Calcium receptor stimulates chemotaxis and secretion of MCP-1 in GnRH neurons in vitro: potential impact on reduced GnRH neuron population in CaR-null mice

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Chattopadhyay N, Jeong K-H, Yano S, Huang S, Pang JL, Ren X, Terwilliger E, Kaiser UB, Vassilev PM, Pollak MR, Brown EM. Calcium receptor stimulates chemotaxis and secretion of MCP-1 in GnRH neurons in vitro: potential impact on reduced GnRH neuron population in CaR-null mice. Am J Physiol Endocrinol Metab 292:E523–E532, 2007. First published September 26, 2006; doi:10.1152/ajpendo.00372.2005.—The factors controlling the migration of mammalian gonadotropin-releasing hormone (GnRH) neurons from the nasal placode to the hypothalamus are not well understood. We studied whether the extracellular calcium-sensing receptor (CaR) promotes migration/chemotaxis of GnRH neurons. We demonstrated expression of CaR in GnRH neurons in the murine basal forebrain and in two GnRH neuronal cell lines: GT1-7 (hypothalamic derived) and GN11 (olfactory bulb derived). Elevated extracellular Ca$^{2+}$ concentrations promoted chemotaxis of both cell types, with a greater effect in GN11 cells. This effect was CaR mediated, as, in both cell types, overexpression of a dominant-negative CaR attenuated high Ca$^{2+}$-stimulated chemotaxis. We also demonstrated expression of a β-chemokine, monocyte chemoattractant protein-1 (MCP-1), and its receptor, CC motif receptor-2 (CCR2), in the hypothalamic GnRH neurons as well as in GT1-7 and GN11 cells. Exogenous MCP-1 stimulated chemotaxis of both cell lines in a dose-dependent fashion; the effect was greater in GN11 than in GT1-7 cells, consistent with the higher CCR2 mRNA levels in GN11 cells. Activating the CaR stimulated MCP-1 secretion in GT1-7 but not in GN11 cells. MCP-1 secreted in response to CaR stimulation is biologically active, as conditioned medium from GT1-7 cells treated with high Ca$^{2+}$-promoted chemotaxis of GN11 cells, and this effect was partially attenuated by a neutralizing antibody to MCP-1. Finally, in the preoptic area of anterior hypothalamus, the number of GnRH neurons was ~27% lower in CaR-null mice than in mice expressing the CaR gene. We conclude that the CaR may be a novel regulator of GnRH neuronal migration likely involving, in part, MCP-1.

chemokine; G protein-coupled receptor; gonadotropin-releasing hormone; CC motif receptor-2; monocyte chemoattractant protein-1; calcium-sensing receptor

IN THE DEVELOPING BRAIN, the majority of postmitotic neurons migrate from their site of origin to their final destination (for review, see Ref. 15). In mammals, neurons synthesizing gonadotropin-releasing hormone (GnRH) display similar migratory properties, migrating from the anterior nasal compartment to their final destination scattered within the basal forebrain to become integral components of the hypothalamic-pituitary-gonadal axis (for review, see Ref. 46). Chemokines are small 8- to 10-kDa secreted proteins that act as chemoattractants for various immune and nonimmune cells, including neurons (for review, see Ref. 6). Among these chemokines, monocyte chemoattractant protein-1 (MCP-1) has been shown to promote migration of rat neural stem cells (45) and to be secreted from a human neuronal cell line (NT2.N), suggesting that neurons themselves can produce chemokines (10). MCP-1 signals via its G protein-coupled receptor, CC motif receptor-2 (CCR2), which is constitutively expressed in rat neuronal and astrocytic cells (3).

Many neuronal processes are controlled by a great diversity of Ca$^{2+}$ signals. In the neurons, Ca$^{2+}$ signals depend on Ca$^{2+}$ entry and/or Ca$^{2+}$ release from internal Ca$^{2+}$ stores. The biology of GnRH neurons is critically dependent on extracellular Ca$^{2+}$ (Ca$^{2+}$). The episodic mode of GnRH release from perfused hypothalamic cells and immortalized GnRH neurons is highly dependent on Ca$^{2+}$, which is controlled by Ca$^{2+}$ entry through plasma membrane Ca$^{2+}$ channels (25, 37). In addition, Ca$^{2+}$ has been shown to be required for initiating GnRH neuronal migration, and these events are partially dependent on N-type voltage-sensitive calcium channel signals (40).

