Developmental dynamics shaping excitatory/inhibitory balance and stress-related gene expression in the central extended amygdala of chicken

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TESI DOCTORAL

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List of abbreviations

ac, Anterior commissure

Acb, Nucleus accumbens

Aco, Core or intermediate part of arcopallium

AcS, Shell of Acb

Ad, Dorsal arcopallium

AHi, Amygdalo-hippocampal area

AO, Anterior olfactory area

APH, Parahippocampal area

APHcl, Caudolateral APH

APHl, Lateral APH

APHm, Medial APH

APir, Amygdalo-piriform area

Arc, Arcopallium

Bas, Nucleus basorostrallis

BasSh, Shell of Bas

BMC, Basal magnocellular corticopetal complex

BST, Bed nucleus of the stria terminalis

BSTL, Lateral BST

BSTM, Medial BST

BSTM1, Dorsal subdivisión of BSTM

BSTM3, Ventral subdivision of BSTM

CCS, Caudocentral septal nucleus

CoS, Commissural septal nucleus

csm, Cortico-septo-mesencephalic tract

DG, Dentate gyrus

CeC, Capsular central amygdala

Ceov, Oval central amygdala nucleus

DB, Diagonal band nuclei

DLA, Anterior dorsolateral thalamic nucleus

DLI, Anterior dorsointermediate thalamic nucleus

DLM, Anterior dorsomedial thalamis nucleus

DLP, Dorsolateral pallium

E, Entopallium

EA, Extended amygdala

EAce, Central EA

Ec, Entopallial core

Ent, Entorhinal cortex

Es, Entopallial shell or belt

GP, Globus pallidus

Gr, Granular cell layer of OB

H, Hyperpallium

HA, Apical hyperpallium

Hb, Habenula

HD, Densocellular hyperpallium

HF, Hippocampal formation

HiC, Nucleus of the hippocampal commissure

IGL, Intergeniculate leaflet

IHA, Interstitial nucleus of HA

INP, Intrapeduncular nucleus

L, Auditory field L (in NCM)

lac, Lateral limb of ac

LAN, Laminar pallial nucleus (lateral to HD and dorsal to MD; it extends into a superficial area, often referred as superficial or lateral hyperpallium)

lfb, Lateral forebrain bundle

LOT, Nucleus of the lateral olfactory tract

LSt, Lateral Striatum

M, Mesopallium

MC, Caudal mesopallium

MD, Dorsal mesopallium

Me, Medial amygdala

MF, Frontal mesopallium

Mi, Mitral cell layer of OB

MI, Intermediate mesopallium

MIF, Mesopallial island field

MIV or MV, Ventral MI

MPA, Medial preoptic area

MPO, Medial preoptic nucleus

MVCo, Core nucleus of MIV

MVM, Medial MIV

MSt, Medial striatum

N, Nidopallium

NC, Caudal nidopallium

NCIF, Island field of NC

NCL, Lateral NC

NCM, Medial NC

NF, Frontal nidopallium

NFL, Lateral NF

NI, Intermediate nidopallium

NIM, Medial NI

NIS, Superficial NI

OA, Anterior olfactory area

OB, Olfactory bulb

Pa, Paraventricular hypothalamic nucleus

PG, Pregeniculate prethalamic nucleus

pINP, Peri/post-INP island field

Pir, Piriform cortex (olfactory cortex)

PO, Preoptic region

PPir, Prepiriform cortex

pRot, Perirotundic belt

RB, Retrobulbar area

RtV, Reticular prethalamic nucleus, ventral part

Se, Septum

SFi, Septofimbrial nucleus

SLI (or LSI), Intermediate subdivision of lateral septum

SLV (or LSV), Ventral subdivision of lateral septum

SO, Supraoptic nucleus

SPa, Septopallidal transition area

StC, Striatal capsule

SuPO, Subpreoptic area (part of the telencephalonopto-hypothalamic domain or TOH)

Th, Thalamus

vaf, ventral amygdalofugal tract

VDB, Vertical subnucleus of DB

VP, Ventral pallidum

Summary

In this PhD project we focused on two major aspects of the brain relevant for regulating emotions and stress responsivity in chicken. The first one is the development of the glutamatergic neurons in the extended amygdala, involved in the excitatory/inhibitory regulatory networks, which unbalance is behind some neurodevelopmental disorders in humans. The second is the development of stress-related corticotropin releasing factor (CRF) receptors and peptides during embryonic and post hatching periods.

GABAergic and glutamatergic neurons are found in the extended amygdala of chickens, but the origin of the glutamatergic cells was unknown. To address whether these cells are telencephalic or extratelencephalic, we used the telencephalic transcription factor Foxg1. The results showed that the glutamatergic cells include two subpopulations: one with Foxg1 and another without Foxg1. Glutamatergic cells with Foxg1 appear to originate in the telencephalon-opto-hypothalamic (TOH) embryonic domain, recently described in mice, which coexpresses Otp and Sim1 with Foxg1. During development, Otp/Foxg1 and Sim1/Foxg1 cells of the TOH form a cell corridor that extends to and overlaps with the glutamatergic cells in the medial extended amygdala. Regarding glutamatergic cells without Foxg1, part of them appear to originate in the core region of the supraopto-paraventricular hypothalamic domain, distinguished by its Otp and Sim1 single labeled cells (i.e. without Foxg1). We also observed Sim1 cells, with and without Fogx1, invading the central extended amygdala. These results demonstrate the complex development of GABAergic and glutamatergic neurons of the extended amygdala, which balance is critical for the correct regulation of emotions in different vertebrates.

Regarding the CRF systems, we studied their development using in situ hybridization for CRF, its two receptors and the binding protein. The results demonstrate the early development of CRF and its receptors in pallial areas involved in sensory processing, sensorimotor integration, and cognition, and a late development in subpallial areas involved in regulating the stress response. In contrast, the CRF buffering protein appears first in the subpallium and develops late in the pallium. The study suggests that stress regulation becomes more sophisticated with age. These results help to understand the mechanisms underlying the negative effects of noise and visual stress during prehatching stages in chicken, and open new venues for studies aiming to understand the role of CRF systems in stress regulation.

Resumen

En esta tesis, estudiamos dos aspectos del cerebro relevantes para entender la regulación de las emociones y la respuesta de estrés en pollo. El primero fue el desarrollo de las neuronas glutamatérgicas de la amígdala extendida, implicadas en redes de excitación/inhibición, cuyo desequilibrio causa algunos trastornos del neurodesarrollo en humanos. El segundo fue el desarrollo de receptores y péptidos del factor liberador de la corticotropina (CRF), implicados en estrés.

Se han encontrado neuronas GABAérgicas y glutamatérgicas en la amígdala extendida de pollo, pero se desconoce el origen de estas últimas. Para saber si se originan o no en el telencéfalo, usamos el factor de transcripción telencefálico Foxg1. Nuestros resultados muestran que hay dos grandes subpoblaciones: unas con Foxg1 y otras sin Foxg1. Las neuronas glutamatérgicas con Foxg1 parecen originarse en el dominio telencéfalo-opto-hipotalámico (TOH), recientemente descrito en ratón, que coexpresa Otp y Sim1 con Foxg1. Durante el desarrollo, células del TOH con coexpresión de Otp/Foxg1 y Sim1/Foxg1 invaden la amígdala medial extendida. Las células glutamatérgicas sin Foxg1 parecen derivar en parte de la zona central del dominio hipotalámico supraopto-paraventricular, que expresa Otp y Sim1, pero no Foxg1. Celúlas Sim1 con o sin Foxg1 también invaden la amígdala central extendida. Los resultados muestran el complejo desarrollo de las neuronas GABAérgicas y glutamatérgicas de la amígdala extendida, cuyo equilibrio es crítico para la correcta regulación de las emociones en distintos vertebrados.

Con respecto a los sistemas CRF, estudiamos el mRNA de CRF, sus receptores 1 y 2, y la proteína ligante de CRF. Los resultados muestran un desarrollo temprano de CRF y sus receptores en áreas paliales implicadas en procesamiento sensorial, sensoriomotor y cognitivo, y un desarrollo tardío de áreas subpaliales implicadas en regulación del estrés. Por el contrario, la proteína ligante de CRF (que tampona su efecto) aparece temprano en el subpalio, pero tarde en el palio. Esto sugiere que la regulación del estrés se vuelve más sofisticada con la edad. Los resultados ayudan a comprender los mecanismos subyacentes a los efectos negativos del estrés prenatal en pollo, y sienta las bases para estudiar la función de los sistemas CRF en la regulación del estrés.

Resum

En aquesta tesi estudiem dos aspectes del cervell rellevants per entendre la regulació de les emocions i la resposta d'estrès en pollastre. El primer és el desenvolupament de les neurones glutamatèrgiques de l'amígdala estesa, implicades en xarxes reguladores d'excitació/inhibició, el desequilibri de les quals causa alguns trastorns del neurodesenvolupament en humans. El segon aspecte és el desenvolupament de receptors i pèptids del factor alliberador de la corticotropina (CRF), implicats en estrès.

S'han trobat neurones GABAèrgiques i glutamatèrgiques a l'amígdala estesa de pollastre, però se'n desconeix l'origen. Per saber si s'originen o no al telencèfal, fem servir el factor de transcripció telencefàlic Foxg1. Els nostres resultats mostren que hi ha dues grans subpoblacions: unes amb Foxg1 i altres sense Foxg1. Les neurones glutamatèrgiques amb Foxg1 semblen originar-se en el domini telencèfal-opto-hipotalàmic (TOH), recentment descrit en ratolí, que coexpressa Otp i Sim1 amb Foxg1. Durant el desenvolupament, cèl·lules del TOH amb coexpressió d'Otp/Foxg1 i Sim1/Foxg1 formen un corredor cel·lular que se solapa amb les neurones glutamatèrgiques de l'amígdala medial estesa. Pel que fa a les cèl·lules glutamatèrgiques sense Foxg1, una part sembla derivar de la zona central del domini hipotalàmic supraopto-paraventricular, distingit per expressar Otp i Sim1, però no Foxg1. A més, cèl·lules Sim1 (amb i sense Foxg1) envaeixen l'amígdala central estesa. Els resultats mostren el complex desenvolupament de les neurones GABAèrgiques i glutamatèrgiques de l'amígdala estesa, l'equilibri de les quals és crític per a la correcta regulació de les emocions en diferents vertebrats.

Pel que fa als sistemes CRF, n'estudiem el mRNA de CRF, els seus receptors 1 i 2, i la proteïna lligant de CRF. Els resultats mostren un desenvolupament primerenc de CRF i els seus receptors en àrees palials implicats en processament sensorial, sensoriomotor i cognitiu i un desenvolupament tardà en àrees subpalials implicades en regulació de l'estrès. Per contra, la proteïna lligant de CRF (que tampona el seu efecte) apareix d'hora al subpali, però es desenvolupa tard al pal·li. Això suggereix que la regulació de l'estrès és més sofisticada amb l'edat. Els resultats ajuden a comprendre els mecanismes subjacents als efectes negatius de l'estrès prenatal en pollastre, i estableixen les bases per estudiar la funció dels sistemes CRF en la regulació de l'estrès.

1. Introduction

In biology, stress is a protective mechanism for animal adaptation to internal or external stressor cues, which aim is to prepare the body to challenging physiological and/or environmental conditions. Among the first to study the stress was Hans Selye, who introduced the notion that stress is not inherently negative buy can have both positive and negative implications depending on the duration and intensitiy. Salye's work revolved around the hypothesis that stress is crucial in adaptation and survival – "general adaptation syndrome", term he coined to explain body's response to stressors. (H. Selye, 1974, 1976). However, when stress becomes chronic, it has a negative impact on physical and mental health (Roozendaal et al. 2009). Upon a stressful event, three major responses are activated: 1) the autonomic nervous system, 2) the endocrine system, 3) the behavioral response. This implies the release of adrenaline and cortisol, which activates metabolism to prepare the body to the challenging situation (Phelps and LeDoux, 2005, Zhang et al., 2021). In particular, the hypothalamus plays an essential role in the control of both the autonomic nervous system (by way of descending projections to sympathetic related centers) and the endocrine system (by way of the hypothalamo-pituitary-adrenal -HPA- axis) (Juruena et al 2020). This immediately activates signal transduction pathways resulting in the instant regulation of specific genes engaged in stress coping processes. Hypothalamic function is in turn regulated by several telencephalic structures, among which the amygdala plays a critical role (Zhang et al., 2021). The amygdala is recognized as the master regulator of the stress response and plays a key role in social behavior and cognition (Phelps and LeDoux, 2005).

