in duplicate using the CFX Connect Real-Time System (Bio-Rad, Hercules, California). The qPCR conditions were as follows: initial denaturation and enzyme activation at 95°C for 10 minutes followed by 40 cycles of denaturation (95°C, 15 seconds), annealing, and reading (60°C, 30 seconds). Melt curve analyses were conducted after each qPCR to demonstrate the presence of a single amplicon. In addition, qPCR products were visualized by agarose gel electrophoresis.

Table 2. Primer Sequences Used in the PCR Experiments

<table>
<thead>
<tr>
<th>Name</th>
<th>Amplicon Size, bp</th>
<th>Direction</th>
<th>Primers (5’ → 3’)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPR101</td>
<td>182</td>
<td>Forward</td>
<td>TGGCCCCGAGAAAGGTGGTCAGA</td>
<td>NM_001033360.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TTAGTGCAGCTGGTGGTGCCATTG</td>
<td></td>
</tr>
<tr>
<td>GPR119</td>
<td>177</td>
<td>Forward</td>
<td>TGCCAGGGGAGGTGGCAC</td>
<td>NM_181751.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCTTGCGAGCCCTTACGGTACC</td>
<td></td>
</tr>
<tr>
<td>GPR173</td>
<td>187</td>
<td>Forward</td>
<td>CAGCTAGGGAGAAGGCTGCT</td>
<td>NM_027543.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TGGTAGCTACACCTCGCAATGGG</td>
<td></td>
</tr>
<tr>
<td>GnRH</td>
<td>200</td>
<td>Forward</td>
<td>CCCCCTGTGCTCCCCCATC</td>
<td>NM_008145.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGGTTCTGCGGATCCGAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CAAAACCAAAACGGTCCCC</td>
<td>NM_008907.1</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>94</td>
<td>Forward</td>
<td>TGCCTTCTTTCACCTCCAAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer sequences used for PCR and qPCR and product sizes are listed for GPR101, GPR119, GPR173, GnRH, and cyclophilin A. Also listed are the accession numbers.
phoresis (1%) using the GelStar Nucleic Acid Gel Stain under UV light and verified by sequencing. Primers specific to GPR173 and the reference gene cyclophilin A are shown in Table 2. qPCR conditions were optimized to produce greater than 95% efficiency for both primer pairs as determined by a 10-fold serial dilution of cDNA (29). The ΔΔCt method was used to measure fold change in GPR173 expression relative to levels obtained from VEH-treated cells. This experiment was repeated over 3 consecutive passages.

Mouse brain and nasal tissue

Experiments using RNA from animal tissues were samples from previously completed studies. For adult mouse brain, RT-PCR (with subsequent sequencing) was conducted on hypothalamic RNA extracted from an 8-week-old C57BL/6 male mouse (The Jackson Laboratory, Bar Harbor, Maine). In brief, the mouse was killed by carbon dioxide overdose with rapid decapitation. The brain was quickly removed, and the whole hypothalamus was dissected on ice. The RNA was immediately extracted by TRIZol reagent and processed as described in our RT-PCR protocol.

RNA samples from mouse noses were obtained at embryonic day (E) 11.5 or E12.5. These samples were generously donated by Dr. Susan Wray (National Institutes of Health, Bethesda, Maryland) and described previously by Wray and colleagues (30, 31). All experimental procedures were conducted in accordance with the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, revised 1996) and were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