One target of Ca$^{2+}$ is the cell surface calcium-sensing receptor (CaR), a G protein-coupled receptor that was originally cloned from the parathyroid gland (4). Following its cloning from parathyroid gland and kidney, the CaR was cloned from a rat striatal library by homology-based cloning (35). In neurons, the CaR resides in nerve endings (35). Although CaR expression has been shown in all central nervous system (CNS) cell types (for review, see Ref. 52) and in diverse regions of brain (34), the biological roles and importance of the CaR in the CNS are still unknown. Interestingly, polyamines, such as spermine and, to a lesser extent, spermidine (31), as well as amyloid-β (Aβ) (53), all of which are present in the CNS, have been shown to be CaR agonists, presumably by virtue of their positive charges. Therefore, the CNS, despite its separation from the systemic circulation by a blood-brain barrier, possesses components well suited for CaR action.
In this report, we investigated whether the CaR is expressed in GnRH neurons and participates in modulating their functions, such as chemotaxis. We first studied the expression of the CaR, MCP-1, and its receptor CCR2 in GnRH neurons in vivo and in vitro. For in vitro studies, we used olfactory-derived (GN11) (32) and hypothalamus-derived (GT1-7) (27) cells. We next determined the effect of the CaR and MCP-1 on the chemotaxis of GnRH neurons in vitro and then studied the regulation of MCP-1 secretion by the CaR in GnRH cell lines. Last, we assessed the impact of the lack of a functional CaR on the GnRH neuronal population in vivo in mice with targeted deletion of the CaR gene (22).

MATERIALS AND METHODS

Materials. All routine culture media were obtained from Invitrogen (Carlsbad, CA). The murine JE/MCP-1 enzyme-linked immunosorbent assay (ELISA) kit, mouse JE/MCP-1, and anti-MCP-1 neutralizing antibody were from R&D Systems (Minneapolis, MN); goat anti-GnRH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-GnRH antibody was from Immunostar (Hudson, WI); Alexa Fluor 488-conjugated donkey anti-goat IgG and Alexa Fluor 594-conjugated donkey anti-rabbit IgGs were from Invitrogen; Vectastain ABC kit (rabbit IgG) was from Vector Laboratories (Burlingame, CA); the RT-PCR and SYBR green quantitative PCR kits were from Qiagen (Chatsworth, CA); protease inhibitors were from Boehringer Ingelheim (Indianapolis, IN); and the enhanced chemiluminescence kit was from Pierce (Rockford, IL). We used charcoal-dextran-stripped FBS (Hyclone, Logan, UT) for various experimental conditions, as described below. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

RNA extraction and RT-PCR. GN11 and GT1-7 cells were maintained in DMEM with 10% FBS. Aliquots of 2 μg of total RNA from 80% confluent cultures of both cell lines were subjected to one-step RT-PCR as described before (9). Primer pair for murine CaR (accession no.: NM_13803) was 5'-TGCAAGACGGGCGAAAA-3' (sense, nt. 1603–1619) and 5'-CCAATTCCTTGAACAC-AATGG-3' (antisense, nt. 483–503). The optimal temperature cycling protocol was 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s in 35 cycles. Bidirectional sequencing of the PCR products was performed employing the same primer pair as described above.

Quantitative real-time PCR. SYBR green chemistry was used to measure the mRNAs of MCP-1, CCR2, and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (to normalize differences in RNA isolation and the efficiencies of the RT), following a previously optimized protocol (39). The sequences of the primers were as follows: MCP-1/CCL2 (accession no.: NM_011333), 5'-TGCAATCTGCCCTAAGTCTCTC-3' (sense, nt. 403–423), 5'-AAGTGCTTG-AGTTGGTGTTGG-3' (antisense, nt. 438–503); CCR2 (accession no.: NM_009915), 5'-GAAGAGGACGT-GGATTACC-3' (sense, nt. 60–82), 5'-TGATGGCGTGAGATGAACTGAGG-3' (antisense, nt. 155–175); and mouse GAPDH, 5'-TCTAATGAGGATGACTAGC-3' (sense), 5'-TACACCCATTGTAGGTACGC-3' (antisense). The temperature profile of the reaction was 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The size of the PCR product was first verified on a 1.5% agarose gel, followed by melting-curve analysis.

Immunocytochemistry. Immunocytochemical studies were performed following an optimized protocol (9, 19, 20). Cells cultured on coverslips for 3 days were fixed with 4% formaldehyde. After blocking of endogenous peroxidase, cells were incubated with an affinity-purified rabbit anti-CaR antibody that was diluted 1:250 from a 5-μg/ml stock. For negative controls, cells were incubated with the same antibody after preabsorption with 10 μg/ml of the specific peptide to which it was raised; a 1:500 dilution of anti-rabbit second-ary antibody conjugated with horseradish peroxidase (HRP) was used for color development with the 3-aminoo-9-ethyl-carbazole substrate system (DAKO, Carpinteria, CA).

Determination of MCP-1 in cell culture supernatants. GN11 and GT1-7 cells were grown to 70–75% confluence in 24-well plates. This medium was replaced with Ca2+-free DMEM (reconstituted with 0.5 mM Ca2+ (see figure legends for details). The effects of elevated Ca2+ were studied by culturing the cells overnight in medium containing 0.5 mM Ca2+ with 1% FBS (charcoal-dextran stripped and heat inactivated). MCP-1 in the conditioned medium was measured by ELISA (R&D Systems). Protein concentrations were measured with the Micro BCA protein kit (Pierce). Data are expressed as picograms per milliliter per milligram protein (pg·ml⁻¹·mg protein⁻¹).