Researchers often consider the amygdala as a mosaic of multiple nuclei and areas with different neuronal types, neurochemical profiles, connections, and functions (Canteras et al., 1995; Swanson and Petrovich, 1998; Martínez-García et al., 2002). The vast majority of these studies have been done in mammals, and usually the mammalian amygdala is divided into the basolateral amygdala (BLA), the medial amygdala (MeA) and the central amygdala (CeA). These studies point to the central amygdala as the main actor in the regulation of the stress response, by way of direct and indirect projections to the hypothalamus and brainstem (Phelps and LeDoux, 2005; Davis et al., 2010). The indirect projections go through the lateral bed nucleus of the stria terminalis (BSTL), forming together with the CeA the central extended amygdala, and this indirect pathway plays a major role in activation of the HPA. Both CeA and BSTL include neuron subpopulations expressing corticotropin releasing factor (Crf) that are involved in sustained fear responses akin to anxiety (Davis et al., 2010). The function of the central extended amygdala and HPA is under the influence of both the basolateral amygdala

and the medial amygdala (Zhang et al., 2021). The latter plays a major role in social behavior and, through its connections with the central amygdala and indirectly the HPA, it modulates the psychogenic and social aspects of stress (Ulrich-Lai and Herman, 2009).

In the clinical context, studies in humans showed that exposure to an acute or chronic stress can induce morphological and functional changes in amygdala nuclei, which remarkably differ from what it happens in the prefrontal cortex (PFC) and the hippocampus (Roozendaal et al., 2004; Kim et al., 2015; Price and McCool, 2022). The predisposition of the amygdala to respond to emotional stimuli might influence the individual susceptibility to anxiety disorders. This is determined during development based on genetic factors, but also on the environment and experience during development, which induce permanent epigenetic modifications (Nestler, 2016). For example, stress experienced during prenatal and early postnatal development is known to affect the way humans and animals behave and respond to stressors later in life (Spencer, 2017). In order to understand these stress-related disorders, it is critical to study the brain centers regulating stress, such as the amygdala, throughout ontogeny.

Knowledge on the mechanisms for amygdalar regulation of stress rely on experimental research done mostly in rodents. However, so far these studies have had little impact in psychiatry, due to the poor success of clinical trials. This may be due to differences between rodents and humans, and nowadays more and more researchers recognize the need of increasing the number of species used in neuroscience in order to extract general principles that can extrapolate to humans and other animals (Preuss, 2000; Bolker, 2019; Striedter, 2019). Moreover, for having a deep knowledge of the neural mechanisms regulating stress, we need to consider both ontogeny and phylogeny.

In addition, chronic and developmental stress are also major problems in farm animals, including chicken. Like in mammals, in chicken the stress response implies the activation of the HPA axis, which is regulated by hypothalamic CRF cells (Carsia et al., 1986; Vandenborne et al., 2005; Smulders, 2021). Domestic chicken is a well-established model system for the neurobiological research. Among the advantages are brain similarities with the mammals (including similarities at the level of hypothalamus, basal ganglia and hippocampal formation), well studied genome, and availability of chicken (Reiner et al., 2004; Jarvis et al., 2005, 2014; Belgard et al., 2013). Like mammals, chicken exhibit behavioural imprinting and vocal learning (reviewed by Rosa-Salva et al., 2015) and are highly social (Csillag et al., 2020), which makes them a suitable model system for behavioural studies as well. Moreover, chicken and

other birds rely more on visual and auditory stimuli than in olfaction, making them more similar to primates than rodent (Clayton and Emery, 2015; Rosa-Salva et al., 2015; Mayer et al., 2017). Finally, as chicken embryos develop externally this allows detailed investigation of the chicken brain during the development (Gobes et al 2017, Flores Santin et al 2021). However, very little is known on the amygdala of birds. The aim of this thesis is to study the extended amygdala of chicken throughout ontogenesis, to try to understand variations in stress response during critical aspects of development.

1.1. Evolutionary developmental biology approach in comparative neurobiology

One problem derived from using different species for studying the brain is that the comparison is not always easy, especially when trying to compare phylogenetically distant species as chicken versus mouse or rat. In addition to the need of analyzing more species of different lineages to trace phylogenetic continuity, one excellent approach is to study the development of the system, since similarity is higher at early stages (reviewed by Medina et al., 2017, 2022). Moreover, many of the regulatory genes expressed at early developmental stages are highly conserved, making easy the comparison of expression domains across species. This approach is based on evolutionary – developmental biology (evo-devo), which is an approach in modern biology focused on studying various developmental mechanisms behind conserved, divergent or convergent evolutionary patterns (Moczek et al., 2015). By comparing the development of different organisms, this can help to determine ancestral relationships between them and to elucidate developmental processes involved in the formation of its divisions/subdivisions evolved (Wilkins, 2013). The focus of the evo-devo approach is thus on the embryonic development and the modifications during developmental stages that may produce new evolutionary features. The core concept of this approach is that some fundamental developmental processes are preserved in the evolution of species.

Apart from its application in evolutionary biology, the evo-devo approach is becoming crucial in illuminating the morpho-functional organization of the brain, and the changes that underlie many human diseases and disorders (in particular, those caused by a developmental alteration). The brain is an extremely complex organ, with multiple divisions and subdivisions, each with numerous different neurons and connections. Understanding the anatomo-functional organization of the brain is highly challenging. These general principles of brain organization can be solved using a developmental approach because the embryonic origin of the cells

determines much of their phenotype later in the development, including neurotransmitter/neuropeptide and receptor expression, as well as connections (reviewed by Medina et al., 2017, 2022). During development, neurons with the same embryonic origin and birth date express similar sets of transcription factors and molecules involved in axonal formation, and we have proposed that these neurons show a trend to become inter-connected (even if some of them migrate to distant areas) (Medina et al., 2017). This is the case of telencephalic neurons that originate in the ventrocaudal pallidal/diagonal division, with expression the transcription factor Lhx6, which populate the principal subnucleus of the BSTM and the posterodorsal subnucleus of the medial amygdala and become interconnected (García-López et al., 2008; Sokolowski and Corbin, 2012; Medina et al., 2017; 2022). Moreover, we also suggested that the evo-devo approach allows to understand connections between neurons that have dissimilar embryonic origin but a partial expression of same transcriptional factors involved in cell maturation and axonal formation, such as neurons of the thalamus and pallium expressing Lhx9 (Medina et al., 2017). Therefore, developmental biology provides a complementary view of the physiology of the mature brain, and when these studies are done across different species it is easier to extract common organizing principles. This approach has been particularly useful to study complex brain areas like the amygdala (Medina et al., 2017).

1.2. Classical anatomy of the amygdala in mammals and developmental-based organization

The amygdala (pl. amygdalae) was first described in 1822 by Friedrich Burdach as an almond shaped gray matter area in the anterior part of the human temporal lobe (Burdach, 1819–1822). In 20th century the term "amygdaloid complex" was introduced that united large number of nuclei under the name amygdala (Johnston 1923). To date, the amygdala remains the subject of intensive research in terms of internal organization and function of different cells and circuits, and regarding their developmental and evolutionary origins, as it is a much more complex structure than previously thought.

Classical studies view the amygdala as a multinucleated complex made up of 13 distinct nuclei and areas (Pitkanen et al., 1997; Swanson and Petrovich, 1998; Calhoon and Tye, 2015). The three major groups are the basolateral nuclear amygdalar complex, the cortical amygdalar areas, and the centromedial nuclei. Each of these nuclei/areas are further divided into specific divisions and subdivisions based on cytoarchitecture, histochemistry, neuronal types and

connections.

Based on its developmental origin, the amygdala can be divided into two parts derived from two separate embryonic compartments of the telencephalon: 1) pallial amygdala (includes the basolateral amygdalar complex and the cortical areas); and 2) subpallial amygdala (with central and medial nuclei originating from the ganglionic eminences and preoptic area) (Puelles et al., 2000, 2016a; Medina et al., 2004; García-López et al., 2008; Medina et al., 2017). These major divisions also include cell subpopulations with other origins, as explained in more detail below.

Pallial and subpallial divisions express different sets of transcription factors (TFs) during development, which are involved specification and/or production and differentiation of GABAergic or glutamatergic neurons (Osório et al., 2010; reviewed by Medina et al., 2017, 2022). These studies have helped to understand why the amygdalar nuclei and areas derived from the pallium are rich in glutamatergic neurons, while those derived from the subpallium are rich in GABAergic neurons. In the early stages of neural development, the pallium is expressing Pax6, Dbx1, Emx1/2, Neurogenins and other regulatory genes in the ventricular/subventricular zone. Progenitor cells from the ventricular zone divide and produce neuroblasts that will migrate to their final locations (e.g. specific layers or subdivisions in the mantle). During this migration they express Tbr2 and as they reach their destination, they will express Tbr1, involved in differentiation of glutamatergic neurons (Hevner et al., 2001). In contrast, subpallial progenitor cells express TF such as Dlx1/2, Nkx2.1, Mash1, Pax6 etc. Derived neuroblasts migrate and reach their destination, and during this process they will coexpress other TFs, such as Mash1 and Dlx5/6, involved in the production and differentiation of GABAergic neurons (Osório et al., 2010; Li et al., 2017).

1.3. Pallial amygdala

1.3.1. Basolateral nuclear complex of the amygdala (BLA)

BLA complex (also known as deep nuclei) is composed of the lateral nucleus (LA), basal or basolateral nucleus (B or BL) and the accessory basal or basomedial nucleus (AB or BM) (Pitkanen et al., 1995; Swanson and Petrovich, 1998). The LA makes the dorsal part of the BLA, and can be subdivided into dorsolateral, ventrolateral and medial subdivisions. The basal nucleus is on the ventral side of the LA and, based on the cellular composition, it is further subdivided into: 1) anterior subdivision (populated by the magnocellular neurons) and the posterior subdivision (populated by the parvocellular neurons). The AB is located ventrally to

the basal nucleus and based on the cell types can be further divided into magnocellular, intermediate and parvocellular subdivisions.

In mammals, the BLA complex contains mostly glutamatergic projection neurons and the rest (approximately 10-20%) are GABAergic inhibitory interneurons (Pitkanen et al., 1997). The BLA receives thalamic input and is extensively and reciprocally connected with the hippocampus, the neocortex, the insular cortex, the olfactory cortex and the perirhinal cortex, and it projects to nucleus accumbens, the centromedial amygdala and the bed nucleus of the stria terminalis (Pitkanen et al., 1997; Swanson and Petrovich, 1998; Calhoon and Tye, 2015).

1.3.2. Cortical amygdalar areas

The cortical amygdalar areas (also known as cortico-like nuclei or superficial nuclei) owe their name to the histological characteristics similar to those of the cortices (they are positioned on the brain surface and contain cell layers). This complex is composed of the nucleus of the lateral olfactory tract (NLOT), bed nucleus of the accessory olfactory tract (BAOT), and several cortical amygdalar areas that include the anterior cortical area (CoA or ACo), the posteromedial cortical area (PMCo, sometimes referred as posterior cortical nucleus or CoP), and the posterolateral cortical area (PLCo, sometimes referred as periamygdaloid complex or PAC) (Swanson and Petrovich, 1998). In frontal sections, the NLOT is located at the rostral part of the cortical amygdala and on its lateral side is the CoA, which continues caudally. The caudal part of this complex is the CoP, bordering with the amygdalohippocampal area deeply (AHi or AHA, also named posterior amygdala) and the PAC area laterally (Pitkanen et al 1995; Swanson and Petrovich, 1998). The cortical amygdalar areas are mostly located superficially to BLA and AHi. Like the BLA, the cortical amygdalar areas contain glutamatergic projection neurons. They receive olfactory input and project to the BLA (in particular, the AB), the AHi, the medial amygdala and the central amygdala.

1.4. Developmental-based organization of the pallial amygdala of mammals

In mammals, the pallial amygdala derives from caudal parts of the so-called ventral pallium and from the ventrocaudal pallium and it comprises the basolateral nuclear complex (BLA) and some cortical amygdalar areas (reviewed by Medina et al., 2017, 2021). Cells in these areas originate from the pallial proliferative zone that expresses Dbx1 (ventral pallium) or Emx1 (ventrocaudal pallium), among other transcription factors (TFs) (Medina et al., 2004, 2017).

During differentiation they express the TF Tbr1, but also Lhx9 and COUP-TFII (Abellán et al., 2009; Tang et al., 2012). It appears that cells derived from Dbx1-expressing progenitors show a trend to locate in anterior parts of BLA and cortical areas of the amygdala (as shown by studying Dbx1-LacZ and Dbx1-GFP reporter mice; Puelles et al., 2016), while cells derived from Emx1 progenitors concentrate caudally but distribute throughout the whole pallial amygdala (Gorski et al., 2002; these cells presumably originate in the ventrocaudal pallium, as discussed by Medina et al., 2017).

