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Different distributions of preproMCH and hypocretin/orexin in the forebrain of the pig (*Sus scrofa domesticus*)

S. Chometton [a], G. Franchi [a], C. Houdayer [a], A. Mariot [a], F. Poncet [a], D. Fellmann [a], Y. Tillet [b], P.Y. Risold [a,*]

[a] EA3922, UFR Sciences Médicales et Pharmaceutiques, SFR-FED 4234, Université de Franche-Comté, Besançon 25000, France
[b] UMR 7247 INRA/CNRS/Université François Rabelais de Tours/IFCE, SFR-FED 4226, Centre INRA Val de Loire, Nouzilly, Tours 37380, France

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ABSTRACT

Neurons producing melanin-concentrating hormone (MCH) or hypocretin/orexin (Hcrt) have been implicated in the sleep/wake cycle and feeding behavior. Sleep and feeding habits vary greatly among mammalian species, depending in part of the prey/predatory status of animals. However, the distribution of both peptides has been described in only a limited number of species. In this work, we describe the distribution of MCH neurons in the brain of the domestic pig. Using *in situ* hybridization and immunohistochemistry, their cell bodies are shown to be located in the posterior lateral hypothalamic area (LHA), as expected. They form a dense cluster ventro-lateral to the fornix while only scattered cells are present dorsal to this tract. By comparison, Hcrt cell bodies are located mainly dorsal to the fornix. Therefore, the two populations of neurons display complementary distributions in the posterior LHA. MCH projections are, as indicated by MCH-positive axons, very abundant in all cortical fields ventral to the rhinal sulcus, as well as in the lateral, basolateral and basomedial amygdala. In contrast, most of the isocortex is sparsely innervated. To conclude, the distribution of MCH cell bodies and projections shows some very specific features in the pig brain, that are clearly different of that described in the rat, mouse or human. In contrast, the Hcrt pattern seems more similar to that in these species, *i.e.* more conserved. These results suggest that the LHA anatomic organization shows some very significant interspecies differences, which may be related to the different behavioral repertoires of animals with regard to feeding and sleep/wake cycles.

Abbreviations: ac, anterior commissure; ACB, nucleus accumbens; AD, anterodorsal nucleus thalamus; AHN, anterior hypothalamic nucleus; AMY, amygdala; arb, arbor vitae; ARH, arcuate hypothalamic nucleus; AV, anteroventral nucleus thalamus; BL, basolateral complex amygdala; BLA, basolateral amygdalar nucleus; BM, basomedial complex amygdala; BMA, basomedial amygdalar nucleus; CA1-3, field CA1-3, Ammon's horn; cc, corpus callosum; Cd, caudate nucleus; CER, cerebellum; CG, central gray; Cing, cingulate region; CLA, claustrum; CoA, cortical amygdalar nucleus; CPu, caudoputamen; cpd, cerebral peduncle; DG, dentate gyrus; DH, dorsal horn, spinal cord; DMH, dorsomedial hypothalamic nucleus; ENT, entorhinal area; fi, fimbria; fx, columns of the fornix; GP, globus pallidus; GRN, gigantocellular reticular nucleus; HYP, hypothalamus; IA, intercalated nucleus amygdala; ic, internal capsule; IF, interfascicular nucleus raphé; IG, induseum griseum; INC, interstitial nucleus of Cajal; IO, inferior olivary complex; LA, lateral amygdalar nucleus; LD, lateral dorsal nucleus thalamus; LHAd, lateral hypothalamic area, dorsal region; LHAv, lateral hypothalamic area, ventral region; lot, lateral olfactory tract, body; lv, lateral ventricle; MB, mammillary body; MD, mediodorsal nucleus thalamus; MDRN, medullary reticular nucleus; ME, median eminence; MEA, medial amygdalar nucleus; mfb, medial forebrain bundle; MG, medial geniculate complex; MRN, midbrain reticular nucleus; MSN, medial septal nucleus; mtt, mammillothalamic tract; ND, nucleus of Darkschewitsch; NDB, diagonal band nucleus; NRT, reticular nucleus thalamus; NSO, supraoptic nucleus, proper; NTS, nucleus of the solitary tract; opt, optic tract; OT, olfactory tubercle; PAG, periaqueductal gray; PERI, perirhinal area; PH, posterior hypothalamic nucleus; PIR, piriform area; PMd, dorsal premammillary nucleus; Pu, putamen nucleus; PVH, paraventricular hypothalamic nucleus; PVT, paraventricular thalamic nucleus; py, pyramidal tract; Re, nucleus reuniens; rf, rhinal fissure; Rh, rhomboid nucleus; RL, rostral linear nucleus raphe; RM, raphe magnus; RN, red nucleus; RSP, retrosplenial area; SC, superior colliculus; SN, substantia nigra; st, stria terminalis; STN, subthalamic nucleus; Sub d, subiculum, dorsal part; Sub v, subiculum, ventral part; SubPVH, subparaventricular zone hypothalamus; SUM, supramammillary nucleus; VAL, ventral anterior-lateral complex thalamus; VCO, ventral cochlear nucleus; VH, ventral horn spinal cord; VMH, ventromedial hypothalamic nucleus; ZI, zona incerta; 3v, third ventricle; 4v, fourth ventricle proper; V, motor nucleus of the trigeminal nerve; VIIn, facial nerve; XII, hypoglossal nucleus.