Gene delivery by recombinant adeno-associated virus. A recombinant adeno-associated virus (rAAV)-based gene delivery method was used, following a previously published protocol (9, 38, 39). Dominant-negative human CaR (R185Q) (1, 2) and the same vector containing the cDNA for the control β-galactosidase protein (referred to hereafter as BG) were under the control of a cytomegalovirus immediate-early (CMV-IE) promoter element (47). Cells at 60–70% confluency were seeded in either 24- or 6-well plates for assay of MCP-1 secretion and chemotaxis, respectively. For the 24-well plates, cells were cultured overnight in 0.25 ml of growth medium; for the 6-well plates, 0.8 ml of medium was used. Each well was infected with ~1,000 virus particles/cell as described before (9, 38, 39). Experiments with low or high calcium concentrations were performed as described below.

Determination of chemotactic activity with a chemotaxis bioassay. Chemotactic activity was determined in a Blind Well Boyden chamber system (Neuroprobe, Gaithersburg, MD) with a polivinlypyrrolidone-free polycarbonate membrane (Neuroprobe) as described previously (48, 49, 52). GN11 cells were cultured until they reached 70–80% confluency, and GT1-7 cells were grown until 40–50% confluency. This difference in confluency was the result of the rapid and isolated mode of growth of GN11 cells, in contrast to the slow, colonized growth of GT1-7 cells. Before the assay, the cells were serum-starved overnight in medium containing 0.5 mM Ca2+ with 1% FBS (charcoal-dextran stripped and heat inactivated). Then, increasing concentrations of Ca2+ in the same medium were freshly prepared and loaded into the lower chamber, which was separated from the upper well by a 5-mm membrane with 8-μm pore size; 100-μl aliquots from 1 × 105 cells/ml were added to the upper chamber and incubated at 37°C for 5 h in a CO2 incubator. The membranes were fixed with methanol and stained with Giemsa. Stained cells on the membranes were counted in six high-power fields (×400). The data were normalized as the fold increase in cellular chemotaxis relative to that in the control.

To determine whether MCP-1 secreted from GT1-7 cells on stimulation by high Ca2+ induced chemotaxis of GN11 cells, we used a previously described protocol following minor modifications (52). We first incubated GT1-7 cells with 0.5 or 3.5 mM Ca2+ as described above; 200 μl of the conditioned medium were incubated with 0.5 μg/ml of either anti-mouse MCP-1 neutralizing antibody or control mouse IgG at room temperature for 2 h. One hundred microliters of these media were used to test chemotaxis of GN11 cells as described above.

In vivo studies. All mice were maintained on a 12:12-h light-dark cycle in a normal pathogen-free environment. All experiments were approved by the standing committee on animals in the Harvard Medical School Center for Animal Resources and Comparative Medicine. For localizing CaR, MCP-1, and CCR2 in GnRH neurons, 4- to 5-wk-old female 129/svj mice were used. To study the effect of deletion of CaR gene on GnRH neuronal populations, mice heterozygous for the CaR mutation with the parathyroid hormone (PTH)-null background (CaR+/−;PTH−/−) were bred to generate CaR+/−;PTH−/− and CaR−/−;PTH−/− mice and genotyped as de-
scribed before (22). Because these mice lack the PTH gene, normocalcemia was maintained by giving 1% CaCl₂ in drinking water.

Tissue preparation and sectioning. For immunofluorescence and immunoperoxidase histochemistry, mice were anesthetized, and tissues were fixed by transcardial perfusion with 4% formaldehyde and 0.2% picric acid. Brain samples were embedded in OCT (Miles, Elkhart, IN) after cryoprotection in 25% sucrose at 4°C; 30-μm sagittal sections were made through the preoptic area (POA) of the anterior hypothalamus using a Zeiss HM 505 E cryostat. Floating sections were used for all experiments with the exception of CaR immunofluorescence, for which the sections were mounted on slides.

Immunofluorescence histochemistry. Two-color immunofluorescence was used to localize CaR in GnRH neurons. Polyclonal antibodies against the CaR (rabbit) or GnRH (goat) were used. For the CaR immunofluorescence, antigen retrieval was performed as described before (7, 8, 33). Autofluorescence was blocked by incubation of the slides with 0.1% glycine-PBS solution for 20 min at room temperature. Sections were incubated with CaR (1:200 dilution) and GnRH antibodies (1:1,000 dilution) in a buffer containing PBS, 0.5% BSA, and 0.1% Triton X-100 at 4°C overnight. Alexa Fluor 488-conjugated donkey anti-goat and Alexa Fluor 546-conjugated goat anti-rabbit IgGs (both 1:200 dilution) were used as secondary antibodies that were applied to sections for 1 h at room temperature. For localizing MCP-1 and CCR2 in GnRH neurons, the staining procedure was essentially the same as that described above except for the primary and secondary antibodies used. For MCP-1 and GnRH colocalization, primary antibodies were goat anti-MCP-1 antibody (1:500 dilution) and rabbit anti-GnRH antibody (1:2,000 dilution) (Immunostar), and secondary antibodies were Alexa Fluor 488-conjugated donkey anti-goat and Alexa Fluor 546-conjugated goat anti-rabbit IgGs (both 1:200 dilution).