The classical divisional scheme of BLA and cortical areas of the amygdala explained in the previous section has been challenged based on DiI radial mapping and gene expression patterns during development, leading to the proposal of five radial histogenetic subunits in the caudal pallium that produce different subdomains of LA, B/BL and AB/BM, each with different cortical areas at the surface (García-Calero et al., 2020). Moreover, based on its development, location and connections, the AHi is also considered a subdivision of the amygdala, which belong to the same radial unit than the PMCo (Swanson and Petrovich, 1998; García-Calero et al., 2021). This new divisional scheme of the pallial amygdala opens new venues for more accurate studies on the connections and functions of neurons derived from each radial unit. Different embryonic origins may help to explain the finding of distinct subpopulations of projection neurons within BLA (for example, Hagihara et al., 2021; Zhang et al., 2021). However, since connectivity studies on the amygdala did not consider this new divisional scheme, in the description below we follow the classical view.

1.5. Subpallial amygdala

1.5.1. Centromedial amygdalar nuclei

The centromedial nuclear complex is composed of the central (CeA) and medial (Me) amygdala. The CeA is located in the rostral part of this nuclear complex and borders the BLA laterally, the caudal pole of the caudate-putamen complex dorsally, and the globus pallidus (GP) and stria terminalis medially. The CeA can be further divided into capsular subdivision (CeC), lateral subdivision (CeL), intermediate subdivision (CeI) and medial subdivision (CeM) (Jolkkonen et al 1998). The Me is located ventromedially with respect to the CeA, and medially with respect to the ACo and the AB. The Me is divided into anterior (MeA), posterodorsal (MePD) and posteroventral (MePV) subdivisions (Canteras et al., 1995). Both the CeA and the Me are rich in GABAergic neurons and give rise to inhibitory projections to the preoptic area,

hypothalamus and brainstem, with differences in the targets of CeA versus Me (Swanson and Petrovich, 1998; Swanson, 2000). However, the medial amygdala also contains glutamatergic projection neurons (Csáki et al., 2000; Johnson et al., 2021; Morales et al., 2021).

1.5.2. Accessory amygdalar nuclei

The amygdalar complex also includes the anterior amygdalar area (AAA) and the intercalated nuclei (ITC). The AAA contains dorsal and ventral subdivisions, located dorsal or medial to NLOT, respectively. The intercalated nuclei are small clusters of GABAergic cells, mostly interposed between BLA and CeA, located adjacent to the fibers of the external and amygdalar capsules. They act as intermediaries between the prefrontal cortex and the BLA with either the CeA or the Me (Paré and Smith, 1993, 1994; Paré et al., 2004; Paré and Durvaci, 2012).

1.6. Extended amygdala

The centromedial amygdala nuclei are continuous rostromedially, through the substantia innominata, with the bed nuclei of the stria terminalis (BNST or BST), forming the centromedial extended amygdala (Alheid and Heimer, 1988). This was based on the following observations.

Knowing that amygdala innervates the sublenticular regions of the substantia innominate (i.e. the area below the globus pallidus and putamen, classically known as lenticular nuclei) and the BNST, it was proposed by Alheid and Heimer (1988) that the centromedial amygdala should be extended to the so-called substantia innominate, partially overlapping the ventral pallidal areas and the corticopetal cell groups, where afferent and efferent connections of the centromedial amygdalar complex project. Thus, a term "extended amygdala" was coined and it is based on the following discoveries: 1) the sublenticular substantia innominata is innervated by projections originating from the medial and central amygdala that merge with the BNST. There are two subdivisions: the medial amygdala is continuous and projects to the ventral corridor of sublenticular cells, and both preferentially innervate the medial bed nucleus of the stria terminalis (BSTM); and the central amygdala is continuous and preferentially projects to the dorsal sublenticular cells, and both preferentially project to the lateral bed nucleus of the stria terminalis (BSTL) (Alheid and Heimer, 1988). 2) Cells along each of these cell subcorridors share similar neurotransmitter and neuropeptide contents, distinguishing the two divisions of the extended amygdala: for example, the central amygdala-BSTL cell corridor

is enriched in galanin, but the medial amygdala-BSTM cell corridor is not (Alheid and Heimer, 1988), Therefore, the term medial extended amygdala was coined to unite the nuclei of the medial cell corridor (medial amygdala, medial sublenticular extended amygdala and the medial bed of the stria terminalis or BSTM). Similarly, the central extended amygdala includes central amygdala, central sublenticular extended amygdala and lateral bed nucleus of the stria terminalis or BSTL. Together medial extended and central extended divisions are collectively known as extended amygdala (Swanson and Petrovich, 1998; Alheid, 2003; Heimer, 2003). Although there is a trend for a segregation of connections separating central and medial extended amygdala, both subcorridors interact by way of projections from the medial amygdala and from the BSTM to the medial part of the central amygdala (Canteras et al., 1995; Bienkowski et al., 2013).

It has been suggested that the neurons along extended amygdala cell corridor behave like striato-pallidal projection systems (Alheid and Heimer, 1988; Swanson, 2000). According to this view, the central and medial amygdalar nuclei are striatal-like, while the BST nuclei are pallidal-like. However, developmental data show that this is more complicated than that, as explained next.

1.7. Developmental-based organization of the centromedial extended amygdala of mammals

Developmental data point to the existence of multiple cell subcorridors along the central and medial extended amygdala, each consisting of cells of different embryonic origin and molecular profile (García-López et al., 2008; Bupesh et al., 2011a,b; reviewed by Medina et al., 2017). These cellular subcorridors are partially intermingled, making it difficult to study their specific connections and functions using classical methodological approaches. Based on developmental data, some of the subcorridors contain striatal-like cells (derived from the lateral ganglionic eminence) while others contain pallidal-like cells (derived from the medial ganglionic eminence and diagonal/peduncular domain), preoptic-like cells or cells with other origins (see more details below). Moreover, each cell subcorridor extends from the centromedial amygdala to BSTL/BSTM, providing support for the existence of a cellular continuum as proposed by Alheid and Heimer (1988), but consisting of multiple subcorridors (Bupesh et al., 2011a,b; Medina et al., 2017). These data also help to integrate the ITC and the anterior amygdala as part of the extended amygdala.

Regarding the central extended amygdala, we have the following subcorridors with dorsal striatal, ventral striatal or pallidal origins: A) Neurons from the dorsal lateral ganglionic eminence (LGEd or dorsal striatal embryonic subdivision), which express Pax6, mainly colonize lateral parts of the central extended amygdala, including ITC, dorsal anterior amygdala, and capsular/lateral parts of the CeA (CeC, CeL), but a few also reach sublenticular parts of the extended amygdala and the BSTL (Bupesh et al., 2011b). In addition, ITC also includes many cells derived from LGEd expressing FoxP2 (Kaoru et al., 2010). B) Neurons from the ventral lateral ganglionic eminence (LGEv or ventral striatal embryonic subdivision), which express Islet1, mainly colonize laterointermediate parts of the central extended amygdala, including CeL and CeM (Waclaw et al., 2010; Bupesh et al., 2011b), but some also reach sublenticular parts of the extended amygdala and BSTL (Bupesh et al., 2011b). C) Neurons with pallidal origin (derived from the medial ganglionic eminence or MGE), which express the TF Nkx2.1, mainly colonize medial parts of the central extended amygdala, including BSTL (Xu et al., 2004; García-López et al., 2008). However, a group of them, originating in the ventrocaudal MGE (also named anterior peduncular or diagonal domain), which expresses Lhx6, also reach CeM and CeL (García-López et al., 2008; Bupesh et al., 2011b). These different embryonic origins may explain the different subtypes of GABAergic neurons found in the central extended amygdala, as explained in a different section.

With respect to the medial extended amygdala, it was shown to receive neurons mainly from pallidal and preoptic progenitor zones of the subpallium, extending from the ventral anterior amygdala and medial amygdala to the BSTM (García-López et al. 2008; Hirata et al., 2009; Carney et al., 2010; Bupesh et al., 2011a), which would account for the GABAergic cells found in these areas. During development, ventrocaudal pallidal (or diagonal) cells express Lhx6, while preoptic cells express Shh. Both cell types populate the anterior and posterior parts of the medial amygdala but are segregated to distinct subdivisions in the posterior part: MePD is rich in Lhx6 pallidal cells, while MePV is rich in Shh preoptic cells. There is a trend for segregation of both cell types also in BSTM, although this is less clear than in MeP (García-López et al. 2008; Medina et al., 2017). In addition, the medial amygdala and BSTM were found to include subpopulations of glutamatergic cells, which originate in the ventral/ventrocaudal pallium (García-López et al., 2008; Bupesh et al., 2011a), the supraopto-paraventricular domain of the hypothalamus (SPV; García-Moreno et al., 2010), and the prethalamic eminence (PThE; Abellán et al., 2010; Ruiz-Reig et al., 2017). It used to be thought that GABAergic subpallial neurons of the medial amygdala and BSTM are predominant, while

glutamatergic cells are less abundant (Swanson and Petrovich, 1998). However, this view has been challenged, as the glutamatergic neurons are also very abundant in the medial extended amygdala (Hong et al., 2014; Morales et al., 2021). Moreover, the majority of them appear to originate in a new radial embryonic division of the telencephalon, located near the border with the hypothalamus, called the telencephalon-opto-hypothalamic domain or TOH (Morales et al., 2021). The TOH was previously included as a dorsal part of the hypothalamic SPV based on its expression of the TF Orthopedia (Otp) (Bardet et al., 2008; Morales-Delgado et al., 2011). However, during development the TOH expresses the telencephalic TF Foxg1 in the ventricular zone, and derived cells from TOH coexpress both Foxg1 and Otp and are aligned with radial glial fibers to reach the posterior BSTM and the medial amygdala (Morales et al., 2021). These cells are glutamatergic, and in the medial amygdala they mostly concentrate in the ventral part of the MeA (MeAV), and in the medialmost part of MeP. In addition, minor TOH-derived cell subpopulations spread dorsally and laterally reaching other parts of the medial amygdala and the pallial amygdala, possibly by tangential migration (see more details in Morales et al., 2021). Interestingly, while GABAergic neurons of the medial amygdala with different origins and molecular profiles promote different types of social interactions (Lischinsky et al., 2017; discussed by García-López et al., 2008; Medina et al., 2011, 2017, 2022; Sokolowski and Corbin, 2012), glutamatergic neurons appear to promote asocial behavior (Hong et al., 2014; Johnson et al., 2021).

1.8. The mammalian amygdala as a multisensory center

Brain functions are mirrored though connections via afferent and efferent projections between different brain regions. In the amygdala, each nucleus has a distinct set of inputs and outputs that define the functions in which that amygdalar nucleus is involved. Connections of the amygdala have been investigated using tract tracing studies, and the function of each pathway were commonly investigated after lesions, by pharmacological manipulation, or by analyzing functional activation in the brain following different behavioral reactions (for example, in front of exposure to a predator odor, as done by Choi et al., 2005). More recently, this is also studied using optogenetic modulation of the activity of specific cells and pathways (Ciocchi et al., 2010). These studies are allowing to identify the neuroanatomical bases of emotion and behavior.

The lateral nucleus of the amygdala (LA, which belongs to the BLA complex) is recognized as a sensory system gatekeeper (Erlich et al 2012). It is a convergent point of the sensory systems (auditory, somatosensory, olfactory, taste and visual systems), coming from the medial geniculate and paralaminar thalamic nuclei (previously known as posterior intralaminar nuclei; Linke et al., 1999, 2000) and from several cortical areas, including the olfactory cortex, the insular cortex and associative sensory areas of the neocortex (reviewed by Swanson and Petrovich, 1998; Phelps and LeDoux, 2005). The LA integrates the information and projects to the central amygdala directly or by way of B/BL and the intercalated amygdalar cells. However, the central amygdala also receives direct sensory input from the paralaminar thalamus (including several modalities), the parabrachial nucleus (including pain and taste) and the solitary nucleus (taste and visceral) (Swanson and Petrovich, 1998), and is considered an integration center for fast responses related to threatening stimuli (Amaral, 2003).

Auditory inputs have been studied the most thoroughly, especially as a part of fear conditioning (Phelps and LeDoux, 2005). Briefly, the auditory inputs originating from the inferior colliculus reach the auditory thalamus (medial geniculate nucleus and paralaminar nuclei), which then projects to the lateral amygdala (but note that the projection also reaches the basomedial amygdala) and the auditory cortex. Thalamic projections to the extralemniscal areas encode frequency properties and provide rapid and imprecise auditory signal processing. More precise auditory processing is provided through the inputs from the cortical areas (auditory cortex and associative regions) which allow a more detailed sound encoding (Choi et al 2021). Importantly, thalamic projections to the lateral amygdala from the medial geniculate and paralaminar nuclei are not only unimodal, but also include multimodal inputs (from thalamic neurons responding to both auditory and somatosensory stimuli), which is needed for the associative learning involved in auditory fear conditioning (Linke et al., 1999). The association that takes place in the lateral amygdala is critical for evaluation of threats and for providing an emotional significance to stimuli (affective valence), while the reciprocal connections of the lateral amygdala with the neocortex (including prefrontal cortex), hippocampal formation, and entorhinal cortex are important for contextualization of the dangers, and for emotional saliency-driven modulation of attention, memory formation, and decision making (Richter-Levin, 2004; Roozendaal et al., 2004; Phelps and LeDoux, 2005; reviewed by Medina et al., 2017, 2022).