Western blot analysis

Total protein lysates were harvested from cells with lysis buffer (O’Dell lysis buffer containing 10 mM EGTA, 10 mM EDTA, 80 μM Na2MoO4, 5 mM NaPO4, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 4 mM p-nitrophenyl phosphate, 1% Triton X-100, and Sigma Inhibitor Cocktail I and II and Roche Complete Protease Inhibitor [Roche, Indianapolis, Indiana]). Cell lysates were briefly sonicated, and supernatant (20 μg) was subjected to SDS-PAGE (4%–12%, Lonza) after centrifugation (20 000g for 20 min). After electrophoresis, the proteins were transferred onto a polyvinyl difluoride membrane (Bio-Rad). Western blot analysis was conducted using methods established previously (32). In brief, the membranes were sequentially incubated with specified primary antibodies (pSTAT3, 1:2000; STAT3, 1:2000; and caspase-7, 1:2000 [Cell Signaling Technology, Danvers, Massachusetts] or β-actin, 1:2000 [Sigma-Aldrich, St Louis, Missouri]) overnight at 4°C followed by incubation at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (Bio-Rad). The blots were visualized with a chemiluminescent signal (Immobilon Western HRP Substrate Peroxide Solution; Millipore Corp, Billerica, Massachusetts) and subsequently digitized (LAS-3000 imager; Fujifilm, Stamford, Connecticut) and analyzed (Image Gauge; Fujifilm, Valhalla, New York).

Immunofluorescent studies

After treatments, GN11 cells were washed with ice-cold 0.1 M phosphate-buffered solution containing 0.9% saline (PBS) and fixed with buffered 4% paraformaldehyde (pH 7.4; Sigma-Aldrich) for 10 min. Subsequently, cells were washed 3 times for 5 minutes with PBS and incubated with methanol for 10 min. After the formalin-methanol fixation, the cells were sequentially washed with PBS (3 times, 5 minutes each), followed by blocking with 5% normal goat serum (1 hour; Sigma-Aldrich) at room temperature, overnight incubation at 4°C with pSTAT3 primary antibody (1:1000; Cell Signaling Technology), and additional washes, and were incubated with Alexa Fluor 488 conjugated secondary antibody (1:1000; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) for 2 hours. After washing with PBS, the cells were coverslipped. Nuclear staining was performed with a 4,6-diamidino-2-phenylindole counterstain (Invitrogen, Eugene, Oregon). Images were taken with a Leica AF6000 microscope (Leica, Allendale, New Jersey).

Time-lapse imaging system

To better visualize the effect of GnRH-(1-5) on GN11 cellular migration, a wound closure assay was examined by time-lapse video microscopy using a Leica AF600 time-lapse microscope equipped with CO2 and temperature controls. GN11 cells were cultured on a Lab-Tek II Chamber Slide System (Nunc, Rochester, New York) following the same protocol for the wound closure assay as mentioned earlier. However, the scratch was made with a 200-μL sterile pipette tip to see the width of the wound at the magnification used. Cells treated with VEH or 100 nM GnRH-(1-5) were run in parallel, and pictures of the same wound area were taken every 10 minutes for 24 hours using Leica AF600 imaging software. All pictures were converted to a Quicktime AVI file and processed with Adobe Premier Pro (Adobe Systems, San Jose, California). The resultant video is labeled as Supplemental Video 1.

Statistical analysis

All statistical analyses were conducted using SPSS (version 16.0; IBM Corp, Armonk, New York). Wound closure assay data were analyzed by a 1-way or 2-way ANOVA, depending on the experiment, followed by the Fisher least significant difference (LSD) post hoc test. Proliferation assay data were analyzed by a 2-way ANOVA followed by an LSD post hoc test. Western blot and qPCR data were analyzed by a 1-way ANOVA followed by an LSD post hoc test. A value of P < .05 was considered significant.

