



Chemokine CXCL14-like immunoreactivity in the α MSH-producing cells and PRL-producing cells of the flat-tailed house gecko pituitary

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ABSTRACT. The distribution pattern of chemokine CXCL14-immunoreactive cells was examined by immunohistochemistry in the pituitary of the gecko *Hemidactylus platyurus*. Immunoreactive cells were observed in the pars intermedia and pars distalis of the pituitary, but not in the pars nervosa. All α -melanocyte-stimulating hormone (α MSH)-producing cells were immunoreactive for CXCL14 in the pars intermedia. The CXCL14-immunoreactive cells corresponded to prolactin (PRL)-producing cells but not to other adenohipophyseal-hormone-producing cells in the pars distalis. CXCL14 secreted from α MSH-producing cells and PRL-producing cells may regulate insulin release from β cells in the pancreatic islets as well as glucose uptake in the muscle cells together with α MSH and/or PRL. In addition, secreted CXCL14 with α MSH and/or PRL may act as a bioactive factor regulating hormone release in the adenohipophyseal cells of the reptilian pars distalis.

KEY WORDS: CXCL14, gecko, pituitary, reptile

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Chemokines are bioactive peptides that indicate chemotactic activity for leukocytes and lymphocytes. Their molecular weight is in the range of 8–14 kDa. With respect to the amino acid residue in the N-terminal region, chemokines can be classified into four subfamilies: C, CC, CXC, and CX3C [6]. The CXC subfamily is characterized by two cysteine residues interposing a single amino acid residue in the N-terminal region; 17 members, CXCL1 to CXCL17, have been identified so far [41]. These CXCLs are classified into two groups, the ELR⁺ and ELR⁻ subclasses, based on whether they have or lack the Glu-Leu-Arg sequence at the N-terminal region [13]. Generally, ELR⁺ chemokines are potent promoters of angiogenesis, whereas ELR⁻ chemokines are potent inhibitors of angiogenesis [29]. CXCL14 is a member of the ELR⁻ chemokines, independently isolated as BRAK [14], BMAC [27], or MIP-2 γ [5] from human tumor cells. A transwell migration assay demonstrated that CXCL14 significantly stimulates the migration of human blood monocytes [19] and natural killer cells [28]. Unlike other chemokines, the CXCL14-like substance is localized in a wide range of nervous tissues and endocrine cells, suggesting that it acts as a neuromodulator and/or hormone [1, 30, 33, 40]. Identification of CXCL14 cDNA in fish [15] suggests that this chemokine is widely conserved not only in mammals but also in other vertebrates. Distribution of CXCL14-like substance has been reported in the amphibian pituitary gland [32]. Because mammals, birds, and modern reptiles all evolved from early reptile stock, reptiles occupy a central position in the evolution of tetrapods [38]. Although the hypothalamo–adenohipophyseal systems of modern reptiles vary, reflecting the phylogenetic position of the reptile groups, understanding the distribution of CXCL14 in the reptilian pituitary is essential for understanding phylogenetic changes in the functions of CXCL14, as in the case of proopiomelanocortin [35]. Among reptiles, the geckos are one of representative groups belonging to the order Squamata, which we and others have utilized as an experimental animal because they are easy to obtain, keep, and breed [7, 16, 17, 31]. We believe that this is the first study that examines distribution of the CXCL14-like substance in the pituitary of a reptile, the flat-tailed house gecko (*Hemidactylus platyurus*).

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Care and Use Committee for University of Teacher Education Fukuoka.

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Table 1. Primary antibodies

Antigen	Host	Source and code no.	Optimal dilution
CXCL14	Rabbit	PeptoTech, Rocky Hill, NJ, USA; 500-P237	0.5 $\mu\text{g/ml}$
αMSH	Sheep	Millipore, Temecula, CA, USA; AB5087	1:10,000
PRL	Goat	R&D Systems, Minneapolis, MN, USA; AF1445	2 $\mu\text{g/ml}$
ACTH	Sheep	Fitzgerald Industries International, Acton, MA, USA; 20-AS01	5 $\mu\text{g/ml}$
GH	Goat	R&D Systems, Minneapolis, MN, USA; AF1566	2 $\mu\text{g/ml}$
LH	Goat	UCB-Bioproducts, Brussels, Belgium; A506/G4H	1:5,000
TSH	Goat	UCB-Bioproducts, Brussels, Belgium; A503/G4H	1:5,000

αMSH , α -melanocyte-stimulating hormone; PRL, prolactin; ACTH, adrenocorticotropic hormone; GH, growth hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone.