Somatosensory information reach amygdala from the thalamus (as mentioned above; Linke et al., 1999, 2000), but also via the insular cortex that has direct projections to all three nuclear complexes, BLA, CeA and Me. This input includes pain related information, which is relevant

for conditioning. The lateral nucleus projects to CeA directly and by way of B/BL and ITC. In mouse, a pathway originating from GABAergic neurons of the central nucleus and targeting glutamatergic neurons in the parafascicular thalamic nucleus, which in turn projects to the somatosensory cortex (SC), is involved in chronic pain (Zhu et al. 2019).

Visual cues from the paralaminar thalamus (including the suprageniculate nucleus; Linke et al., 1999, 2000) and from the associative visual cortex terminate in the dorsal portions of the LA, CeL and the basomedial amygdalar nucleus. As for the auditory, visual thalamic input to the BLA includes unimodal and multimodal inputs. In humans, the BLA is reciprocally connected with the fusiform gyrus, which is involved in facial recognition (Amaral, 2002; Herrington et al., 2011). One study suggests that the amygdala increases the neural activity in the fusiform gyrus allowing visual cues of affective valence to reach awareness (Duncan et al. 2007).

Olfactory projections to the amygdala originate in the main and accessory olfactory bulbs, which project to the periamygdaloid and cortical amygdalar nuclei, as well as the medial nucleus of the amygdala (Mohedano-Moriano et al., 2007; Pro-Sistiaga et al., 2007; Martínez-Marcos, 2008). The projections include vomerolfactory pathways, involved in processing pheromonal information, which are relevant for regulating social behaviors. The olfactory or piriform cortex also has efferent connections to all three above mentioned parts of the amygdala, therefore cementing the role of olfaction in the amygdaloid complex (Abellan et al. 2013). The integration that takes place in the medial amygdala is important to modulate social and feeding behaviors (Swanson, 2000; Choi et al., 2005), while the projections from the cortical amygdalar areas to the orbitofrontal cortex contribute to the impact of odors on affective states (Soudry et al., 2011). Functionally, the amygdala provides salience of the olfactory signals by refining the olfactory stimuli via amygdalo-corticofugal projections (Oboti et al. 2020). In COVID-19 patients suffering from the loss of smell function, amygdala showed upregulation of the interferon - related neuroinflammation genes and specific profile of downregulation in synaptic expression (CAMK2B and COL1A2 being two key genes in neuroinflammatory response) (Piras et al. 2021).

Gustatory associative areas of the cortex show efferent connections to the dorsal LA, basal and central amygdalar nuclei (Lin et al. 2021). Moreover, projections from the solitary tract (notably taste, chemo and mechanoreceptors of the visceral afferent pathway) target the CeL (Riccardo et al. 1978, van der Kooy et al. 1984). The gustatory cortex (in the insular cortex)

and the BLA complex are involved in deciphering the palatability of the food. It is postulated that the BLA organizes the behavioral taste dynamics (Lin et al. 2021). LA and BLA also play a role in taste aversion, and by projection to CeA and BSTL, this pathway is also involved in regulating ingestion (Swanson, 2000). The latter pathway is also modulated by olfactory information, and this is at least partially mediated by projections from olfactory-recipient areas of the amygdala, including cortical areas and medial amygdala, to CeA (Swanson and Petrovich, 1998; Swanson, 2000).

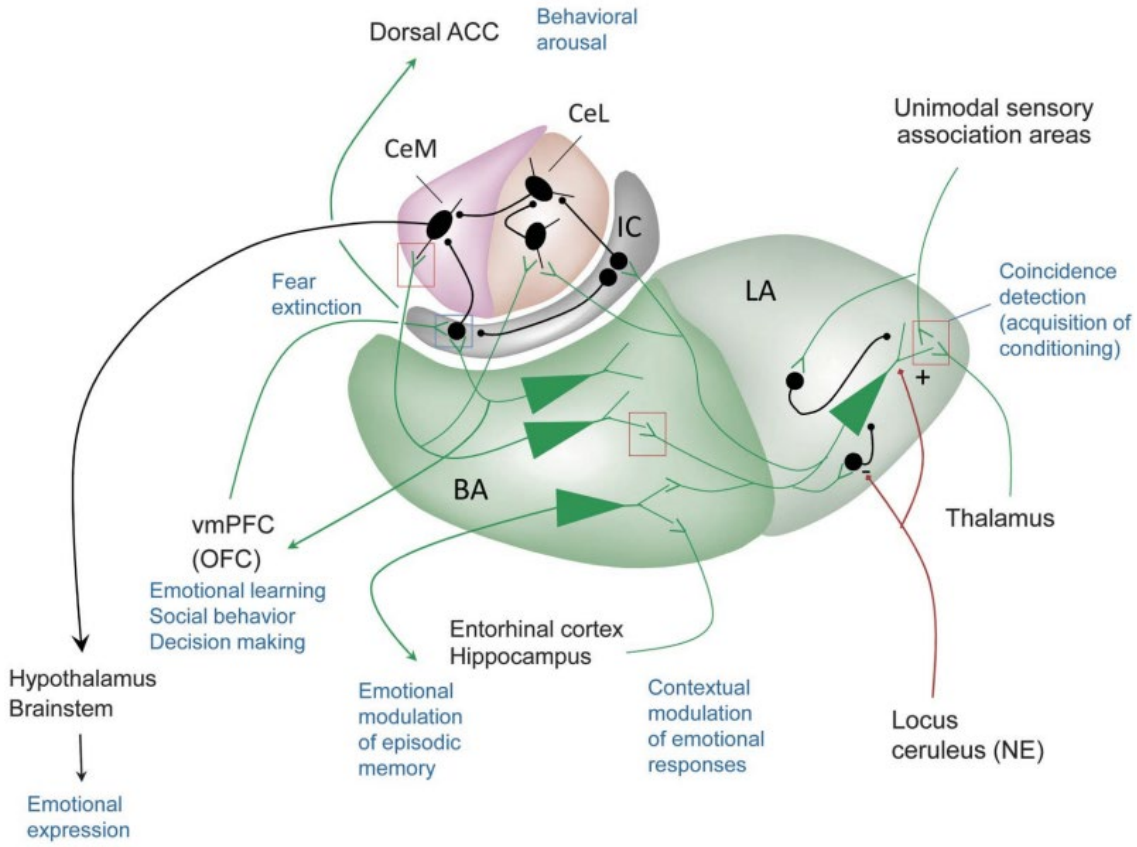
In summary, the amygdalar complex receives sensory cues from all the modalities. Traditionally, the basolateral complex is considered the main input division of the amygdala (with the basal nucleus being the main target of the prefrontal cortex) while the centromedial nuclei are considered the main output division. However, it should be noted that it is not a simple relay center of signals but a structure with multiple internal and external circuitries that evaluates and integrates the sensory information and assigns appropriate emotional dimension, valence, intensity, and approachability (Simic et al. 2021). Moreover, the amygdalar complex is able to modulate how the rest of the brain works on the basis of the emotional saliency of stimuli, the context, and previous experience.

1.9. Role of the mammalian amygdala in fear

One of the first experiments describing the role of amygdala comes from the lesion studies of the temporal lobe in monkeys first performed in the 1888 by Brown and Schafer where the dissociation of the temporal lobe led to the docile behavior in monkeys. Lesions in the parts that correspond to the amygdalar complex led to the absence of fear, asocial behavior, hypersexuality, and impaired visual processing. These behavioral symptoms, later named "taming effect" were observed in different animal models, provided the insight in the role of amygdala in the emotional processing and recognition of fear. Unlike other emotional responses, fear (whether conditioned or unconditioned) can be quantitatively measured with the parameters of the autonomic nervous system: increase in the heart rate, elevation of the blood pressure, release of the stress-related hormones and behavioral changes (startle, vocalization, freezing) (Phelps and LeDoux, 2005). These fear responses are evolutionary conserved and electrical stimulation of the amygdala causes fear responses across different species. These findings opened the venue for investigating the role of amygdala in fear and fear conditioning, by means of laboratory experiments mainly using rats (Dolan et al. 2002).

Fear conditioning is happening in three phases: 1) acquisition of the conditional stimulus (CS) when it becomes associated with the noxious stimulus; 2) memory trace formation when the noxious signal was paired with the CS; and 3) retrieval, when the repetition of the noxious signal causes a defensive response (Krabbe et al 2018, Cooper et al 2021). Early lesion studies showed that the amygdala is crucial in the second phase of the fear conditioning, thus implying its role in fear learning. CS and noxious cues converge in BLA (in particular, in LA), where the signal is processed. Repetitive exposure to the associated CS and noxious stimulus causes activation of these circuits in the BLA implying learning, and that the information is possibly stored there as well (Rabinak et al. 2009). Projections from the BLA to the CeA are activated during the conditioned fear response, leading to freezing, flight or defensive behaviors. An overview of this circuits is given in the scheme 1.

In rodents, the central amygdala contains two distinct GABAergic cell populations that become active (on cells) or inactive (off cells) during the conditioned fear response (Ciocchi et al., 2010; Haubensak et al, 2010). These two cell types inhibit each other and project mainly unidirectionally to output cells, thus providing a sophisticated regulation of stress (Ciocchi et al., 2010; Haubensak et al, 2010; reviewed by Medina et al., 2017, 2022). These two cell types express either protein kinase C-delta (PKCd; many of these also enkephalin or ENK) or somatostatin (SST) (Haubensak et al., 2010; Pompolo et al. 2005). Based on their location, these two neuron subtypes were suggested to originate in different embryonic domains of the subpallium: PKCd/ENK (off) neurons were suggested to originate in the dorsal striatal embryonic division or LGEd, since during development they clearly overlap with LGEd-derived cells that express the transcription factor Pax6; in contrast, SST (on) neurons were shown to originate in the ventrocaudal pallidal (diagonal) embryonic domain, which produces Nkx2.1/Lhx6 cells during development (Bupesh et al., 2011b; Medina et al. 2017, 2022). The central extended amygdala (CeA and BSTL) also contains another GABAergic neuronal population that expresses corticotropin-releasing factor (CRF) and is involved in sustained fear responses, similar to anxiety (Davis et al., 2010). These have been suggested to originate in the ventral striatal embryonic division (LGEv; Bupesh et al., 2011b). Thus, it appears that neurons with different neuropeptide content and function have different embryonic origins. However, the central extended amygdala contains additional neuron subtypes, including some that coexpress several neuropeptides (McCullough et al., 2018), and more studies are needed to investigate the function and embryonic origin of the different neuron subtypes of the central amygdala.



Scheme 1: Main subdivisions of the amygdala in mammals in the context of fear conditioning. Pyramidal neurons in the lateral nucleus (LA) play a crucial role by receiving excitatory inputs conveying conditioned and unconditioned stimuli, driving synaptic plasticity during conditioning. GABAergic inhibition locally regulates this process, while monoamine neurotransmitters, such as norepinephrine (NE) released from terminals originating in the locus coeruleus, enhance excitatory synaptic plasticity in LA neurons through direct and indirect effects on GABAergic interneurons. LA neurons project to the basal or basolateral nuclei (BA), which, in turn, influence the output neurons of the centromedial nucleus (CeM) through direct or indirect pathways involving GABAergic neurons in the intercalate mass (IC) or centrolateral nucleus (CeL). Control over the CeM involves multiple disinhibitory networks mediated by distinct subsets of GABAergic neurons in the IC and CeL. A subset of BA neurons project to the dorsal anterior cingulate cortex (dACC), which is engaged in fear conditioning and behavioral arousal. Fear extinction involves an interactive network comprising the amygdala, ventromedial prefrontal cortex (vmPFC) including the orbitofrontal cortex (OFC), and anterior hippocampus. The vmPFC contributes to fear extinction through an excitatory input from the OFC to GABAergic neurons in the IC, which modulate the BA inputs to the CeM. (adapted from Benarroch et al 2015)