Results

Effect of GnRH-(1-5) on migration

The ability of GnRH-(1-5) to regulate GN11 neuronal migration was examined using a wound closure assay. GN11 cells treated with 1 and 100 nM GnRH-(1-5) significantly (P < .05) delayed wound closure at 24- and 48-hour treatments (Figure 1A). In addition, we wanted to better visualize cell morphology and movement so we generated a time-lapse video of the wound closure assay in cells treated with VEH and 100 nM GnRH-(1-5). Again, there is a delay in wound closure with GnRH-(1-5) treatment, showing that GN11 cells seem to have a lower pro-
pensity to extend their filopodia and migrate into the wound (Supplemental Video 1). We also confirmed the effect of GnRH-(1-5) on migration using the secondary method, the Boyden chamber assay. A significant dose-dependent decrease \((P < 0.05)\) in migration across the porous membrane was observed in GN11 cells treated with 100 nM GnRH-(1-5) compared with VEH treatment (Figure 1B). Although there was a reduction in migration with 1 nM GnRH-(1-5) treatment, this difference did not reach statistical significance (Figure 1B). In addition, the inhibitory effect of GnRH-(1-5) on migration was independent of cellular proliferation as determined by the MTS-based colorimetric assay, which showed no statistical differences in growth between VEH- and GnRH-(1-5)–treated cells at 24- and 48-hour treatments (Figure 1C).

**Screening for putative GnRH-(1-5) receptors**

The primary screen of the panel of orphan human GPCRs showed that GnRH-(1-5) had increased binding activity for GPR101, GPR119, GPR148, and GPR173, whereas negligible activity was observed in cell lines expressing other GPCRs (Table 1). The cell lines used initially for the primary screen displayed high constitutive activity (DiscoveRx, personal communication) that resulted in inverse agonism for the receptors. Of the 4 receptors identified in the primary screen, GPR148 was omitted because this receptor is not present in rodents (32). Only GPR173 mRNA was present in GN11 cells (see below). Thus, GPR173 was the focus for the secondary screen. GPR173-expressing cells were treated for 15 minutes with GnRH-(1-5) (0.01-1000 nM) to construct a binding curve. In this screen, GnRH-(1-5) caused a dose-dependent increase in β-arrestin binding as measured by increased β-gal activity with an EC\(_{50}\) of 1.9 nM GnRH-(1-5) (Figure 2D).

To determine whether the GPCRs identified to bind GnRH-(1-5) in the primary screen were expressed in the GN11 cell line, we generated specific primers against each GPCR (Table 2), measured their expression by RT-PCR, and subsequently visualized them by agarose gel electrophoresis (Figure 2A), along with RNA obtained from mouse hypothalamic tissue as positive controls (Figure 2A). Interestingly, we found that only GPR173 was expressed in GN11 cells, whereas GPR101 and GPR119 were not detected (Figure 2A). No bands were observed in PCR samples in which the RNA was not treated with reverse transcriptase (−RT) (Figure 2A).

**GPR173 expression in embryonic nasal tissue**

To examine the physiological relevance of GPR173 in GnRH neuronal migration, RNA from E12.5 mouse nasal tissue and E11.5 nasal explant tissue cultured 3 days in vitro were used to detect the expression of GPR173 in addition to that of GPR101 and GPR119 (Figure 2B). These time points have been previously characterized to contain migrating GnRH neurons (30). Furthermore, we confirmed GnRH expression at both time points by RT-
Interestingly, only GPR173 mRNA was highly expressed in the nasal tissues examined but not GPR101 or GPR119 mRNAs. Effect of GPR173 silencing on GnRH-(1-5)-mediated inhibition of GN11 cellular migration

Because it was determined that GPR173 is expressed in GN11 cells and in nasal tissue containing migrating GnRH neurons, we examined whether down-regulation of GPR173 blocks the effect of GnRH-(1-5) on GN11 cellular migration. We used an siRNA-based approach to silence GPR173 expression in GN11 cells. The efficacy of GPR173 knockdown was measured by qPCR using primers that span exons 2 and 3 of the full-length GPR173 mRNA (Figure 2C) in GN11 cells treated with either the transfection reagent only (VEH), the GPR173 siRNA pool (25 nM), or a nontargeting siRNA pool (25 nM). GN11 cells treated with the GPR173 siRNA pool had an approximately 80% reduction in GPR173 mRNA levels relative to those of cells treated with VEH and with the nontargeting siRNA (Figure 2E). We also conducted a wound closure assay to examine whether GPR173 silencing blocked GnRH-(1-5) inhibition of GN11 cellular migration. Cells treated with 100 nM GnRH-(1-5) or 100 nM GnRH-(1-5) + nontargeting siRNA had significantly ($P < .05$) reduced wound closure relative to that of control cells (Figure 3). Conversely, wound closures in GN11 cells treated with 100 nM GnRH-(1-5) and GPR173 siRNA were statistically indistinguishable from those of control cells (Figure 3).