Five male and five female *H. platyurus* (body length, 10.5–11.5 cm) were purchased from local dealers in Osaka Prefecture, Japan, in July and December 2019. They were kept in cages at 24°C for more than 1 week under a 12-hr (06:00–18:00) light/12-hr (18:00–06:00) dark cycle and fed with cricket larvae. They were anesthetized with an isoflurane inhalation solution (Pfizer, New York, NY, USA) until their active movement disappeared, then deep anesthesia was induced by intraperitoneal injection of pentobarbital sodium (35 mg/kg) (Kyoritsu Seiyaku, Tokyo, Japan). The heart was dissected and transcardially perfused with 0.9% NaCl, followed by 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB, pH 6.9). The pituitary and brain, along with the skull, were rapidly dissected and fixed for 2 days in the same fixative at 4°C. After fixation, the samples were stored in PB. The samples were decalcified in 10% ethylenediaminetetraacetic acid-2Na for 1 week, then immersed in 20% sucrose, cut into 6- μm sagittal sections using a cryostat (HM525; Microm, Walldorf, Germany), and thaw-mounted on gelatin-coated glass slides.

A routine method [31] was used for immunostaining. Briefly, the sections were rinsed in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS) overnight and incubated with rabbit anti-human CXCL14 antibody (code no. 500-P237; PeptoTech, Rocky Hill, NJ, USA) diluted in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 (PBS-BSAT) for 24 hr at 4°C (Table 1). The immunogen of this antibody is *Escherichia coli*-derived recombinant human CXCL14. The amino acid sequence of gecko CXCL14 has been identified in *Gekko japonicus* (XP_015279537.1, NCBI). A BLASTP search of the GenBank database indicated that the sequence conforms to human CXCL14 (20–99) with 85% sequence homology. After the sections were rinsed with PBS, they were incubated in a secondary antibody, biotinylated goat anti-rabbit IgG (BA-1000; Vector Laboratories, Burlingame, CA, USA) diluted to 1:100 in PBS-BSAT for 1 hr at room temperature. The sections were rinsed with PBS and reacted with avidin-biotin-horseradish peroxidase complex (ABC, PK-6100; Vector Laboratories) at a dilution of 1:200 in PBS-BSAT for 30 min at room temperature. After a final rinse in PBS, the sections were reacted with 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer solution (pH 7.4). The sections were counterstained with thionin, dehydrated in a graded alcohol series, cleared with xylene, and coverslipped using Malinol (Muto Pure Chemicals, Tokyo, Japan). The control experiments for the staining profiles were prepared by omitting the antibody in the first incubation or by using an antibody pre-absorbed with recombinant human CXCL14 (5 $\mu\text{g/ml}$, code no. 300-50; PeptoTech). For general histologic examination of the pituitary visualizing the pars intermedia, the sections were immunostained with sheep anti- α -melanocyte-stimulating hormone (αMSH) antibody (Table 1), biotinylated rabbit anti-sheep IgG (BA-6000; Vector Laboratories), and ABC. Immunoreactivity was then detected by DAB deposition.

To identify the CXCL14-immunoreactive cell types, we performed double immunofluorescence staining using the anti-CXCL14 antibody in combination with antibodies against six adenohypophyseal hormones: αMSH , prolactin (PRL), adrenocorticotropic hormone (ACTH), growth hormone (GH), luteinizing hormone (LH), and thyroid-stimulating hormone (TSH). The antibodies used against these adenohypophyseal hormones are shown in Table 1. Immunoreactivity was visualized by fluorescein-conjugated donkey anti-rabbit IgG (AP182F; Millipore, Temecula, CA, USA) for CXCL14, rhodamine-conjugated donkey anti-sheep IgG (AP184R; Millipore) for αMSH and ACTH, and rhodamine-conjugated donkey anti-goat IgG (AP180R; Millipore) for PRL, GH, TSH, and LH.