Immunoperoxidase histochemistry. For comparison of the number of GnRH-positive cells in the POA, 4- to 5-wk-old female CaR+/−;PTH−/− and CaR−/−;PTH−/− mice (4 in each group) were subjected to procedures for fixing and harvesting brains as described above. All sections through basal forebrain were collected and stained by the floating method using anti-rabbit GnRH antibody (1:2,000 dilution). GnRH neurons were visualized by the avidin-biotin-peroxidase complex (ABC) method using the Vectastain ABC kit (rabbit IgG). Diaminobenzidine tetrahydrochloride was used as the chromogen. Only the cell bodies stained with GnRH antibody were counted. Two researchers blinded to the identities of the samples counted the GnRH-positive neurons from all the sections encompassing the POA in the anterior hypothalamus.

Statistics. Data are presented as means ± SE of the indicated number of experiments. Data were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison test or Student’s t-test when appropriate. A P value < 0.05 was taken to indicate a statistically significant difference.

RESULTS

Expression of CaR in GnRH neurons in vitro and in vivo. CaR mRNA expression in GN11 and GT1-7 cells was demonstrated by RT-PCR (Fig. 1A). Sequencing of the PCR-amplified product was 100% identical to the corresponding
segment of the mouse CaR cDNA, suggesting that it was derived from a bona fide CaR mRNA.

The two cell types showed positive immunoreactivity with the CaR antibody (Fig. 1B). Preincubation of the antibody with the peptide to which it was raised eliminated the staining (Fig. 1B), suggesting the specificity of the immunostaining.

We next studied the expression of the CaR in GnRH neurons in the mouse POA. Figure 2 shows that the CaR antibody-labeled neurons (red, Fig. 2A) appeared to also be labeled for GnRH (green, Fig. 2B) using a goat polyclonal antibody. CaR staining was on the membrane as well as intracellular. Merging of the images in Fig. 2, A and B, confirmed that the same cells that were positive for GnRH also expressed the CaR (merged, Fig. 2C). Although CaR expression was also observed in various other cells (data not shown), we selected the field positive for GnRH neurons for localizing CaR in these neurons.

Expression of MCP-1 and CCR2 in GnRH neurons in vitro and in vivo. Comparison of MCP-1 mRNA levels by quantitative real-time PCR (QPCR) revealed significantly higher mRNA levels in GN11 than GT1-7 cells (Fig. 3). However, the depolarizing condition (30 mM KCl) stimulated MCP-1 secretion by twofold in GT1-7 cells but had no effect on GN11 cells (Fig. 3). In addition, both GT1-7 and GN11 cells expressed CCR2 mRNA (MCP-1 receptor); however, GN11 cells had 7.2 ± 2.4-fold (P < 0.05) higher CCR2 mRNA levels than GT1-7 cells.

We next studied expression of MCP-1 and CCR2 in hypothalamic GnRH neurons. Using a goat polyclonal antibody to MCP-1 and a rabbit antibody to GnRH, we found MCP-1 expression in GnRH-positive neurons in the POA (Fig. 4A). Neurons exhibiting MCP-1 immunofluorescent staining (green) were also stained for GnRH (red), and the staining for MCP-1 and GnRH was colocalized when images were merged. Although MCP-1 expression was also observed in various regions of the brain (data not shown), we selected the field positive for GnRH neurons for localizing MCP-1 in these neurons.

Fig. 2. Immunofluorescent localization of CaR in GnRH neurons. Thirty-micrometer cryostat sections from the preoptic area (POA) of the anterior hypothalamus of mice were subjected to antigen retrieval and stained for CaR and GnRH as described in MATERIALS AND METHODS. A section labeled with the CaR antibody (red filter) was photographed (A), and the same field, when photographed under a green filter, detected GnRH-positive cells (B). Merging the red and green colors in the same field shows the localization of the CaR in GnRH-positive cells (C). Preincubating the primary antibody with the peptide to which the antibody was raised eliminated the CaR staining (D). Scale bar, 30 μm.

Fig. 3. GN11 cells secrete ~3 times more monocyte chemoattractant protein-1 (MCP-1) than do GT1-7 cells cultured in DMEM containing 0.5% FBS (basal medium, BM). Culturing the cells in BM + 30 mM KCl [depolarizing condition (DC)] increased secretion of MCP-1 from GT1-7 but not from GN11 cells. ** > *; P < 0.05.
Likewise, CCR2 expression was observed in GnRH neurons. CCR2 expression was seen in GnRH-positive neurons in the POA (Fig. 4B). A goat polyclonal antibody to CCR2 and a rabbit antibody to GnRH revealed CCR2 immunofluorescent staining of neurons (green) that also stained for GnRH (red), and staining for MCP-1 and GnRH was colocalized when the images were merged.