1.10. Role of the mammalian amygdala in stress and anxiety

Due to the overlap between the brain networks that regulate fear conditioning and stress responses (acute and sustained/chronic), the amygdala is also a focus in studying chronic stress

and anxiety. In humans, chronic stress and anxiety are mental states of high apprehension in the absence of immediate threats (Woo et al. 2021). Anxiety disorders represent the most common psychiatric disorder affecting approximately 28% of the population (Penninx et al 2021). Although the role of amygdala in emotional processing (especially fear processing) is well studied in rodents and primates, complete understanding of anxiety and stress circuits remains unclear, largely due to extensive inter and intra connections in amygdalar nuclei, but also to the high cellular heterogeneity in each single nucleus or area of the complex. So far, the connections between the amygdala, prefrontal cortex (PFC) and ventral hippocampus (vHPC) and their role in stress regulation and memory formation are well studied (Roozendaal et al., 2004; Phelps and LeDoux, 2005). Briefly, the amygdala serves as a main relay center between the PFC and vHPC, having an extensive network of reciprocal connections with the two structures. The bidirectional connections between amygdala (BLA) and hippocampus are the basis for a highly dynamic regulation of memory-related long-term plasticity in both directions, down-top and top-down (Richter-Levi, 2004). Projections from the BLA neurons that target the vHPC originate in both anterior and posterior parts of the BLA. These projections play a prominent role in behavioral aspects of stress response; optogenetic suppression of either anterior or posterior part of the BLA suppresses anxiety/stress response in the maze exploration behavioral experiments. On the other hand, optogenetic stimulation of the vHPC neurons that project to the BLA reduces the anxiety response in the same behavioral paradigm (Besteher et al 2019). This BLA-vHPC pathway is considered a stress mediating pathway that complements the BLA-CeA intraamygdalar pathway, which it is postulated that is involved in the aversive and anxiety like behaviors (Duvarci et al 2014, Sun et al. 2020). Corticotropic releasing factor (CRF) neurons of CeA and BSTL are known to be critical for anxiety, and regulate the activation of HPA (Davis et al., 2010). In addition, CRF expressing neurons in the CeA were recently found to project to the locus coeruleus (Wolfe et al. 2019), which is part of the ascending arousal system (Saper and Fuller, 2017). Alterations in these cells and their projections lead to anxiety disorders (Davis et al., 2010), which causes appear to be multifactorial including exposure to early life stress (Spencer, 2017). Moreover, the CeA and the associated BSTL are reciprocally connected with the ventral tegmental area, which contains dopaminergic neurons that play a critical role in motivation and reward (Swanson, 2000; Hasue and Shammah-Lagnado, 2002). A dysregulation of these functional pathways may be behind some mood and anxiety disorders (Lebow and Chen, 2016).

Chronic stress is known to impair long term memory formation and working memory, and this is correlated with a decrease in dendritic branches and spines in the hippocampus and PFC (Roozendaal et al., 2004; Kim et al., 2015). Unlike the hippocampus and the PFC, upon stress the amygdalar complex can exhibit significant structural remodeling, with increased dendritic branches and spines at least in males (Price and McCool, 2022). Prolonged exposure to stress is known to increase the spine density in the pyramidal neurons of the BLA in males (but not females), while acute restraint stress paradigm delays the synaptogenesis by up to 10 days and shows an increase in the anxiety-like behavioral patterns (Price and McCool, 2022). Chronic stress exposure shows irreversible dendritic hypertrophy and spine outgrowth in the BLA interneurons, dendritic hypotrophy in the MeA and overall increase in size of the BLA complex (Zhang et al. 2020, Sun et al. 2020).

Chronic restraint stress (as well as chronic cold stress) leads to the overall disinhibition of the BLA complex (Zhu et al. 2018). However, the exact causes of this are unclear. One of the explanations could be a sustained release of glutamate from the dorsomedial PFC (part of the top-down amygdala regulation) or the hypoactivity of the inhibitory neurons in the amygdala. This shift in the excitatory state of amygdala, via optogenetic experiments, showed the importance of the inhibitory interneural networks of amygdala in the stress and anxiety like behavior (Zhang et al. 2019). The role of inhibitory-excitatory neural network in the amygdala, therefore, is essential for understanding the stress response in healthy and unhealthy conditions. Interactions between excitatory and inhibitory neurons do not only occur in the BLA, but also in the extended amygdala. The latter mostly contain inhibitory neurons that are regulated by excitatory inputs from BLA and other parts of the pallium (Swanson and Petrovich, 1998). However, some data also point to the existence of glutamatergic neurons in the extended amygdala, including the central amygdala, the medial amygdala, and the BST (Csáki et al., 2000; Hong et al., 2014; Morales et al., 2021). Better knowledge of the distribution and the origin of these neurons in the amygdalar complex could help to elucidate the complete brain networks that regulate stress and anxiety. If this is done in different species, including non-mammals, it could also provide meaningful insights into general organizing principles of the neural basis and the evolution of the stress response (Hennequin et al. 2017).

1.11. CRF system

As noted above, a complementary system that needs to be considered for studying stress and anxiety is the corticotropin releasing factor network, comprised of corticotropin releasing factor (CRF), its receptors (CRFR1 and CRFR2) and CRF binding protein (CRFBP) (Vasconcelos et al. 2020).

CRF is a peptide hormone that is released in stress in the portal blood vessels connecting the median eminence with the adenohypophysis. The origin of CRF are parvocellular neurons in the paraventricular nucleus of the hypothalamus, which project to the median eminence and release the hormone in the hypophyseal portal vein network. Through this vein system, it is carried to the anterior part of the pituitary gland (adenohypophysis) where it stimulates the release of adrenocorticotropic hormones (ACTH) in the blood stream. Their final target is the adrenal gland which releases cortisol into the blood stream. Thus, the axis between hypothalamus via pituitary and adrenal gland is called hypothalamic-pituitary-adrenal (HPA) axis (Nestler et al., 2008; Herman et al. 2016). Its role in mediating the stress response through activation of the HPA is well established.

However, CRF neurons and circuits are also known in other brain regions and systems: nucleus accumbens, central nucleus of the amygdala, sublenticular and rostral parts of the substantia innominata (in areas now included as part of the extended amygdala), BST (mainly its lateral part), dorsomedial and supramammillary hypothalamus, and several brainstem nuclei known to be part of the ascending reticular activating system (mainly parts of the periaqueductal gray, parabrachial nucleus, and locus coeruleus), among other areas (Swanson et al., 1983). These Crf cells give rise to complex ascending and/or descending pathways (Swanson et al., 1983). Minor Crf expressing neuron subpopulations are also found in the hippocampus and superficial areas of the neocortex, which appear to be local-circuit cells or interneurons (Swanson et al., 1983; Gallopin et al., 2006; Chen et al., 2010). This wide array of CRF networks implies a role in modulating different brain functions, but the effects of CRF are complex and depend on the neural system, dose and time of exposure, and the age (Chen et al., 2004, 2010; Vandael et al., 2021). For example, in the mature hippocampus, short-term application of CRF leads to an activation of principal neurons, increase of long-term potentiation (LTP, a process related to memory formation), and promotion of adult neurogenesis (Chen et al., 2010; Koutmani et al., 2019; Vandael et al., 2021). However, application of CRF during longer time leads to decrease in LTP, and this contributes to the detrimental effects of chronic stress on hippocampal

structure and function, and on memory formation (Vandael et al., 2021). During hippocampal development, CRF is transiently expressed in Cajal-Retzius-like cells and was shown to play a role in dendritic differentiation of principal neurons: dendritic arbors of hippocampal neurons become more exuberant when CRF transmission is inhibited (Chen et al., 2004). This suggests that over-exposure to CRF during critical developmental periods may have a negative impact on hippocampal function, explaining the negative effects of early-life stress.

CRF acts via two receptors and its function can be modulated by a binding protein (Schreiber et al. 2018). In rats, CRF receptor 1 or its mRNA (Crfr1) is expressed in many areas of the central nervous system, including strong expression in the olfactory bulb and cortex, many areas of neocortex, some subdivisions of the hippocampus, basolateral and medial amygdala, dorsomedial hypothalamus, adenohypophysis, superior colliculus, cerebellar cortex and pontine nuclei, among other areas (Chen et al., 2000, 2004). Crfr1 expression is low or moderate in the septum and BST. In contrast, expression of CRFR2 mRNA (Crfr2) is more restricted, occurring mostly in specific parts of the pallium, subpallium and hypothalamus (Zuo et al., 2021). Crfr2 is strongly expressed in the lateral septum, cortical amygdala and supraoptic and ventromedial hypothalamus, and it is moderate in the BST, medial amygdala, olfactory bulb, parts of the hippocampus, and paraventricular hypothalamus (Dedic et al. 2018).

Both receptors belong to the G coupled family receptors but exert opposing intracellular and physiological effects. It is often considered that CRFR1 has an anxiogenic effect, while the CRFR2 has an anxiolytic effect (Ventura-Silva et al. 2020), but this is more complex since it depends on the specific areas, cells and circuits (Henckens et al., 2016). CRF binding protein (CRFBP) has sequestering effect on the CRF; it binds CRF with high affinity and decreases the bioavailability of the CRF in the synaptic cleft. Another mechanism by which CRFBP modulates the CRF system is allosteric modification of the CRFR2 where the CRFBP binds CRFR2 intracellularly and promotes its release on the surface (Slater et al. 2016, Curley et al. 2021).

All members of the CRF gene (Crf) family, including their isoforms, show high level of evolutionary conservation (Cardoso et al., 2016). The Crf network includes five paralogous genes in the Gnathostomata taxa: Crf (also known as Crf1), Crf2 (lost in placental mammals, teleost fish and chicken [although the latter was recently challenged by the finding of Crf2 in chicken by Bu et al., 2019]), urocortin1 (Ucn1, known as urotensin 1 in fish; this is missing in chicken), urocortin2 (Ucn2) and urocortin 3 (Ucn3) (Lovejoy et al. 2013; Cardoso et al., 2016, 2020). It appears that genes of this family are organized in two subgroups: one containing Crf1,

Crf2 and Ucn1 genes, and another one containing Ucn2 and Ucn3 genes (Cardoso et al., 2016). All the peptides encoded by these genes can bind to CRFR1 and/or CRFR2 and have similar pharmacological actions in different physiological processes related to stress and anxiety (revised by Cardoso et al., 2016). CRFR1 are expressed by principal neurons of the hippocampus and mediate the action of endogenous CRF during the stress response, as explained above. Their role in stress response is also exhibited by their ability for modulating excitatory activity in the amygdala, as explained in next section.

1.12. CRF systems involving the amygdala

Amygdala is both a target and the site of synthesis of CRF. CRF synthesis was observed in the central nucleus, which contains a subpopulation of CRF -containing neurons, but the receptors distribution is low in this nucleus. The opposite stands for the basolateral complex, poor in CRF containing cells (Baumgartner et al. 2021). In the BLA, CRF disrupts the memory formation, indicating its role in synaptic plasticity, although the mechanism it not clear (Baumgartner et al. 2021). There is a significant overlap between the GABAergic and CRF expressing cells in the CeA, where this neuronal population coexpresses both and is responsible for a tonic GABAergic activity. This contrasts with the CRF activated presynaptic glutamate release while maintaining the levels of postsynaptic concentrations low in the same neuronal circuits (Kirby et al. 2008). Thus, CRF is able to modulate both GABAergic and glutamatergic transmission in CeA (Wolfe et al., 2019).

In rats, CRF cells of CeA were shown to be involved in fear learning and to project to the BSTL (Gray, 1993; Pomrenze et al., 2019a,b). The latter also contains a subpopulation of CRF projection neurons, which in turn target CRF cells of the paraventricular hypothalamic nucleus, as well as autonomic centers in the lateral hypothalamus and brainstem. This dense CRF producing network is responsible for the anxiety-like response (Davis et al., 2010). Studies in rodents show that anxiety-like behavior depends on the CRF projections from CeA to BSTL, on CRF cells of BSTL and on the activation of CRFR1 (Pomrenze et al., 2019); CRF released from the terminals of the CeA projections bind the CRFR1 in the BSTL, and prolonged stress exposure leads to the increase in the CRF receptors expression (Pomrenze et al. 2019). This could imply a positive feedback loop where chronic stress leads to the overexpression of the CRF and its receptors, while the activity of the other GABAergic neurons that are targets of the CRF terminals is reduced, leading to the overall hypoactivity of the cells in the BST.

Deletion of the α1-subunit from CRFR1-expressing neurons in the amygdala, BNST, and paraventricular nucleus resulted in a prominent anxiety behavior during the elevated zero maze and open field tests (Wang et al. 2020).

Dysregulation of the CRF system is observed in anxiety disorders, clinical depression, PTSD, and addiction, and thus this serves as a promising target for these disorders. However, although preclinical data clearly supported the role of CRF networks in the stress responses, the clinical studies using antagonist of the CRF receptors have mostly been unsuccessful. Experimental and clinical studies show low levels of the lasting symptom reduction and propose several reasons for such consequences, such as differences in CRF receptors expression between humans and experimental animals, and differences in organization and functional regulation of synapses between experimental animal models (mostly rodents) and humans. Moreover, prenatal stress induces changes in CRF expression, as well as in structure and function of hippocampus and amygdala (Chen et al., 2004; Wolfe et al.2019). These changes, happening during the development can also be transmitted to the future generations by means of epigenetic histone modifications (Lautarescu et al. 2020).