RESULTS

Figure 1a shows a sagittal section through the pituitary immunostained with anti- αMSH antibody. CXCL14-immunoreactive cells were observed in the pars intermedia and pars distalis (Fig. 1b). We were unable to detect any CXCL14-immunoreactive structures in the pars nervosa. All endocrine cells in the pars intermedia were immunoreactive for CXCL14 in all animals. The cells were columnar in shape, with a height of 15–20 μm and a width of approximately 10 μm (Fig. 2a). The CXCL14-immunoreactive cells were scattered in the pars distalis of all animals (Fig. 1b). These cells were 8–13 μm in diameter and round or ovoid in shape. Pre-absorption of the CXCL14 antibody with recombinant CXCL14 abolished these staining profiles (Fig. 1c). There were no differences between the five males and five females in pituitary staining profiles.

Double immunofluorescence staining revealed that all CXCL14-immunoreactive cells in the pars intermedia were immunopositive for αMSH and that all αMSH -immunoreactive cells in the pars intermedia were immunopositive for CXCL14

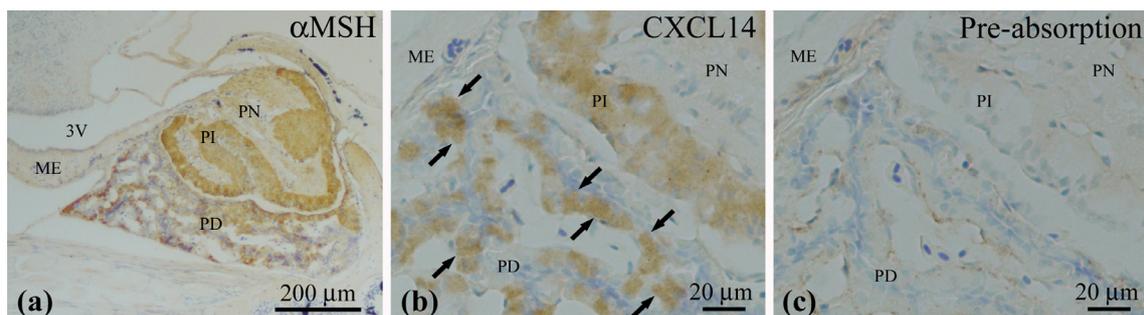


Fig. 1. Low-magnification micrograph of a pituitary immunostained with α -melanocyte-stimulating hormone (α MSH) antibody in sagittal section (a). Moderately-magnified micrographs of the pituitary immunostained with CXCL14 antibody (b) and showing disappearance of these staining profiles by pre-absorption with recombinant CXCL14 (c) in serial sagittal sections. Arrows in (b) indicate CXCL14-immunoreactive cells scattered in the pars distalis. The anterior portion of the pituitary is to the left. Abbreviations: ME, median eminence; PD, pars distalis; PI, pars intermedia; PN, pars nervosa; 3V, third ventricle.