**High Ca\(^{2+}\), acting via the CaR, induces chemotaxis of GN11 and GT1-7 cells.** In pilot studies, elevating Ca\(^{2+}\) significantly induced chemotaxis of both GN11 and GT1-7 cells in a Boyden chamber chemotaxis assay (data not shown). Adenovirus-mediated infection of the other cells with a naturally occurring dominant-negative CaR (R185Q) (1) attenuated and right-shifted high Ca\(^{2+}\) concentration stimulation of other biological effects (9, 36, 38, 39), which served as evidence for mediation of this action by the CaR. To determine whether the effect of elevated Ca\(^{2+}\) on the chemotaxis of these cell lines was mediated by the CaR, we overexpressed R185Q (DNCaR) (1, 2) and BG by the rAAV method as described in MATERIALS AND METHODS. Figure 5A shows that, in BG-transfected GN11 cells, chemotaxis was increased by 81 ± 7 (P < 0.05) and 183 ± 8% (P < 0.05) at 3.5 and 7.5 mM Ca\(^{2+}\), respectively, compared with 0.5 mM Ca\(^{2+}\). DNCaR-transfected GN11 cells failed to show any significant stimulation of chemotaxis at 3.5 mM Ca\(^{2+}\) compared with 0.5 mM Ca\(^{2+}\).

Similarly, Fig. 5B shows that, in BG-transfected GT1-7 cells, chemotaxis was increased by 38 ± 5 (P < 0.05) and 77 ± 11% (P < 0.05) at 3.5 and 7.5 mM Ca\(^{2+}\), respectively, compared with 0.5 mM Ca\(^{2+}\). DNCaR-transfected GT1-7 cells failed to show any significant stimulation of chemotaxis at 3.5 mM Ca\(^{2+}\) compared with 0.5 mM Ca\(^{2+}\). We therefore conclude that the CaR mediates the effect of high Ca\(^{2+}\) on the chemotaxis of these cell lines. Significant increases in chemotaxis of DNCaR-transfected GN11 and GT1-7 cells at 7.5 mM Ca\(^{2+}\) compared with 0.5 mM Ca\(^{2+}\), albeit to a much lesser extent than those observed with BG-transfected cells, are consistent with the right-shifted response to elevated Ca\(^{2+}\) reported for CaR-mediated biological functions with other cell types (36, 38).

Fig. 4. Immunofluorescent localization of MCP-1 and CC motif receptor-2 (CCR2) in GnRH neurons. Thirty-micrometer cryostat sections from the POA of the anterior hypothalamus of mice were stained for MCP-1, CCR2, and GnRH as described in MATERIALS AND METHODS. A: localization of MCP-1 in GnRH neurons. From left to right: hypothalamic neurons showing labeling with MCP-1 antibody (green filter); the same field photographed under a red filter for detecting GnRH-expressing cells; green and red color merged in the same field showing localization of the MCP-1 in GnRH-positive cells. B: localization of CCR2 in GnRH neurons. From left to right: hypothalamic neurons showing labeling with CCR2 antibody (green filter); the same field photographed under a red filter for detecting GnRH-positive cells; green and red color merged in the same field showing localization of the CCR2 in GnRH-positive cells. Scale bar, 50 μm.

Fig. 5. CaR activation promotes chemotaxis of GnRH cell lines. A: elevated Ca\(^{2+}\) stimulated chemotaxis of GN11 cells transfected with β-galactosidase (BG, vector control); chemotaxis was significantly attenuated when cells were transfected with DNCaR. ** > * > # > !; P < 0.05. B: elevated Ca\(^{2+}\) stimulated chemotaxis of GT1-7 cells transfected with BG; chemotaxis was significantly attenuated when cells were transfected with DNCaR. ** > * > #; P < 0.05. Cells that migrated to the side of the membrane on which CaCl\(_2\) had been added 5 h earlier were counted as described in MATERIALS AND METHODS. Data are pooled from 4 independent experiments.
MCP-1 stimulates chemotaxis of GN11 and GT1-7 cells. Because MCP-1 is known to induce chemotaxis of various cells and GnRH neurons express the MCP-1 receptor CCR2, we next investigated the effect of exogenous MCP-1 on the chemotaxis of GN11 and GT1-7 cells. Figure 6 shows that both GN11 and GT1-7 cells exhibited chemotaxis in response to exogenous MCP-1, and GN11 cells were more responsive than GT1-7 cells.