This implies that it is necessary to map out the distribution of neurons and colocation of the different receptors and proteins involved in the stress/anxiety response in different model animals to precisely understand what is evolutionary conserved and what is different, and to be able to extract general organizing principles that can help to understand better how the CRF systems mediate the stress response in all animals. This can be done by way of evolutionary developmental biology studies, which facilitate comparisons across species throughout ontogeny, and can help to fill the gap between the embryonic origin of neurons with their adult phenotype and functional networks in which they are engaged. Below I will focus on what is known in chicken and other sauropsids regarding their amygdala and the CRF systems in relation to the stress response, which study is the aim of this thesis.

1.13. The avian amygdala based on evo-devo

1.13.1. Avian pallial amygdala

Based on topological position and developmental genoarchitecture, the ventral and ventrocaudal pallia have been identified in sauropsids (birds and reptiles) and give rise to ventral and ventrocaudal parts of the dorsal ventricular ridge DVR (Puelles et al., 2000; Medina

et al., 2017, 2021). These include the nidopallium and arcopallium in birds, and corresponding parts of the anterior and posterior DVR in reptiles. In sauropsids, this structure contains Lhx9, COUP-TFII and/or Emx1 expressing neurons claimed to be homologous to those of the pallial amygdala of mammals, which appear to be highly conserved in amniotes (Puelles et al., 2000; Abellán et al., 2009; Chen et al., 2013; Desfilis et al., 2018; Medina et al., 2017, 2021). In contrast, pallial amygdala cells derived from Dbx1 progenitors are only found in mammals. Although the sauropsidian DVR and pallial amygdala derive from homologous pallial sectors and express similar sets of transcription factors during embryonic development, they also show a great morphological and molecular divergence later on, as revealed by a global transcriptome study in juvenile/adult chickens (Belgard et al., 2013). While differences exist, more recent single cell transcriptome studies in reptiles and zebra finches showed that glutamatergic neurons of DVR and pallial amygdala are highly similar (Tosches et al., 2018; Colquitt et al., 2021), especially at the level of transcription factor expression, but differences are related to activity-related genes (Colquitt et al., 2021). Regarding the activity-related genes, there is convergence in their expression between glutamatergic cells of DVR and those of the neocortex (Colquitt et al., 2021, 2022).

In spite of their divergence, the pallial amygdala and the DVR also show similarities at the level of connections and functions, likely inherited from the most recent common ancestor (reviewed by Medina et al., 2022). Like the BLA of mammals, the sauropsidian DVR is a multisensory area that receives input from the collothalamus (including subdivisions comparable to paralaminar, suprageniculate and medial geniculate nuclei of mammals), is widely connected with other parts of the pallium (including the dorsal pallium or Wulst and hippocampus), and projects to the subpallial amygdala, hypothalamus and brainstem (Bruce and Neary, 1995; reviewed by Medina et al., 2019, 2022). Moreover, the sauropsidian DVR, including nidopallium and arcopallium, is involved in several socio-emotional functions (Medina et al., 2022). For example, the avian arcopallium is involved in fear/avoidance behavior (Saint-Dizier et al., 2009), in food aversion (Tokarev et al., 2011), and social facilitation (Xin et al., 2017). The avian caudal nidopallium plays a role in associative learning and decision making (Güntürkün, 2005) and in value coding in relation to reward (Dykes et al., 2018). Some authors have discussed these findings in the context of comparing the DVR with the neocortex (for example, Güntürkün, 2005). However, the pallial amygdala (the natural homologue of the DVR based on their common origin) also plays a role in these functions (Pessoa et al., 2019; Medina et al., 2022). Thus, it makes more sense to do the comparison

between DVR and pallial amygdala in order to extract general organizing principles that we can extrapolate to different animals.

1.13.2. Avian extended amygdala

Previous studies from our laboratory using an evo-devo approach have identified a nuclear complex in the ventral telencephalon (the subpallium) of chicken and zebra finches that seems comparable to the central extended amygdala (EAce) of mammals based on their similarity in embryonic origin, transcription factor expression and topological location (Vicario et al., 2014, 2015, 2017; Pross et al., 2022). This region includes cell populations expressing the transcription factors Pax6, Islet1 or Nkx2.1 during development, and migration assays have shown that the Pax6 cells are derived from the dorsal striatal embryonic domain (Std, comparable to mouse LGEd), the Islet1 cells derive from the ventral striatal embryonic domain (Stv, comparable to mouse LGEv), while Nkx2.1 cells derive from the pallidal embryonic division (Pa, comparable to mouse MGE) (Vicario et al., 2014, 2015). Like in mammals, Pax6 cells derived from Std show a trend to occupy lateral and intermediate positions (capsular and peri-intrapeduncular subdivisions), Islet1 cells derived from Stv mostly occupy intermediate areas (peri-intrapeduncular and oval subdivisions), while Nkx2.1 cells mostly occupy medial parts (BSTL) (Vicario et al., 2014, 2015). Moreover, we found a subpopulation of ITC-like cells, derived from Std and expressing Pax6 and/or FoxP2 (Vicario et al., 2014, 2015, 2017). In addition, the avian central amygdala contains a subpopulation of glutamatergic neurons, which origin and function are unknown. Like the EAce of mammals, this whole area in birds appears to receive inputs from pallial amygdala-like areas, including the nidopallium and arcopallium, and give rise to descending projections to the hypothalamus and brainstem (Atoji et al., 2016; Hanics et al., 2017; reviewed by Medina et al., 2017). Moreover, at least its medial part, including the BSTL, has been implicated in chronic stress in chicken (Nagarajan et al., 2014). However, the implication of other newly identified subdivisions of the central extended amygdala of chicken in the stress response in unclear.

Regarding the medial extended amygdala, in birds this includes the BSTM and medial amygdala (Kuenzel et al., 2011). In chicken and zebra finch, BSTM and medial amygdala include neurons with embryonic origins and transcription factor and other regulatory protein expressions similar to those of the mammalian BSTM and medial amygdala (Abellán and Medina, 2009; Abellán et al., 2010; Vicario et al., 2017). This includes neurons derived from the ventrocaudal pallidal (diagonal) division, which express Nkx2.1/Lhx6 during development,

and neurons derived from the preoptic region which express Shh and Lhx5. Like in mammals, neurons of the avian medial extended amygdala derived from the subpallium appear to be GABAergic (Abellán and Medina, 2009; Abellán et al., 2013; Medina et al., 2017). In addition, the avian medial extended amygdala also contains several subpopulations of glutamatergic neurons, presumably derived from different sources, including the ventral/ventrocaudal pallium (expressing Lhx9), the hypothalamic SPV (expressing Otp) and the prethalamic eminence (expressing Lhx5 and other TFs) (reviewed by Abellán et al., 2014). This appears to be similar in turtles (Moreno et al., 2010, 2012). While recognizing all these different contributions, a recent study in mouse found that most glutamatergic neurons of the medial extended amygdala originate in a new radial embryonic division of the telencephalon, which is different from pallium and subpallium and was previously included as part of the hypothalamus (Morales et al., 2021). This division produces neurons coexpressing Otp and the telencephalic transcription factor Foxg1. These glutamatergic cells are projection neurons (Morales et al., 2021), and in contrast to the GABAergic cells, they appear to promote asocial behavior (Hong et al., 2014; Johnston et al., 2021). So far, it is unclear whether this glutamatergic neuron population of the EAme is present in non-mammalian amniotes. Like in mammals, the medial amygdala and BSTM of birds receives olfactory input, express gonadal hormone receptors and contain neurons that become active following sexual encounters (reviewed by Kuenzel et al., 2011; Medina et al., 2019, 2022). However, more studies are needed to discern the specific phenotype and function of GABAergic and glutamatergic neuron subpopulations with different embryonic origin, and know how these different cell subtypes interact with each other. Like in mammals, the avian medial amygdala projects to the BSTL (Atoji et al., 2016), and appears able to regulate stress responses based on social stimuli. However, many aspects of the neural basis of this control are unknown in birds.

1.14. CRF systems in birds

Like in mammals, in chicken CRF plays a role in initiating activation of the HPA axis during the stress response, leading to release of ACTH by the pituitary gland and to an increase in plasma corticosterone (Carsia et al., 1986; Vanderborne et al., 2005). In the brain, CRF is expressed in neurons of the paraventricular hypothalamic nucleus that project to the median eminence (Richard et al., 2004). In addition, subpopulations of CRF expressing cells are found throughout many areas of the telencephalic pallium (including hippocampal formation,

hyperpallium, mesopallium, nidopallium, and arcopallium), in the BSTL and adjacent areas of the extended amygdala, in the perimammillary hypothalamus and in several areas of the brainstem, including subdivisions of the ascending reticular activating system, such as the periaqueductal gray, the parabrachial nucleus and the locus coeruleus (Richard et al., 2004). However, information of the avian non-hypothalamic CRF functional systems/networks is still poor, including what specific cells of these brain areas are expressing CRF (for example, if they are interneurons or projection neurons), through which receptor systems they act, where are these receptors expressed in the brain and what is the effect of CRF activation in each region, including the amygdala and the hippocampus. In the avian central amygdala, it is unclear whether there is a group of CRF neurons connected with those of the BSTL, like found in mammals. Moreover, in mammals the role of CRF is age dependent, but nothing is known on the development of the non-hypothalamic CRF systems in birds.

2. Hypothesis and Objectives

As explained above, stress exposure leads to an imbalance between GABA- and glutamate-mediated neurotransmissions, as well as the activation of corticotropin releasing factor pathways connecting the extended amygdala with the hypothalamus, which finally activates the HPA axis that produces cortisol release to the blood system. Anxiety and stress-related disorders are a major problem not only in humans, but also in farm animals, including poultry, the most extended and intensive farming system in Europe (Augère-Granier and EU Parliament Member's Research Service, 2019). Chronic stress has a negative impact in the health of animals and on productivity (De Haas et al., 2021). Understanding the neural basis of the regulation of the stress response in farm animals, and how environment and experience affect this, can help to design better ways of housing and having animals in farming industry. At the same time, such studies can help to extract general organizing principles in those neural systems that might extrapolate to other animals, including humans. However, most data on neural systems regulating the stress response come from studies in mammals, mainly rodents, and studies in non-mammals are scarce.

Knowing this, the major aim of this thesis project is to do research to better understand the brain systems that regulate stress in chicken. In particular, I will focus in two major aspects that can contribute to better understand the regulation of the stress response: (1) Study the developmental origin and distribution of the glutamatergic cells in the extended amygdala, involved in the excitatory/inhibitory networks, which origin is unknown. (2) Provide a detailed map of the telencephalic expression of peptides and receptors of the corticotropin releasing factor network throughout embryonic and early posthatching development, using in situ hybridization. In posthatching development, I will focus in a pre- and a post-imprinting age (P0 versus P7), in animals both sexes. The ages selected could help to understand changes in neurotransmitter/neuropeptide systems or in other features behind the modifications in fear responses that occur a few days after hatching (Freeman and Manning, 1984): neo-hatched animals (P0) show a general low response to different environmental and social stressors (for example, they are quite confident with strangers), but this changes after a few days. A similar transitory non-responsive period has been observed in mammalian neonates. The mechanisms behind this shift are still under study. In addition to other influences, in birds the shift could be additionally related to or be influenced by the 'imprinting' process (rapidly occurring after hatching in chicken), after which animals become very fearful in front of strangers and novelties (Bolhuis, 1999).

3. Material and Methods

3.1. Tissue collection, fixation and sectioning

All animals were treated according to the regulations and laws of the European Union (Directive 2010/63/EU) and the Spanish Government (Royal Decrees 53/2013 and 118/2021) for the care and handling of animals in research. The protocols used were approved by the Committees of Ethics for Animal Experimentation and Biosecurity of the University of Lleida (reference no. CEEA 08-02/19), as well as that of the Catalonian Government (reference no. CEA/9960_MR1/P3/1 for embryos, and CEA/9960_MR1/P4/1 for post-hatchlings). Fertilized chicken embryos of the *Gallus gallus domesticus* (Leghorn) were incubated in a draft-free incubator at 37.5°C and 50-60% humidity until the desired stage. For the first objective, we used 37 animals, mostly embryos with 8 days of incubation (E8, equivalent to Hamburger and Hamilton's stages HH34/35; N = 21), in addition to a few other ages (from E13 until E18). For the second objective we used 28 embryos at E8, E14 (HH40), E16 (HH42), E18 (HH44), plus 10 post hatchlings of two ages, P0 and P7. Younger embryos were rapidly decapitated and their brain dissected and fixed by immersion in 0.1 M phosphate buffered 4% paraformaldehyde (PFA). Older embryos (E14, E16, E18) and post-hatchlings were first anaesthetized with MS222 (tricaine methanesulfonate) or halothane, followed by sodium pentobarbital (with a euthanasic dose of 100 mg/kg, intraperitoneal), and then they were perfused transcardially with saline followed by PFA. After dissection, the embryonic brains were postfixed at 4°C in phosphate buffered 4% PFA for 24h and subsequently were washed 3X with 1M PBS for 10 min on mild shaking and cryopreserved at -20°C in the prehybridization buffer before further use.