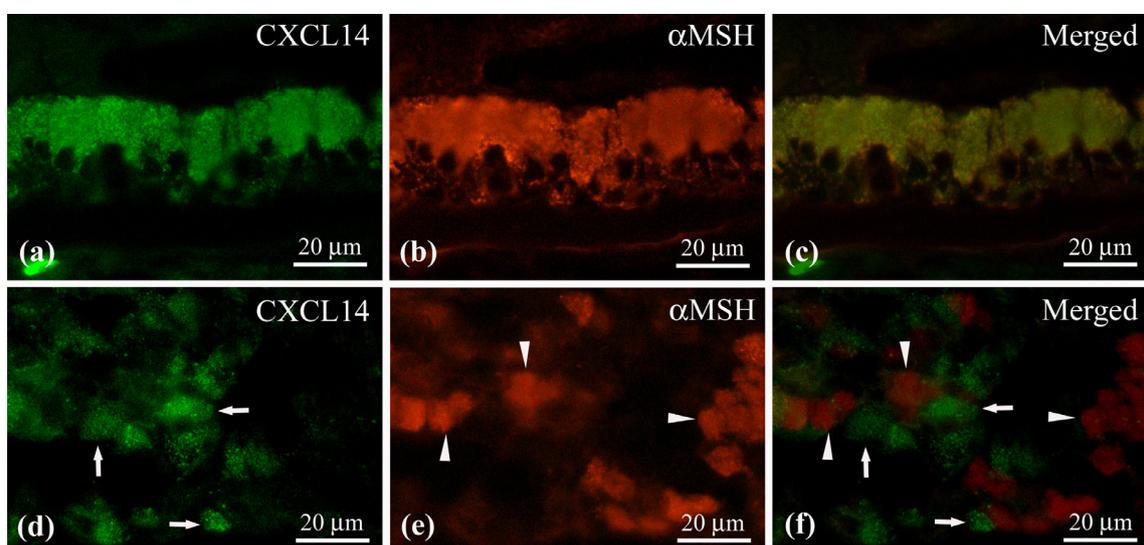


Fig. 2. Fluorescence micrographs of double-stained sections showing CXCL14 (a and d) and α -melanocyte-stimulating hormone (α MSH) (b and e) immunoreactivity. (a and b) are images of an identical section showing the pars intermedia, whereas (d and e) are images of an identical section showing the pars distalis. Panels (c) and (f) are merged photographs of (a and b) and (d and e), respectively. Note that all CXCL14-immunoreactive cells in the pars intermedia are immunopositive for α MSH (a–c). Arrows in (d and f) indicate CXCL14 immunoreactive cells, which are immunonegative for α MSH in the pars distalis. Arrowheads in (e and f) indicate α MSH-immunoreactive cells, which are immunonegative for CXCL14 in the pars distalis.

(Fig. 2a and 2b). These cells were detected as yellowish-colored cells in merged images of the immunofluorescence micrographs (Fig. 2c). Additionally, α MSH-immunoreactive cells were detected in the pars distalis (Fig. 2e). These cells were immunonegative for CXCL14 (Fig. 2d and 2f). Double immunofluorescence microscopy revealed that almost all the CXCL14-immunoreactive cells in the pars distalis were immunopositive for PRL (Fig. 3a–c). These CXCL14/PRL-immunoreactive cells presented with a yellowish color in the merged micrograph (Fig. 3c), which was similar to the appearance of CXCL14/ α MSH-immunoreactive cells (Fig. 2c). PRL-immunoreactive cells were not observed in the pars intermedia (data not shown). The CXCL14-immunoreactive cells were immunonegative for ACTH (Fig. 3d–f), GH (Fig. 3g–i), LH (Fig. 3j–l), and TSH (Fig. 3m–o).

DISCUSSION

In the present study, we first reported the existence of a CXCL14-like substance in the α MSH-producing and PRL-producing cells of the reptilian pituitary. Disappearance of the CXCL14 staining profiles after pre-absorption with recombinant CXCL14 suggests that the anti-CXCL14 antibody specifically recognized the gecko CXCL14. The α MSH-immunoreactive cells observed in the pars distalis must have been ACTH-producing cells, because ACTH includes the amino acid sequence of α MSH [35]. Among reptiles, the gecko has a reduced pars tuberalis, and hence the portal vessels pass from the median eminence to the pars distalis