* Corresponding author. Tel.: +33 363 082223; fax: +33 363 082280.
E-mail address: pierre-yves.risold@univ-fcomte.fr (P.Y. Risold).

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Introduction

Neurons producing melanin-concentrating hormone (MCH) form a conspicuous cell population in the mammalian lateral hypothalamic area (LHA) (Baker, 1991; Bittencourt et al., 1992; Griffond and Baker, 2002; Nahon et al., 1989). In most species, they are described as being located in the dorsal and perifornical regions alongside hypocretin/orexin (Hcrt) producing neurons (Hahn, 2010; Swanson et al., 2005). However, we recently observed that the relative distribution of these cells could show some important interspecies differences (Croizier et al., 2013; Croizier et al., 2010). MCH cell bodies are found dorsal to the fornix and in the dorsolateral LHA in rodents while they are mostly dorsal to the fornix in the cat and are closer to the third ventricle in the dorsal and posterior hypothalamus in human (Croizier et al., 2013; Krolewski et al., 2010; Torterolo et al., 2006). By comparison, MCH neurons formed a dense cluster in the ventrolateral LHA of the sheep (Chaillou et al., 2003; Tillet et al., 1996).

Based mostly in experiments made in rodents, these neurons are involved in several behaviors including the control of REM sleep and feeding (Boutrel et al., 2010; Eggermann et al., 2003; Griffond and Baker, 2002; Hassani et al., 2009; Jego et al., 2013; Qu et al., 1996; Verret et al., 2003). The behavioral repertoires of vegetarian/prey or predatory animals is obviously extremely different with regard to feeding and sleeping habits, and it may be possible to relate interspecies differences in MCH distribution to these behavioral divergences. However, to date, MCH distribution has been analyzed in only a very limited number of large mammals.

The domestic pig is a preferentially vegetarian, omnivorous animal. It evolved from wild pig species that show a short sleep duration, as very often reported in prey animals. The object of the present study was to investigate the distribution of MCH cell bodies in the domestic pig brain. This distribution was compared to that of Hcrt containing neurons that overlap with MCH-expressing cells in many species. The distribution of the pig MCH and Hcrt projections was also researched.

Materials and methods

Animals

Animal care and all procedures were carried out in accordance with French law (authorization A 37801 of the French Ministry of Agriculture).

Four young adult female large-white pigs, weighing 70–80 kg, were killed by a lethal intravenous injection of Nesdonal ® (16 mg/kg). The hormonal status of these animals was unknown, but they had just reached puberty. Heads were perfused through the carotid arteries with two liters of 1% sodium nitrite solution in 0.1 M phosphate buffer (pH 7.4) and four liters of a cold fixative solution made of paraformaldehyde (PFA) dissolved in the same phosphate buffer (40 g/L). The brain was removed, dissected out and sliced into 1 cm thick blocs. Then tissue samples were post-fixed for 24 h in the same fixative.

Tissue cryoprotection

Blocs were cryoprotected in a 15% sucrose solution (Sigma) and 0.1% sodium azide in 0.1 M (pH 7.4) phosphate buffer at 4 °C. Then, they were quickly frozen by immersion in isopentane at −74 °C using the Snap-Frost™ system (Excilone, France). The brains were cut in four series of 30 μm (2 brains) or in three series of 60 μm (2 brains) thick coronal sections, collected in a cryoprotector solution (1:1:2 glycerol/ethylene glycol/phosphate buffered saline or PBS) and stored at −20 °C.

MCH in situ hybridization

A BLAST (Basic Local Alignment Search Tool) was performed to compare pig MCH mRNA with rat MCH mRNA. The percentage of homologies was high (81%) and *in situ* experiments were performed using a MCH RNA probe that was previously obtained in our laboratory (Brischoux et al., 2001).