Immediately before used, brains were embedded in a 4% low- melt agarose matrix and sectioned in frontal, sagittal and horizontal planes using a Leica VT 1000S vibratome (speed 4-5, thickness 100 µm). Sections were collected serially in 0.1M phosphate buffered saline (PBS), pH 7.4, at 4°C. For objective 1, free-floating sections were processed for *in situ* hybridization to study mRNA expression of *Ortopedia homeobox* (*Otp*), *Single-minded homolog 1* (*Sim1*) and *Vesicular glutamate transporter 2* (*vGlut2*) chicken genes, while for objective 2 sections were processed to study expression of chicken genes encoding CRF (*Crf*), CRF receptors 1 and 2 (*Crfr1* and *Crfr2*), and CRF binding protein (*Crfbp*). For objective 1, some of the hybridized sections were then processed for immunohistochemistry or immunofluorescence. I explain these different techniques below.

3.2. Preparation of the riboprobe for *in situ* hybridization experiments

The clones were obtained by BBSRC ChickEST Database (Boardman et al., 2002) and distributed by Source BioScience, except for that of Crf, which was bought to GenScript (Table of clones below).

3.2.1. Plasmid isolation - Midiprep

The clones were used to transform bacteria following the protocol of Invitrogen for DH5α competent bacterial (ref. 18265-017; Subcloning Efficiency™ DH5α Competent Cells; Invitrogen ThermoFisher Scientific). The bacteria containing the plasmid of interest (see Table below) were grown overnight in 50 ml of liquid LB culture medium. Finally, the plasmids were isolated using the Qiagen Plasmid Midi Kit (ref. 12243).

Table 1: Genes and cDNAs employed for in situ hybridization

Gene name	**Gene accession number**	**EST (expressed sequenced tag) code**	**Size (bp) and alignment with accession number**	**Obtained from**
Chicken Corticotropin releasing factor (*Crf*)	NM_001123031.1		504 (165-668)	GenScript
Chicken Corticotropin releasing factor receptor 1 (*Crfr1*)	NM_204321	ChEST704a13	672 (899-1571)	Source BioScience

Chicken Corticotropin releasing factor receptor 2 Variant X2 (*Crfr2*)	XM_015281045.4	ChEST43i8	899 (1674-2387)	Source BioScience
Chicken Corticotropin releasing factor binding protein Variant X2 (*Crfbp*)	5XM_046935644.1	ChEST66a	858 (14-858)	Source BioScience

3.2.2. Plasmid linearization and purification

Most of the plasmids obtained in the previous step were linearized following the Takara protocol:

Reagent	**Quantity**
10X buffer	1 µl
0.1% BSA	2 µl
0.1% Triton X-100	2 µl
Plasmid DNA	3 µl
Sterilized water	Up to 20 µl

The mix was incubated for 2h at 37 °C and the product was cleaned using the UltraClean Purification protocol (MolBio Laboratories). Cleaned product was stored at -20 °C before proceeding with the next step.

To amplify the inserts by PCR were used *Taq* PCR Master Mix (Quiagen; ref 201445) and two primers containing the sequence of T7 (5´-CGTAATACGACTCACTATAGGGCGA-3´) and

T3 (5´-GCAATTAACCCTCACTAAAGGGAAC-3´) promotors, present in the pBlueScript II SK (+) plasmid. The program listed below was used.

Temperature (ºC)	**Step**	**Time**	
95	initial denaturation	1 minute	
95	denaturation	30 seconds	30 cycles
60	annealing	30 seconds	
72	elongation	1 minute/kb	
72	final elongation	10 minutes	

CRF peptide clone was acquired to GenScript, but the vector (pcDNA3.1-C-(k)DYK) does not present the RNA promotor to synthesize antisense RNA but only for sense with T7. This problem was solved amplifying by PCR the insert with a new primer containing the T3 promotor site to synthesize antisense RNA. For it, we used the Phire Hot Start DNA polymerase (FINNZYMES-ThermoFisher; ref. F-120) and the primer 5´-GATATTAACCCTCACTAAAGGGAACCTTCCGATGATTTCCATCAGTTTC-3´, which contains a piece of the Crf gene sequence and the T3 promotor sequence to polymerize antisense RNA. The other primer was T7 as explained above. The size differences between the both primers were solved using two annealing temperatures (51 ºC for 10 cycles and 56ºC for 20 cycles) with the program, as indicated below.

Temperature (ºC)	**Step**	**Time**	
98	initial denaturation	30 seconds	
98	denaturation	5 seconds	
51 and 56	annealing	5 seconds	10 cycles 51º and 20 cycles 56º
72	elongation	20 seconds/kb	
72	final elongation	7 minutes	

Linearized DNA obtained from the previous steps were purified following the instructions of UltraClean PCR Clean-Up Kit (MO BIO Laboratories; ref 12500-100)

3.2.3. Synthesis of the riboprobe

Linearized plasmids obtained from the previous steps were used to synthesize the riboprobe following the T3 RNA polymerase protocol (Roche Diagnostics).

Reagent	**Quantity**
Linearized plasmid	1 µg
Nucleotide (DIG) mix	2 µl
RNA polymerase	2 µl
Transcription buffer 10x	2 µl
Sterilized water	Up to 20 µl

The mix was incubated for 2h at 37°C and the following was added to the mix: 100 µl TE buffer, 5 µl 8M LiCl and 300 µl of absolute ethanol molecular grade pure (Scharlau). The resulting mix was placed at -20°C for at least 30 minutes before proceeding with the purification of the probe.

TE buffer: (recipe for 1ml): 10 µl of 1M Tris-HCl pH 8 (Sigma Aldrich), 2 µl 0.5M EDTA (ethylenediaminetetraacetic acid; Sigma Aldrich) and up to 1 ml volume with sterile water.

3.2.4 Purification of the riboprobe

The mix obtained in the previous step was centrifuged for 30 minutes at 14000g at 4°C, then the supernatant was discarded, the pellet was washed with 200 µl of 70% Ethanol (molecular grade pure Scharlau), then centrifuged again for 10 minutes at 14000g at 4°C. The supernatant was discarded and the pellet was left to dry for 2-5 minutes. In the end the pellet was resuspended in the 50 µl mix of water and Formamide molecular grade pure (1:1). Resuspended riboprobe was stored at -20°C before being added to the hybridization buffer for the *in situ* hybridization procedure.

3.3. *In situ* hybridization

Free floating sections were prehybridized in the prehybridization buffer (see composition of buffers below) for 2h at 58°C and then hybridized in the hybridization buffer containing the riboprobe overnight at 63°C (0,5-1µg/ml depending on the probe). After hybridization, the sections were washed abundantly first with the 1:1 mix of 1X MABT and the prehybridization buffer at 58°C for 20 min and 15 min on mild shaking at room temperature and after with 1X MABT for 2h on mild shaking at room temperature, while exchanging the MABT buffer every 15 minutes. Sections were then blocked in a blocking reagent (Roche), 1X MABT and sheep serum (Sigma) for 4h at room temperature, then incubated in an antibody against digoxigenin (alkaline-phosphatase-coupled anti-digoxigenin; diluted 1:3,500; Roche Diagnostics) overnight at 4°C, followed by washing with 1X MABT, and finally reacted with BM purple (Roche Diagnostics). Sections were kept in 0.1M Tris-HCl pH 7.0 before proceeding with the immunohistochemistry (next step).

Solutions: Prehybridization buffer: 50% formamide molecular (Fisher), 1.3X standard saline citrate (pH 5), 5 mM ethylenediaminetetraacetic acid (pH 8.0; Sigma-Aldrich, Steinheim, Germany), 1 mg/ml of yeast tRNA (Sigma-Aldrich), 0.2% Tween-20 and 100 µg/ml of heparin (Sigma-Aldrich), completed with water (free of RNAase and DNAase; Sigma-Aldrich). **Hybridization buffer:** 50% formamide molecular (Fisher), 10% dextran sulfate (Sigma), 1 mg/ml of yeast tRNA (Sigma-Aldrich), 0.2% Tween-20, 2X Denhardt solution (Sigma Aldrich), 1X salt solution completed with water (free of RNAase and DNAase; Sigma-Aldrich). **1X MABT**:1.2% maleic acid, 0.8% NaOH, 0.84% NaCl and 0.1% Tween-20.

3.4. Immunohistochemistry

Free-floating sections previously hybridized were processed for immunohistochemistry following the protocol used by Morales et al., 2021 (based on protocols commonly used in our lab). Briefly, sections were treated with the peroxidase deactivation buffer (1% H_2O_2 and 2% methanol in 0.1M Tris-HCl) on shaking for 30 minutes at room temperature (RT), washed 3X with 0.1M Trish-HCl, permeabilized with 0.3% PBS with triton (PBST) for 20 minutes, followed by incubation in the blocking solution containing 10% normal goat serum and 2% bovine serum albumin in 0.3% PBST for 1h, on shaking, at RT. Subsequently, the sections were incubated with the primary antibody (Rabbit anti-Foxg1, IgG, 1:1000), diluted in the blocking buffer for 72h at 4°C, on shaking. Then the sections were thoroughly rinsed 3 times

with 0.1M PBS at RT on shaking, for 10 minutes, and then incubated with the secondary antibody (Goat anti-rabbit IgG, biotinylated, 1:200 in PBST) for 2h, at RT, on mild shaking. The sections were then thoroughly washed in PBS 3 times for 10 minutes, on shaking, and incubated with the avidin-biotin complex (Vector Lab) for 1h at RT on shaking. Finally, the sections were rinsed with the 0.1M Tris-HCl and incubated in diaminobenzidine (DAB) diluted in a Tris-buffered solution also containing urea and H_2O_2, until signal of sufficient quality was achieved. The reaction was stopped with a thorough rinse in 0.1M Tris-HCl. Sections were kept in the dark at 4°C, before being mounted with glycerol-gelatin medium.

3.5. Fluorescent in situ hybridization (indirect FISH)

The tissue was permeabilized with 0.3% PBST for 30 minutes, followed with incubation in the prehybridization buffer for 2h at 58°C. After, sections were hybridized overnight at 63°C in the hybridization buffer containing the riboprobe (0.5-1 µg/ml depending on the probe). Then, the sections were thoroughly washed with the prehybridization buffer for 30 min at 58°C, followed with 3X wash in the sodium-citrate buffer (SSC, pH 7.5, 0.02M) for 10 minutes on shaking at RT. Next, samples were treated with the peroxidase inhibition buffer described above followed with three washes in Tris-HCL buffer (TNT: 10% Tris, pH8.0, 0.1 M; 0.9% NaCl; 0.05% Tween-20), for 15 minutes on mild shaking at RT. Subsequently, the samples were treated with the blocking buffer (20% blocking reagent and 20% sheep serum in TNT) for 2h on mild shaking at RT. After, sections were incubated with the primary antibody anti-DIG-POD coupled (Sheep anti-digoxigen, peroxidase conjugated antibody, 1:200, Roche Diagnostics), diluted in the blocking buffer overnight, at 4°C, on mild shaking in dark. The next day, sections were thoroughly rinsed with the TNT buffer three times for 10 min on shaking, at RT, then treated with the TSA working solution (tyramine conjugated to Cy3, 1:50, freshly prepared before the reaction) for 10 minutes, in dark, on mild shaking at RT. Reaction was stopped with a thorough wash with the 0.1M Tris-HCl (3 times for 10 min, on shaking at RT in dark). Sections were then maintained in dark, in 0.1M Tris-HCl at 4°C, before further use (immunofluorescence next step).

3.6. Immunofluorescence

Sections obtained in the FISH experiments were permeabilized and treated with blocking buffers as described in the Immunohistochemistry section. Then, the sections were incubated with the anti-Foxg1 (1:1000, in blocking buffer, for 72h at 4°C on mild shaking). Subsequently, the sections were thoroughly washed as described before and incubated with the secondary fluorescent antibody, in dark, for 2h on mild shaking at RT (goat anti-chicken, coupled to Alexa488, 1:500, Invitrogen). Then, the sections were thoroughly washed with the PBS and maintained in dark before mounting with gelatin and covered with the antifading mounting medium (Vectashield Hardset Antifade mounting medium, Vector Laboratories).