Effect of GnRH-(1-5) on STAT3 phosphorylation

Previous work has implicated the STAT3 pathway in regulation of GN11 cellular migration (14); thus, we examined whether GnRH-(1-5) alters the levels of pSTAT3. GN11 cells were treated for 5, 15, and 30 minutes with VEH or 100 nM GnRH-(1-5), and the relative levels of pSTAT3 (Y705) and STAT3 were measured by Western blot analysis. GnRH-(1-5) decreased the levels of pSTAT3 in a time-dependent manner, reaching significance at 15 minutes (Figure 4A). This effect was specific to GnRH-(1-5) because treatment with the full-length GnRH did not alter the levels of pSTAT3 (Figure 4B).

To further reinforce the significance of STAT3 signaling in GN11 cellular migration, we used a pharmacological inhibitor of STAT3, WP1066 (33). We found that WP1066 treatment decreased pSTAT3 levels (Figure 5A) without inducing apoptosis as measured by cleaved caspase 7 levels at 30-minute, 18-hour, or 24-hour treatments (Figure 5B). Furthermore, WP1066 robustly delayed wound closure at 1 μM similar to GnRH-(1-5)
treatment; however, we did see inhibition at lower concentrations (Figure 5C).

Finally, we investigated whether GPR173 mediates the GnRH-(1-5) decrease in pSTAT3 levels using siRNA to GPR173. We tested this effect using 2 methods: immunofluorescent analysis and Western blots. Our results indicate that the GnRH-(1-5)–mediated decrease in pSTAT3 levels was blocked by silencing of GPR173 expression in GN11 cells (Figure 6, A and B). This effect was specific to GPR173 because treatment with the nontargeting siRNA had no effect on the GnRH-(1-5) decrease in pSTAT3 levels (Figure 6, A and B).

**Discussion**

The present study shows that GnRH-(1-5), a metabolic product of GnRH, regulates the migration of GN11 neurons by attenuating FBS-stimulated GN11 neuronal migration. Furthermore, we have demonstrated that the orphan receptor GPR173 and the STAT3 signaling pathway mediate this inhibition. Our previous studies have shown that GnRH-(1-5) can regulate GnRH mRNA expression (8) and facilitates lordosis behavior in estrogen-primed ovariectomized rats (7). Interestingly, EP24.15 is expressed along the migratory path of GnRH-secreting neurons between the olfactory placode and CNS during development (9). Furthermore, previous studies have shown that migrating GnRH neurons secrete GnRH and that the levels of secretion increase as development progresses, reaching the picogram range (34, 35). Thus, the availability of GnRH-(1-5) mediated by EP24.15 suggests that GnRH-(1-5) may play a role in the development of the GnRH neuroendocrine system. In this study, we tested whether GnRH-(1-5) regulates the migration of GN11 neurons, a GnRH-secreting cell line used extensively to study the mechanism of GnRH neuronal migration (14, 17, 25, 36). GnRH-(1-5) treatment inhibited the ability of GN11 neurons to migrate into a wound in an in vitro wound closure assay. This effect seems to be specific to GnRH-(1-5) because treatment with the full-length GnRH had no effect on GN11 cellular migration compared with that of control cells (data not shown). We performed this type of migration assay because it is a simple experiment that has been used previously to test the motility/mobility of GN11 cells in response to a treatment (37, 38). GnRH neurons during development are exposed to a variety of signaling cues that regulate the rate of migration (2), and the proper coordination of these signals is critical for GnRH neurons to locate their destination in a timely manner dependent on the stage of development (1). Although the local physical and chemical environment of the wound closure assay used in the present study is simpler than the environment encountered by migrating GnRH neurons during development, this assay and the GN11 cell line offer an initial starting point to test the effects of GnRH-(1-5) on migration. Our results suggest that GnRH-(1-5) decreases GnRH neuronal motility, which could potentially serve to balance the effects of other factors that enhance the migration of GnRH neurons during development. This finding is more evident.
Treatment with the full-length decapeptide GnRH had no effect on the levels of pSTAT3. Bars indicate SEM (n = 4). Data were analyzed by a 1-way ANOVA followed by an LSD post hoc test. *P < .05 relative to VEH.