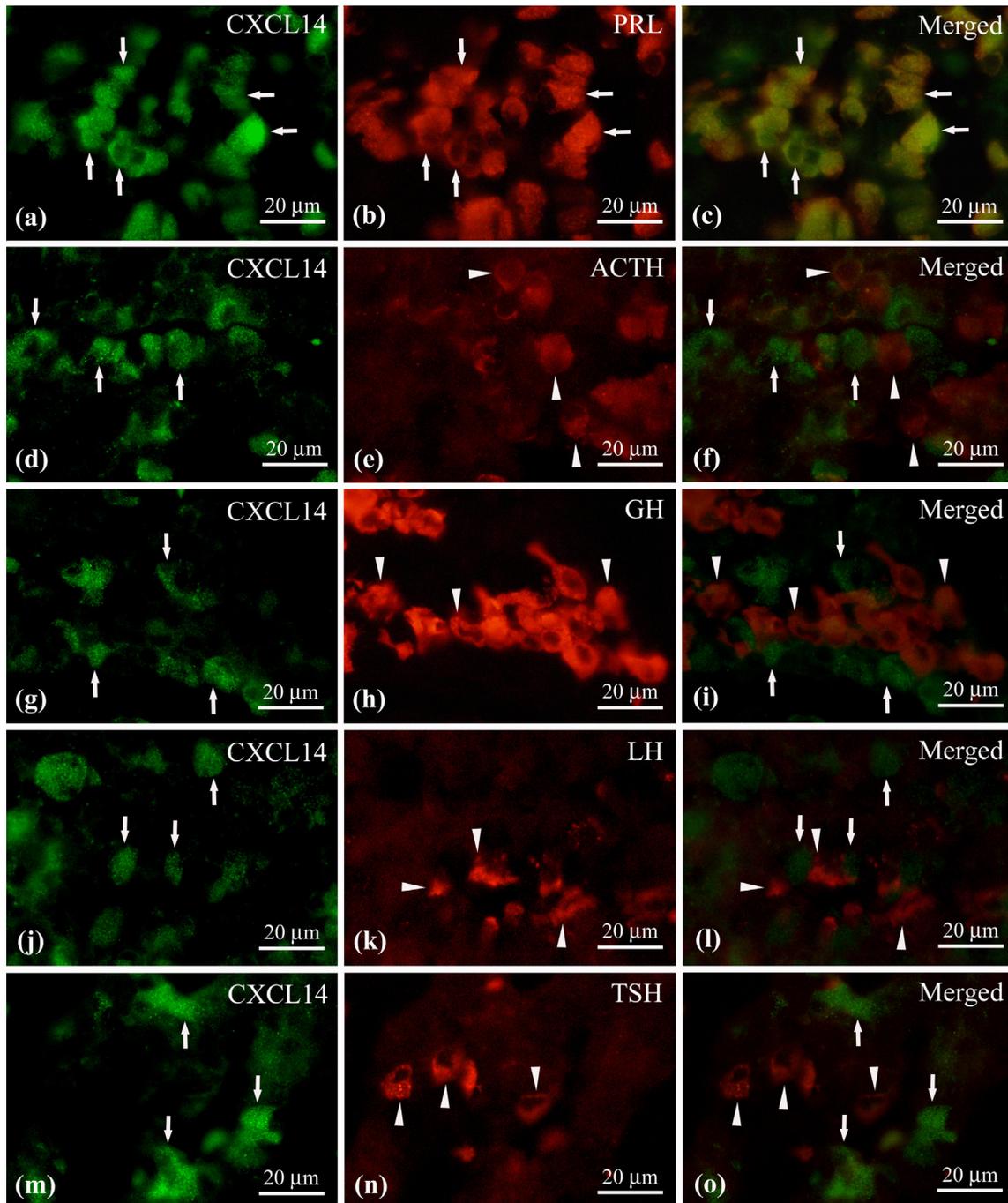


Fig. 3. Fluorescence micrographs of double-stained identical sections (a and b, d and e, g and h, j and k, m and n) showing CXCL14 (a, d, g, j, and m), prolactin (PRL) (b), adrenocorticotrophic hormone (ACTH) (e), growth hormone (GH) (h), luteinizing hormone (LH) (k), and thyroid-stimulating hormone (TSH) (n) immunoreactivity in the pars distalis. Panels (c), (f), (i), (l), and (o) are merged photographs of (a and b), (d and e), (g and h), (j and k), and (m and n), respectively. Arrows in (a–c) indicate CXCL14-immunoreactive cells that are immunopositive for PRL. Note that CXCL14-immunoreactive cells correspond to PRL-immunoreactive cells (a–c). Arrows in (d and f), (g and i), (j and l), and (m and o) indicate CXCL14-immunoreactive cells that are immunonegative for ACTH, GH, LH, and TSH, respectively. Arrowheads in (e and f), (h and i), (k and l), and (n and o) indicate CXCL14-immunonegative cells that are immunopositive for ACTH, GH, LH, and TSH, respectively.

in connective tissue, not via the pars tuberalis as in mammals [3]. The pars distalis in reptilian species contains five secretory cell types, as in mammals, and the pars intermedia in reptiles of the order Squamata, which includes the gecko, surrounds the pars nervosa [3, 9]. However, to date, the presence of CXCL14 in adenocytes has been demonstrated only in urodeles, namely, *Ambystoma mexicanum* and *Hynobius nigrescens*, where GH-producing cells are immunoreactive for CXCL14 [32]. The paucity of data for pituitary CXCL14 makes it difficult to discuss the functions of pituitary CXCL14 in detail, except for the following notes.