High Ca\(^{2+}\) stimulates MCP-1 secretion in GT1-7 cells via the CaR. We investigated the effect of elevated Ca\(^{2+}\) on the production of MCP-1 by GT1-7 and GN11 cells. Elevated Ca\(^{2+}\) increased MCP-1 secretion in GT1-7 cells in a concentration-dependent fashion, with an EC\(_{50}\) = 3.0 mM (Fig. 7A). Spermine, a physiological, polycationic agonist of the CaR (30) that is abundant in the CNS, also stimulated MCP-1 production at 100 and 200 \(\mu\)M in the presence of 0.5 mM Ca\(^{2+}\). High Ca\(^{2+}\) (3.5 mM) also increased mRNA levels of MCP-1 in GT1-7 cells by 3.2 ± 0.65-fold (\(P < 0.05\)) compared with low Ca\(^{2+}\) (0.5 mM). Elevated Ca\(^{2+}\) had no effect on the synthesis and secretion of MCP-1 in GN11 cells (data not shown).

We next asked whether high Ca\(^{2+}\)-induced MCP-1 production in GT1-7 cells is CaR mediated. Using the same approach we used to demonstrate the involvement of the CaR in promoting chemotaxis, we showed that overexpressing the DNCaR in GT1-7 cells abolished the stimulation of MCP-1 production by high Ca\(^{2+}\) (Fig. 7B).

CaR-stimulated MCP-1 secretion from GT1-7 cells is biologically active. We next asked whether the increased secretion of immunoreactive MCP-1 in response to elevated Ca\(^{2+}\) is capable of stimulating chemotaxis of GN11 cells expressing CCR2. We collected conditioned medium from GT1-7 cells incubated overnight with 0.5 or 3.5 mM Ca\(^{2+}\). Before the assay, Ca\(^{2+}\) concentration of the conditioned medium from cells incubated with 0.5 mM Ca\(^{2+}\) was adjusted to 3.5 mM Ca\(^{2+}\) (see MATERIALS AND METHODS). Normalizing the Ca\(^{2+}\) concentration to 3.5 mM in these experiments ensured that all changes in chemotaxis would be due to factors in the conditioned medium, e.g., MCP-1, rather than the Ca\(^{2+}\) concentration per se. In the pilot experiment, we observed that chemotaxis of GN11 cells in response to the conditioned medium obtained from GT1-7 cells treated with 3.5 mM Ca\(^{2+}\), when incubated with 0.25–0.5 \(\mu\)g/ml neutralizing antibody against MCP-1, was inhibited by 18 and 45%, respectively (data not shown). We did not see any further inhibition of GN11 cell chemotaxis by increasing neutralizing antibody concentration from 0.5 to 1.0 \(\mu\)g/ml (data not shown). Figure 8 shows that conditioned medium from GT1-7 cells treated with 3.5 mM Ca\(^{2+}\) containing 0.5 \(\mu\)g/ml mouse IgG1 increased the chemotaxis of GN11 cells more than twofold compared with conditioned medium from GT1-7 cells incubated with 0.5 mM Ca\(^{2+}\) containing the same amount of IgG1. The chemotaxis of GN11 cells in response to the conditioned medium obtained from GT1-7 cells treated with 3.5 mM Ca\(^{2+}\) incubated with a neutralizing antibody (0.5 \(\mu\)g/ml) to MCP-1 was reduced by ~45% compared with similarly obtained conditioned medium incubated with IgG1 (Fig. 8). These results suggest that the stimulation of chemotaxis by the conditioned medium was caused, in part, by the MCP-1 synthesized and secreted by the GT1-7 cells following CaR activation.

GnRH neurons are fewer in CaR gene-deficient mice. We next investigated the effect of deletion of the CaR gene on the number of GnRH neurons in the hypothalamus. Since CaR\(^{−−}\);PTH\(^{−/−}\) mice suffer from severe hyperparathyroidism and hypercalcemia (17), factors that, potentially, could interfere with the interpretation of results from studies of these animals, we used a “rescued” form of mice with a
the CaR is expressed in mouse GnRH neurons in vivo. Whether the CaR is expressed in the migrating population of GnRH neurons during early development is unknown at this stage. However, comparable levels of CaR expression in the premigratory GN11 and the postmigratory GT1-7 cells suggest that the CaR is also expressed in the migrating GnRH neurons in the nasal placode.

Accurate migration of GnRH neurons from their site of origin in the nasal placode to their final destination in the anterior hypothalamic POA is an indispensable, but poorly understood, event for mammalian reproduction. High concentrations of Ca$_{2+}^+$ and type I CaR agonists (neomycin) have previously been shown to promote chemotaxis of mouse osteoblastic (MC3T3-E1) and monocyte/macrophage (J774) cell lines (48–50). Whether high Ca$_{2+}^+$-stimulated chemotaxis of these cells was indeed CaR mediated remains conjectural. Here, we unequivocally demonstrated the involvement of CaR in cellular chemotaxis, as elevated Ca$_{2+}^+$-stimulated migration of GN11 and GT1-7 cells is significantly attenuated by DNCaR in these cells. Our result therefore suggest that, in addition to an N-type voltage-sensitive calcium channel mediating the effects of Ca$_{2+}^+$ in the migration of GnRH neurons (40), CaR too could mediate the chemotaxic effect of high Ca$_{2+}^+$ in GnRH neurons, at least in vitro. Although the CaR promoted migration of both of these cell lines, its effect was more pronounced in GN11 than GT1-7 cells. Higher chemotactic responsiveness of GN cells compared with GT1 cells to other stimuli, such as FBS and hepatocyte growth factor, has been noted previously (12, 26).