Figure 4. Effect of GnRH-(1-5) treatment on pSTAT3 levels. A, GN11 cells were treated for 5, 15, and 30 minutes with 100 nM GnRH-(1-5), and the relative levels of pSTAT3(Y705) and STAT3 were measured by Western blot analysis. GnRH-(1-5) treatment decreased pSTAT3 levels in a time-dependent manner, reaching significance at 15 min. B, Treatment with the full-length decapeptide GnRH had no effect on the levels of pSTAT3. Bars indicate SEM (n = 4). Data were analyzed by a 1-way ANOVA followed by an LSD post hoc test. *P < .05 relative to VEH.

from our time-lapse video demonstrating the effect of GnRH-(1-5) to inhibit radial migration of GN11 cells across the wound area. Furthermore, we used the Boyden chamber assay as an alternative assay to test the effect of GnRH-(1-5) to inhibit migration. These results mirrored our wound closure experiments in which GnRH-(1-5) inhibited the migration of GN11 cells. In both these experiments, charcoal-stripped FBS was used as a stimulator of migration, which leads us to speculate that the effect of GnRH-(1-5) on migration is complex and involves the integration of multiple signaling factors. We also found that the effect of GnRH-(1-5) is directly on migration and not proliferation because our proliferation assay results showed no differences in growth in response to GnRH-(1-5) treatment.

It is well established that many neuropeptides including the decapeptide GnRH bind and activate a GPCR to elicit their function. The orphan receptor GPR173 is a member of the small GPCR family called super conserved receptor expressed in brain, which includes the orphan receptors GPR27 and GPR85 (39). A recent report suggests that GPR27 may regulate insulin production (40), whereas GPR85 has been implicated in neurogenesis (41) and in the development of schizophrenia (42); however, as with GPR173, no known ligands have been implicated in their activation. In our study we sought to determine whether GnRH-(1-5) activated a GPCR to regulate GN11 cellular migration. In our primary screen using a panel of 74 orphan GPCRs, GnRH-(1-5) showed specific activity with GPR101, GPR119, GPR148, and GPR173. In this screen we decided to use a high dose of GnRH-(1-5) (30 μM) because of the nature of the high-throughput assay having constitutive β-arrestin recruitment activity depending on the GPCR. Therefore, GnRH-(1-5) in this system may act as an inverse agonist. Nevertheless, changes in β-arrestin recruitment indicate specific binding to the GPCR. Once it was determined that GPR173 was the only receptor expressed in GN11 cells, a secondary screen was conducted using the GPR173 cell line to induce a concentration-dependent increase in β-arrestin recruitment by GnRH-(1-5), indicating the binding specificity. We further examined whether GPR173 is required for the effect of GnRH-(1-5) on GN11 cellular migration using an siRNA-based approach to block GPR173 expression. Interestingly, we found that the GnRH-(1-5)–mediated inhibition of migration was reversed only when GPR173 expression was silenced. This finding suggests that GPR173 is the signal-transducing element for the effect of GnRH-(1-5) on migration. It is still not clear, however, whether GnRH-(1-5) is the only endogenous ligand for GPR173 because many GPCRs have been known to bind multiple ligands, leading to different biological functions. Furthermore, the other candidate receptors cannot be overlooked, and GnRH-(1-5) may play a tissue-specific role, depending on the expression of certain GPCRs. GPCR ligand promiscuity is not uncommon and such is the case with the osteocalcin-sensing receptor GPRC6A, which is a GPCR with multiple structurally dissimilar ligands (43). A more thorough analysis of the GnRH-(1-5) and GPR173 interaction is underway in addition to the biological relevance of the other candidate receptors.