Free-floating sections were post-fixed in 4% PFA during 20 min at room temperature. Then they were incubated 1 h at 4 °C in 6% hydrogen peroxide (Sigma) in PBS with 1% Tween (PBT). Tissues were rinsed briefly in PBT, incubated in glycine solution (2 mg/ml PBT, Sigma) 10 min and in PBS with 0.3% Triton X100 (PBS-T) 15 min at room temperature. After rinsing in PBT, sections were incubated in proteinase K solution (10 μg/ml PBT, Roche) 15 min at 37 °C then in 4% PFA/0.2% glutaraldehyde (Electron Microscopy Sciences) 20 min at room temperature. Tissues were rinsed briefly in PBT and incubated in TEA (Triethanolamine, VWR) and acetic anhydride (Sigma) solution (TEA 1.33 ml, acetic anhydride 250 μl, HCl 410 μl qsp 100 ml water) 5 min at room temperature. Then, sections were incubated in hybridization buffer (50% formamide, 45% 10× SSC, 5% Denhart 100×, 0.1 mg/ml salmon sperm DNA) at 37 °C during 10 min and overnight at 56 °C in humid chambers with hybridization buffer containing 100 ng labeled MCH RNA probes. The antisense and control sense rat MCH probes were produced by using the RNA transcription kit (Roche) and were DIG-UTP-labeled. After rinsing with solution I (50% formamide, 49% 5× SSC pH 4.5, 1% SDS) and solution III (50% formamide, 50% 2× SSC pH 4.5) for three 30 min washes at 37 °C, sections were incubated in 0.1% blocking reagent (Roche) in PBT 90 min at room temperature. They were incubated in anti-DIG Fab fragments conjugated to alkaline phosphatase (1:1300, Roche) overnight at room temperature and revealed with enzyme substrate NBT (nitro-blue tetrazolium chloride, Roche) BCIP (5-bromo-4-chloro-3′-indolyphosphate phosphate, Roche) 2 h at room temperature. Sections were rinsed, mounted on gelatin-coated slides and cover-slipped with 60:40 glycerol:PBS-T. Control hybridization, including hybridization with sense DIG-labeled riboprobes, was performed.

Immunohistochemistry MCH, NEI, Hcrt

After rinsing in PBS-T, free-floating sections were incubated with the primary antibody in milk solution (PBS-T, 1% bovine serum albumin, 10% lactoproteins and 0.01% sodium azide), during 65 h at 4 °C. The primary antibodies used were anti-MCH [salmon or rat MCH, our laboratory (Risold et al., 1992), made in rabbit, 1:1000], anti-NEI [the MCH precursor neuropeptide glutamic acid-isoleucineamide, our laboratory (Risold et al., 1992), made in rabbit, 1:2000] and anti-hypocretin (Santa Cruz Biotechnology, made in goat, 1:1000). The labeling was then revealed through the avidin-biotin complex system for the rabbit antibodies and through the peroxidase-anti-peroxidase system for the goat antibody. Sections were incubated for 24 h at 4 °C in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories) at a dilution of 1:1000 in PBS-T, or for 8 h at room temperature in a solution of donkey anti-goat IgG (Jackson Immunoresearch) at a dilution of 1:1000 in PBS-T. Then, sections were placed in the mixed avidin-biotin horseradish peroxidase (HRP) complex solution (ABC Elite Kit, Vector Laboratories) for 1 h at room temperature, or in the goat peroxidase anti-peroxidase (PAP) solution (DAKO) at a dilution of 1:1000 for 16 h at 4 °C. The peroxidase complex was visualized by a 6 min exposure to a chromogen solution containing 0.04% 3,3'diaminobenzidine tetrahydrochloride (DAB, Sigma) with 0.006% hydrogen peroxide (Sigma) in PBS. The reaction was stopped by extensive washing in PBS. Sections were mounted on gelatin-coated slides, and then dehydrated and coverslipped with Canada balsam (Roth). An adjacent series was always stained in a solution of 1% toluidine blue (Roth) in water to serve as a reference series for cytoarchitectonic purposes.

Some sections were revealed by immunofluorescence. After the incubation with the primary antibody, the tissue was incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG antibody (Invitrogen, 1:1000) or with Cyanine-3 donkey anti-goat IgG antibody (Jackson Immunoresearch, 1:1500) diluted in PBS-T for 2 h at room temperature. Finally, the sections were washed with PBS-T three times 5 min, were mounted on gelatin-coated slides and coverslipped with 60:40 glycerol:PBS-T.

Antibody characterization

Please see Table 1 for a list of all antibodies used.

The specificities of the sMCH, rMCH and NEI antisera were tested by blotting against the peptides salmon and rat MCH, NEI, NGE, GRF37, and alpha-MSH (Risold et al., 1992). The labeling provided by the two MCH AS on the pig brain sections

Table 1
Table of primary antibodies used.

Antigen	Immunogen	Manufacturer	Dilution used
MCH	Synthetic Salmon MCH; full 17 amino acid sequence	Risold et al. (1992)	1/1000
MCH	Synthetic rat MCH; full 19 aa sequence	Risold et al. (1992)	1/1000
NEI	Synthetic rat NEI; full sequence	Risold et al. (1992)	1/2000
Hypocretin I/Orexin A	Human orexin A, 19 aa of the c-terminal fragment	Santa Cruz Biotechnology, goat polyclonal, # GO 813	1/1000