Chemokines provide migratory signals to diverse immune and nonimmune cells. Neural stem cells express CCR2 and migrate in response to stimulation with MCP-1 (18, 41). In vivo, early GnRH neurons resemble neural stem cells in that they express nestin (a stem cell marker) (23), as do GN11 but not GT1-7 cells (30). In addition, a high level of CCR2 expression has been observed in the POA of the anterior hypothalamus (3), although the neurohormonal phenotype of these CCR2-expressing neurons was not determined. We demonstrated that GnRH-positive neurons in the POA express CCR2 as well as MCP-1. Consistent with our in vivo results, the mRNAs encoding MCP-1 and CCR2 were readily detectable in both GN11 and GT1-7 cells. Our observation of substantially higher levels of both MCP-1 and CCR2 in GN11 cells than in GT1-7 cells is of interest. Whether this difference is reflected in migrating GnRH neurons during development is currently unknown.

**DISCUSSION**

In this report, we demonstrate the physical presence and functional roles of the CaR in GnRH neurons. Both nucleotide- and antibody-based detection methods revealed CaR expression in two GnRH neuronal cell lines. Colocalization of the CaR in GnRH-positive cells of the POA in the anterior hypothalamus by the antibody-based approach demonstrated that CaR$^{-/-}$;PTH$^{-/-}$ genotype in which the severity of the CaR$^{-/-}$ phenotype is reduced (22). Four- to five-week-old CaR$^{-/-}$;PTH$^{-/-}$ female mice were compared with age- and sex-matched CaR$^{+/+}$;PTH$^{-/-}$ mice. Representative staining for GnRH neurons in the POA revealed a reduced number of GnRH neurons in CaR$^{-/-}$;PTH$^{-/-}$ mice compared with CaR$^{+/+}$;PTH$^{-/-}$ mice (Fig. 9). Neurons having cell bodies with axonal processes were counted (white arrows; Fig. 9, A and B). Counting of those GnRH-positive neurons in the POA (see MATERIALS AND METHODS) revealed 27 ± 4% fewer GnRH neurons in CaR$^{-/-}$;PTH$^{-/-}$ mice compared with CaR$^{+/+}$;PTH$^{-/-}$ mice ($P < 0.05$; Fig. 9).

**Fig. 9.** CaR-null mice have a reduced population of anterior hypothalamic GnRH neurons. Representative sections showing GnRH neurons from the POA of the anterior hypothalamus of the brain of CaR$^{+/+}$;PTH$^{-/-}$ (A) and CaR$^{-/-}$;PTH$^{-/-}$ (B) mice stained with GnRH antibody as described in MATERIALS AND METHODS. Pooled data from 4 mice in each group showing that CaR$^{+/+}$;PTH$^{-/-}$ mice had 27 ± 4% fewer GnRH neurons than did CaR$^{-/-}$;PTH$^{-/-}$ mice. $P < 0.05$. AJP-Endocrinol Metab • VOL 292 • FEBRUARY 2007 • www.ajpendo.org
That CCR2 expressed in GnRH neuronal cells is functional was demonstrated by the exogenous addition of MCP-1, which stimulated chemotaxis of GnRH cell lines. GN11 cells exhibited a more vigorous response to MCP-1 than GT1-7 cells. To our knowledge, this is the first demonstration of the involvement of a chemokine in the chemotaxis of GnRH neuronal cells. Considering MCP-1-stimulated chemotaxis to be a bioassay for CCR2, our findings are consistent with the increased levels of CCR2 mRNA expression in GN11 cells compared with GT1-7 cells. Expression of both MCP-1 and CCR2 in GnRH neurons suggests that the action of MCP-1 is autocrine; however, our data showing stimulation of chemotaxis of GnRH cell lines in response to exogenous MCP-1 also indicate a possible paracrine mode of action of MCP-1.

Because MCP-1 is expressed in GnRH neurons in vivo and is copiously secreted by the two cell lines studied here, we next addressed the regulation of its secretion in these GnRH cell lines. Despite the higher levels of secretion of MCP-1 by GN11 cells, exposing these cells to high extracellular K⁺-evoked depolarization (30 mM KCl) did not alter MCP-1 secretion, whereas such depolarization markedly stimulated GT1-7 cells. Depolarizing conditions are known to stimulate GnRH secretion from GT1-7 but not GN11 cells (30). Similarly, CaR activation preferentially stimulated MCP-1 secretion from GT1-7 cells. Lack of regulated secretion of MCP-1 in GN11 cells, in contrast to GT1-7 cells, may be a function of cell maturation (30).