Previous studies have shown that the STAT3 pathway is important for axonal growth (13) and migration in GN11 neurons (14). Therefore, to determine whether GnRH-(1-5) regulates the activation of STAT3, we measured the phosphorylation of STAT3 (pSTAT3) in GN11 neurons after treatment with GnRH-(1-5). Interestingly, GnRH-(1-5) potently reduced the levels of pSTAT3 in a time-dependent manner. This is in contrast to GN11 neurons treated with the decapeptide GnRH, which had no significant effect on pSTAT3 levels. These results suggest that the effect of migration and the inhibition of phosphorylation of STAT3 are specific to GnRH-(1-5). Furthermore, we expected GnRH-(1-5) to decrease pSTAT3 levels because a previous report by Magni et al (14) showed that GN11 cells treated with the cytokine LIF had increased pSTAT3 levels. In addition, they showed that LIF stimulated GN11 neuronal migration (14), which is in agreement with our results showing that GnRH-(1-5) inhibits the phosphorylation of STAT3 and subsequently migration. To further reinforce the link between the
STAT3 pathway and migration, we used a pharmacological inhibitor of STAT3, WP1066, which inhibits the phosphorylation of STAT3 (33). Interestingly, we found that WP1066 not only inhibited the phosphorylation of STAT3 but also delayed GN11 cellular migration in a wound healing assay, thereby mimicking the effect of GnRH-(1-5). We determined whether GPR173 mediates the effect of GnRH-(1-5) to decrease pSTAT3 levels using our siRNA-mediated approach. In accordance with the results from our migration study, silencing GPR173 expression also blocked the GnRH-(1-5)–mediated downregulation of pSTAT3 levels.

The present study indicates that GnRH-(1-5) decreases pSTAT3 levels and inhibits GN11 neuronal migration via GPR173; however, the intracellular mechanism mediated by the interaction of GnRH-(1-5) and GPR173 leading to the dephosphorylation of STAT3 remains to be determined. Previous work with the GPCR, angiotensin II type 1 receptor, showed that it can induce the phosphorylation of certain members of the STAT family including STAT3 when activated by angiotensin II (44, 45). Furthermore, it has been shown that agonist activation of angiotensin II type 1 receptor is mediated in part by the activation of certain Janus kinases and depends on Rac recruitment in smooth muscle cells (45), suggesting that some GPCRs can deviate from the canonical heterotrimeric G protein signaling pathway. However, our results indicate that GnRH-(1-5) decreases pSTAT3 levels, suggesting that GPR173 is probably not interacting with members of the Janus kinase family, which function to phosphorylate and therefore activate STAT proteins (46). In our in vitro model, treatments were administered after dilution in 10% charcoal-dextran stripped FBS to remove endogenous steroid influence; however, GN11 neurons still proliferate in this environment, albeit at a slower rate. We chose this protocol because GnRH-expressing neurons during development are exposed to a variety of factors that aid in their migration before entering the CNS (2). Using a wound closure assay, we showed that GN11 cells invade the wound area rapidly in our culture conditions; however, after GnRH-(1-5) treatment there is a delay in wound closure, suggesting that GnRH-(1-5) is probably attenuating the effect of certain cytokines/growth factors to stimulate migration. Interestingly, the cytokine LIF has been shown to enhance GN11 neuronal migration in part by up-regulating the levels of pSTAT3 (14). Furthermore, LIF expression is present early in development as GnRH neurons are migrating from the nasal placode to the CNS (14, 47); therefore, LIF, among other factors, may act to stimulate migration along the vomeronasal tract while GnRH-(1-5) via GPR173 plays a modulatory role in fine-tuning the migration of GnRH neurons before they enter the CNS through the cribriform plate (9).