Although sex differences in response to a high-fat diet are remarkable in insulin-sensitive *CXCL14*-deficient mice [23], we could not find differences between males and females in the distribution patterns of *CXCL14* in the gecko pituitary. In *CXCL14*-deficient mice, serum insulin levels were significantly lower than those in control mice after an intraperitoneal injection of glucose [23]. *CXCL14* immunoreactivity was localized in the somatostatin-producing cells in the pancreatic islets of normal mice [1, 30]. Suzuki and Yamamoto [30] postulated that *CXCL14* acts as a paracrine agent in the mouse pancreas. Recently, Atanes *et al.* [1] concluded that *CXCL14* inhibits insulin secretion from β cells through decrease in ATP levels in the mouse islets. In cultured myocytes, *CXCL14* significantly inhibited insulin-stimulated uptake of 2-deoxyglucose in a dose-dependent manner [23]. In addition, *CXCL14*-deficient mice have a lower body weight than control mice, mainly due to a decrease in food intake [36]. These data suggest that *CXCL14* participates widely in glucose homeostasis, including appetite control. α MSH acts as a peripheral regulator of glucose and fat metabolism [4], as well as a melanocyte stimulator. A radio receptor assay demonstrated that α MSH inhibits insulin secretion via specific binding to the cultured β cells of hamsters [26]. In cultured muscle cells of mice, α MSH stimulates glucose uptake [8]. On the other hand, PRL stimulates insulin secretion from cultured pancreatic islets of humans and rats [20, 39]. In geckos, *CXCL14* secreted from α MSH-producing cells and PRL-producing cells may regulate insulin release from islet β cells as well as glucose uptake in muscle cells in an endocrine fashion together with α MSH and/or PRL.

Alternatively, *CXCL14* could possibly regulate adenohipophyseal hormone secretion. *CXCL12*, another ELR⁻ CXC chemokine, stimulates GH release from cultured pituitary cells of rats [10, 21]. Although proper receptors for *CXCL14* have not been reported, significant binding of *CXCL14* to CXCR4, a receptor for *CXCL12*, has been reported in cultured human bone marrow cells and leukemia-derived cells [37]. In the ovine pituitary, CXCR4 is localized in GH-producing and LH-producing cells [25]. Matsumura *et al.* [22] suggested that α MSH secreted from the pars intermedia regulates the functions of PRL-producing cells in the pars distalis of the mouse. In the gecko pituitary, *CXCL14* secreted from α MSH-producing cells may modulate hormone release from adenohipophyseal cells in the pars distalis in an endocrine fashion. In addition, *CXCL14* secreted from PRL-producing cells may also modulate pituitary hormone release in a paracrine and/or autocrine fashion in the pars distalis.

The coexistence of *CXCL14* with various bioactive substances has been reported in rodent nervous systems. For example, *CXCL14* coexists with vasopressin and oxytocin in the rat hypothalamus [40], with γ -aminobutyric acid (GABA) in the mouse hippocampus [2], with somatostatin in the mouse alimentary tract [33], and with neuropeptide Y in the rat salivary glands [34]. With respect to endocrine systems, *CXCL14* coexists with GH in urodele pituitaries [32] and with somatostatin in mouse gastro-entero-pancreatic endocrine cells [1, 30, 33]. In addition, the present study demonstrated the coexistence of *CXCL14* with α MSH and PRL in the gecko pituitary. Although all the functions of *CXCL14* are not yet known, the diversity of the coexisting bioactive substances of *CXCL14* suggests that *CXCL14* may be associated with additional functions, such as regulation of the physiological states of cells. For example, association of *CXCL14* with morphogenesis has been postulated in *Xenopus* [24], chick [11, 12], and mouse [11] embryos. In early mouse embryos, *CXCL14* inhibits the outgrowth of trophoblast in a paracrine and/or autocrine fashion [18]. Future studies elucidating these functions are warranted.

In conclusion, we demonstrated the presence of a chemokine, *CXCL14*, in the pituitary of a reptile, the gecko. *CXCL14* coexisted with α MSH in the pars intermedia and with PRL in the pars distalis. *CXCL14* in the gecko pituitary may contribute to the regulation of pituitary hormone secretion and/or glucose homeostasis, in addition to having a chemotactic function. Although studies in reptiles are limited compared with those in mammals, further studies may provide new insights on the function of *CXCL14* in reptiles.

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