In addition to elevated Ca²⁺, another physiological CaR agonist, spermine (31), which is available in the CNS, likewise stimulated MCP-1 secretion from GT1-7 cells, providing further support to the physiological importance of the CaR in these cells. To our knowledge, the role of the CaR in regulating chemokine secretion has not been described previously. Recently, we showed that elevated Ca²⁺ stimulates RANTES (CCL5regulated on activation, normal T cell expressed and secreted) secretion in osteoblasts and osteoclasts (52), but the role of the CaR was not assessed. Although CaR-stimulated MCP-1 secretion in GT1-7 cells could be transcriptional, since elevated Ca²⁺ increased mRNA levels of MCP-1 mRNA, other signaling mechanisms might underlie the stimulation of MCP-1 secretion by the CaR. This question warrants investigation.

Because the CaR stimulates MCP-1 secretion from GT1-7 cells, we next studied whether the stimulated release of MCP-1 is biologically active. Because GN11 cells exhibit more functional CCR2 than do GT1-7, we used GN11 cells to assess the biological activity of CaR-stimulated MCP-1 production by GT1-7 cells and found that MCP-1 secreted by GT1-7 cells on CaR activation promotes migration of GN11 cells. We obtained definitive evidence that the MCP-1 secreted by the GT1-7 cells following CaR stimulation was capable of promoting chemotaxis of GN11 cells by showing that the migratory response to the conditioned medium was attenuated (45% inhibition over control) by a neutralizing antibody to MCP-1. The observed inability of neutralizing antibody to completely block the migration of the GN11 cells might result from upregulation of other chemokines such as CCL7/MCP-3, CXCL-9/monokine induced by interferon-γ), and CXCL-10/interferon-γ-inducing protein-10 (IP-10) by elevated Ca²⁺ in these cells (2a).

Thus far our data have revealed a chemoattractive role for the CaR in addition to its stimulatory effect on the secretion of a chemoattractant, MCP-1, in a GnRH neuronal cell line in vitro. Therefore, lack of the CaR gene might negatively impact the GnRH neuronal population in vivo, likely by affecting their migration. To examine this possibility, we used CaR-deficient mice with a CaR−/−;PTH−/− genotype (22) and compared them with mice with a CaR+/+;PTH−/− genotype to circumvent the problem of the severe hyperparathyroidism and consequent hypercalcemia associated with the CaR−/−;PTH−/+ genotype, resulting in neonatal death (17); 1% CaCl₂ in drinking water maintained normocalcemia in both genotypes. The number of GnRH neurons in the POA of the anterior hypothalamus was 27% lower in the female CaR−/−;PTH−/− mice than in their CaR+/+;PTH−/− counterparts, clearly suggesting that the lack of functional CaR affects the size of the GnRH neuronal population in the adult mice. Whether this effect is due solely to the impact on GnRH neurons of losing the chemoattractive role of the CaR and/or its role in stimulating MCP-1 secretion or to additional mechanisms is currently unknown. For example, the CaR might have a neurotrophic effect on GnRH neurons, whose absence could affect differentiation and/or cellular survival of the GnRH neurons during early development and thus result in fewer GnRH neurons in CaR−/−;PTH−/+ mice. Further studies are required to investigate this possibility.

Despite the loss of 27% of GnRH neurons in the POA of the anterior hypothalamus, CaR−/−;PTH−/− female mice exhibited normal or near-normal fertility. This observation is not unexpected, considering the biological function served by the GnRH neurons that is crucial to species survival. Notable in this regard are findings in hypogonadal (hpq) mice, which are unable to synthesize the mature GnRH peptide because of a deletion in the GnRH gene. The fertility of these mice was restored by the successful transplantation of just one to three detectable GnRH neurons (13, 14, 24), revealing a high degree of redundancy in the number of GnRH neurons. Thus, although reduced, the number of GnRH neurons in CaR−/− mice was clearly sufficient to maintain reproduction under laboratory conditions. However, it is important to point out that, although these animals reproduced, the level of reproductive activity could be compromised compared with controls. To that end, careful study of reproductive parameters, such as age of attaining puberty (as assessed by vaginal opening time and size), duration of estrous cycle, reduction in the size of the first litter, and early reproductive senescence, could shed light on the presumably subtle impact of a reduced GnRH neuronal population in CaR−/− mice, as recently observed in a mouse model where a dominant-negative fibroblast growth factor receptor was targeted to GnRH neurons (42). Future studies can address these issues.

We conclude that, in GnRH neurons, the CaR promotes chemotaxis; stimulates secretion of MCP-1, the chemokine that promotes chemotaxis of GnRH neurons in vitro; and is involved in the maintenance of the GnRH neuronal population at the level of the intact organism.
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