Our findings demonstrate that GPR173 may contribute to the migration of GnRH neurons. Previous work characterizing the GN11 cell line offered evidence for its neuronal phenotype (16, 19); yet it is still considered a heterogeneous population of cells in which the majority are GnRH positive (16, 48). Therefore, in our model it is not clear whether GnRH-(1-5) is acting directly on GnRH neurons via GPR173. We began addressing this issue by detecting the expression of GPR173 in mouse embryonic nasal tissue from

---

**Figure 5.** Effect of WP1066, an inhibitor of STAT3 phosphorylation, on GN11 cellular migration. A, GN11 cells treated for 30 minutes with increasing doses of WP1066 had reduced pSTAT3 levels. B, Doses of WP1066 did not induce apoptosis at 30-minute, 18-hour, or 24-hour treatments because cleaved caspase-7 (Casp7) levels were not detected. C, A wound closure assay was used to determine the effect of WP1066 on migration. GN11 cells treated with increasing doses of WP1066 for 24 hours delayed wound closure with a robust effect seen at 1 μM. Scale bar corresponds to 250 μm.
E12.5 and nasal explant tissue from E11.5 cultured for 3 days in vitro. These tissues have been previously verified to contain migrating GnRH neurons (30). Interestingly, we found that GPR173 was highly expressed at both time points measured, whereas we could not detect GPR101 or GPR119 expression. These results indicate that GPR173 is present during critical periods when GnRH neurons are migrating along the vomeronasal tract and that the GnRH-(1-5) influence on their migration is plausible.

It is possible that GnRH-(1-5) could be acting in an autocrine or paracrine manner to regulate GnRH neuronal migration during development. Our results suggest an autocrine mechanism because we used GN11 cells, which have been used extensively as a model for GnRH neuronal migration. However, because of the immortalization of this cell line, the exact nature of GnRH neurons in vivo may not be reflected. Therefore, GnRH-(1-5) acting as a paracrine regulator via GPR173 to indirectly influence the migration of GnRH neurons cannot be ruled out. GnRH-(1-5) acting on neighboring cells expressing GPR173 may alter the microenvironment by regulating the availability of certain growth factors/cytokines that directly act on GnRH neurons. The selective activation of GPR173 to regulate migration is an important finding because this type of regulation may contribute to the transitioning of migrating GnRH neurons from a radial migratory trajectory to a tangential one as they are leaving the nasal region to enter the CNS. Future work will focus on determining the localization of GPR173 relative to GnRH neurons and alteration of the microenvironment in the absence of GPR173 during development.

In summary, we demonstrate that the GnRH metabolite GnRH-(1-5) regulates GN11 cell migration by acting through the intracellular STAT3 pathway. These processes are dependent on the activation of GPR173 because silencing of GPR173 expression in GN11 cells reversed any GnRH-(1-5) effect. The mechanism of GnRH-(1-5) is probably complex, involving the interaction of other growth factors that serve to stimulate migration. Further investigation is certainly warranted in elucidating the role of GnRH-(1-5) in the development of the GnRH neuroendocrine system and the role played by the orphan receptor GPR173 in the action of this metabolite.

**Acknowledgments**

We thank Dr Sally Radovick for the GN11 cell line, Dr Horacio Novaira for his technical assistance with this cell line, Dr Susan Wray for generously donating RNA from embryonic nasal tissue, Dr Mary Lou Cutler for providing the phase contrast microscope, and Ms Nina Semsarzadeh for her experimental assistance. We also wish to thank Dr Joan Witkin and Dr Robert Handa for providing constructive criticism to the manuscript.

Address all correspondence and requests for reprints to: T. John Wu, PhD, Department of Obstetrics and Gynecology, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, Maryland 20814.

This work was supported by National Science Foundation grants IOB-0544150 and IOS-1052288 (to T.J.W.) and Department of Defense grant TO85FU-01 (to D.O.L. and T.J.W.).

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

**Disclosure Summary:** The authors have nothing to disclose.
References