Primary antibodies				
Antibody name	**Type**	**Antigen recognized**	**Dilution**	**Manufacturer and reference**
Anti-Digoxigenin-AP Fab fragments	Polyclonal	Digoxin	1:3500	Roche, ref 11093274910 RRID:AB_514497
Anti-Digoxigenin-POD Fab fragments	Polyclonal	Digoxin	1:200	Roche, 11207733910
Rabbit anti Foxg1, IgG	Polyclonal	Synthetic peptide corresponding to Human FOXG1 aa 400 to the C terminus conjugated to keyhole limpet haemocyanin	1:2000	Abcam Antibodies, ab18259

Secondary antibodies			
Antibody name	**Type**	**Dilution**	**Manufacturer and reference**
Goat anti-rabbit IgG	Biotinylated	1:200	Vector, BA-1000
Donkey anti-rabbit Alexa fluor 488	Fluorescent	1:500	Invitrogen, A11034

3.7. Digital photographs and figures

Digital microphotographs from conventional in situ hybridization and immunohistochemistry were obtained with a Leica microscope (DMR HC, Leica Microsystems GmbH, Germany) equipped with a Zeiss Axiovision Digital Camera (Carl Zeiss, Germany). Serial images from fluorescent material were taken with a confocal microscope (Olympus FV1000; Olympus Corporation, Japan). Selected digital immunohistochemical images were adjusted for brightness and contrast with Corel PHOTO-PAINT 2019 (Corel Corporation, Canada), while the fluorescent images were adjusted and extracted using Olympus FV10-ASW 4.2 Viewer (Olympus Corporation). Finally, the figures were mounted using CorelDraw 2019 (Corel Corporation, Canada).

3.8. Nomenclature

For the identification of forebrain cell masses, we primarily followed the proposal of the Avian Brain Nomenclature Forum (Reiner et al., 2004) and the chick brain atlas (Puelles et al., 2019). For the developing chicken brain, we followed Puelles et al. (2000) as well as our own publications on the subject (Abellán and Medina, 2009; Abellán et al., 2009, 2010, 2014; Medina et al., 2019), including those in which we identified new subdivisions of the central extended amygdala in developing chicken (Vicario et al., 2014).

4. Results

4.1. Distinct Subdivisions at the Transition Between Telencephalon and Hypothalamus Produce Otp and Sim1 Cells for the Extended Amygdala in Chickens

The extended amygdala, formed by the centromedial amygdala and BST, is usually thought to be mostly composed by GABAergic neurons that originate in the telencephalic subpallium (Alheid and Heimer, 1988; Swanson and Petrovich, 1998; Puelles et al., 2000; García-López et al., 2008; Carney et al., 2010). Nevertheless, different studies have also noted the presence of different subpopulations of glutamatergic cells in parts of the extended amygdala, which originate in the different domains bordering the subpallium, including the ventral/ventrocaudal pallium and the supraopto-paraventricular hypothalamic domain (SPV), among others (García-López et al., 2008; Bardet et al., 2008; García-Moreno et al., 2010; Abellán et al., 2013). However, the SPV has recently been re-defined in mouse with the description of new domain at the boundary between telencephalon and hypothalamus, named the telencephalon-opto-hypothalamic domain (TOH), that coexpresses the telencephalic transcription factor Foxg1 and Otp, a transcription factor commonly used to define the SPV (Morales et al., 2021). Interestingly, this division produces a large subpopulation of glutamatergic neurons of the medial extended amygdala, previously thought to originate in the hypothalamus (García-Moreno et al., 2010). The TOH and the central part of SPV also appear to produce minor subpopulations of cells that invade tangentially the telencephalic subpallium and pallium (García-Calero et al., 2021; Morales et al., 2021, 2022).

An overlapping area between Foxg1 and Otp expression domains was recently found in the developing forebrain of zebrafish (Affaticati et al., 2015), which resembles the mouse TOH (Morales et al., 2021). However, the study in zebrafish did not analyze coexpression in cells of the Foxg1/Otp overlapping area and did not study its relationship to the extended amygdala. Moreover, data on the overlap of Foxg1 and Otp in other vertebrates are missing, making unclear whether the TOH is a conserved, fundamental compartment the vertebrate forebrain or whether it appeared only during evolution of particular lineages.

The objective of this chapter is to elucidate the presence of this newly described domain, the TOH, in chicken. Since in mouse and chicken, the transcription factor Sim1 is expressed in the alar hypothalamus overlapping Otp expression (Michaud et al., 1998; Acampora et al., 2000; Caqueret et al., 2005), we also analyzed the expression of Sim1 in combination with Foxg1 in chicken. Here we confirmed the existence of this new domain, the TOH, where Otp/Sim1 are coexpressed together with the Foxg1 (marker of cells of telencephalic origin). Previous studies

showed that, in mouse and other vertebrates, the Otp and Sim1 cells from SPV and/or TOH eventually populate the extended amygdala during development (Bardet et al., 2008; García-Moreno et al., 2010; Moreno et al., 2012; Biechl et al., 2017; García-Calero et al., 2021; Morales et al., 2021). Then, we examined to what extent the cells from TOH and the adjacent SPV in chicken produce cells that populate the extended amygdala, and examined whether these may be glutamatergic. Therefore, we first analyzed the expression of Sim1 and Otp in relation to Foxg1 and later we compared our results with the VGLUT2 (marker of glutamatergic neurons) to analyze the degree of overlap between TOH-derived cells and the excitatory neuronal populations of the extended amygdala.

Otp versus Fogx1

We first compared the expression domains of Otp and Foxg1 in the forebrain of chicken embryos, in sagittal, horizontal and frontal sections, by doing double labeling as follows: for Otp we carried out chromogenic in situ hybridization to detect the mRNA, which was followed by immunohistochemistry to detect Foxg1 protein (Fig. 1; Otp signal is seen in dark blue, and Foxg1 immunoreactivity is visualized in brown). In sagittal and horizontal (Figure 1A-E) sections, Otp is strongly expressed in the supraopto-paraventricular hypothalamic domain (SPV). Foxg1 expression is high in the telencephalon. An overlapping area is present near the telencephalic-hypothalamic border, with high expression of Otp and Foxg1, which we named TOH due to its resemblance to the homonymous area in mouse. This new domain is extended through the peduncular (p) and the terminal (t, pre-peducular) prosomeric subdivisions (Figure 1A). In the peduncular prosomere, it forms part of the medial bed nucleus of the stria terminalis or BSTM (Figure 1A, E). In the terminal prosomere, the TOH comprises the subpreoptic (SuPO) region that is located ventrally to the subpallial preoptic area (Figure 1C). More detailed views of the ventricular zone of the TOH can be seen in the horizontal and coronal sections where Foxg1 expressing (Foxg1+) cells of the TOH ventricular zone are juxtaposed to the periventricular cells expressing Otp or Foxg1 (Figure 1D, F, G). In coronal sections, the peduncular and pre-peduncular prosomeric subdivisions of the TOH domain can be sequentially observed from anterior to posterior (topologically dorsal to ventral) levels. At the anterior section level only the subpreoptic terminal subdivision can be seen (Figure 1F), while in subsequent posterior sections the peduncular TOH domain can be seen, in Figure 1G, followed by the paraventricular hypothalamic subdivision (in Fig. 1H, belonging to the so

called SPV core). Terminal and peduncular parts of the TOH domain were bounded ventrally by the Otp-rich and Foxg1 poor SPV core domain (Figure 1F-H). The latter included the primordium of the paraventricular nucleus (Pa in Figure 1E,H) and at least part of the supraoptic nucleus. In addition to a major population of Otp cells, the SPV core was observed to contain a minor subset of mostly dispersed Foxg1 immunoreactive cells (Figure 1E,H).

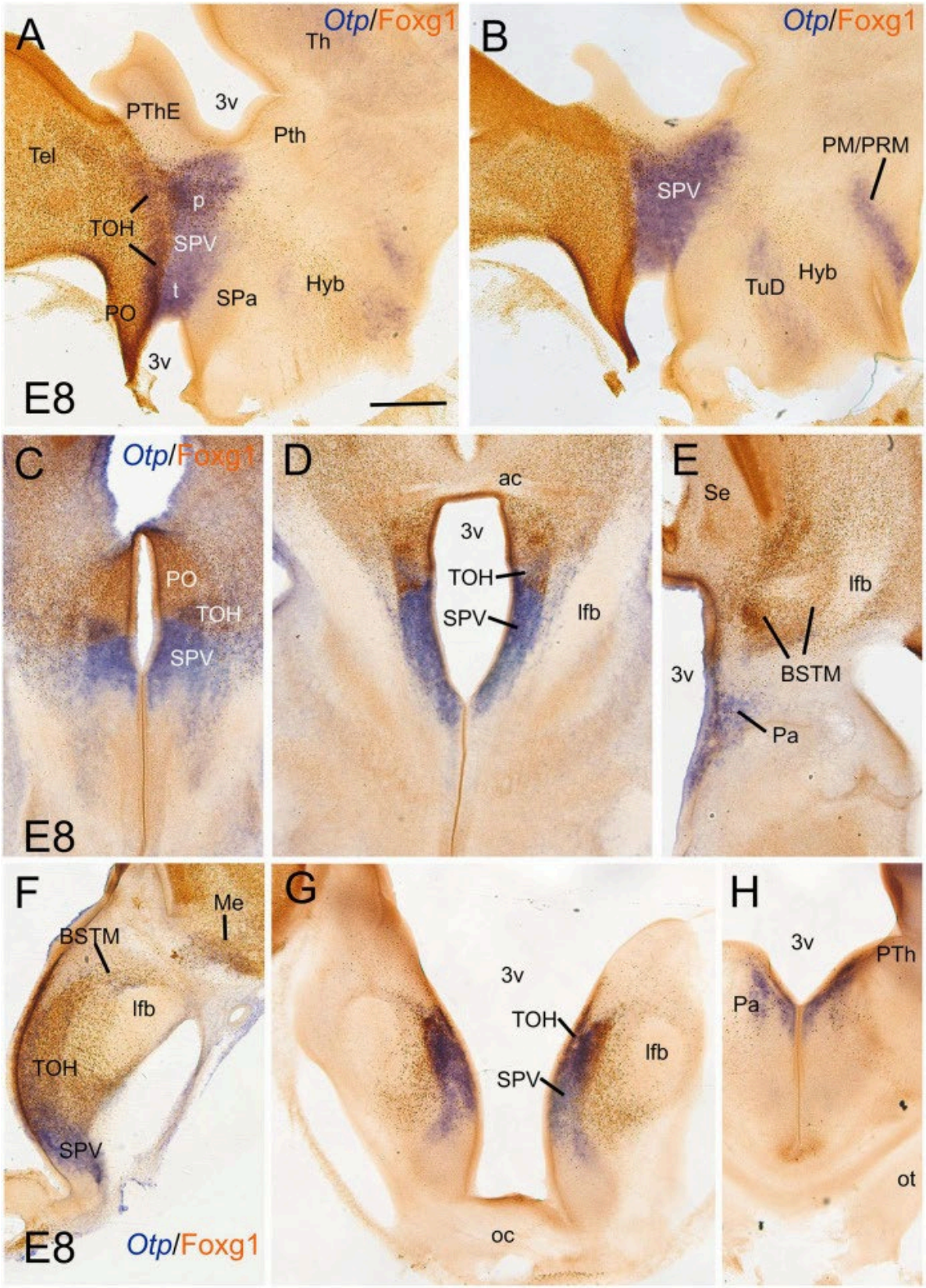


Figure 1. Chromogenic in situ hybridization (Otp in dark blue) with immunohistochemistry (FoxG1 in brown) in sections of the chicken forebrain in sagittal (A,B), horizontal (C-E) and coronal (F-H) planes, at E8. In sagittal sections, the telencephalon (anterior) is to the left, while in the sagittal sections is to the top. In coronal sections, dorsal is to the top; in E and F, medial is to the left. Scale: bar in (A) = 200 µm (applies to all). (Adapted from Metwalli et al. 2022)

immunofluorescence for Foxg1 (Figures 2-6). In the sagittal sections, an Otp/Foxg1 overlapping domain (TOH) was observed intercalated between the subpallium and the SPV core (Figures 2, 3). This new domain was investigated in higher magnification and showed a high density of cells coexpressing both Foxg1 and Otp (Figure 2B, details of the squared area in Figure 2C-C´´´).

The TOH domain with a high concentration of Otp/Foxg1 coexpressing cells stretches from the ventral subdivision of the BSTM (seen in the peduncular level of TOH, Figure 3D), to the subpreoptic region (seen in the terminal levels of TOH in Figure 3A, B, with the details of the subpreoptic region squared in B in Figure 3C-C''). In these sections, a minor subpopulation of scattered Foxg1 cells without the Otp signal can be seen in the SPV core domain, ventral to TOH (Figures 2B, 3D), and a cluster of Foxg1 only expressing cells was observed in the paraventricular nucleus (Figure 3D and pointed with an arrow in the Figure 3E). This island of cells is related to a group of Foxg1 cells that appear to follow the unlabeled fornix-like fiber bundle, travelling from dorsal to ventral (Figure 3A, A´´).

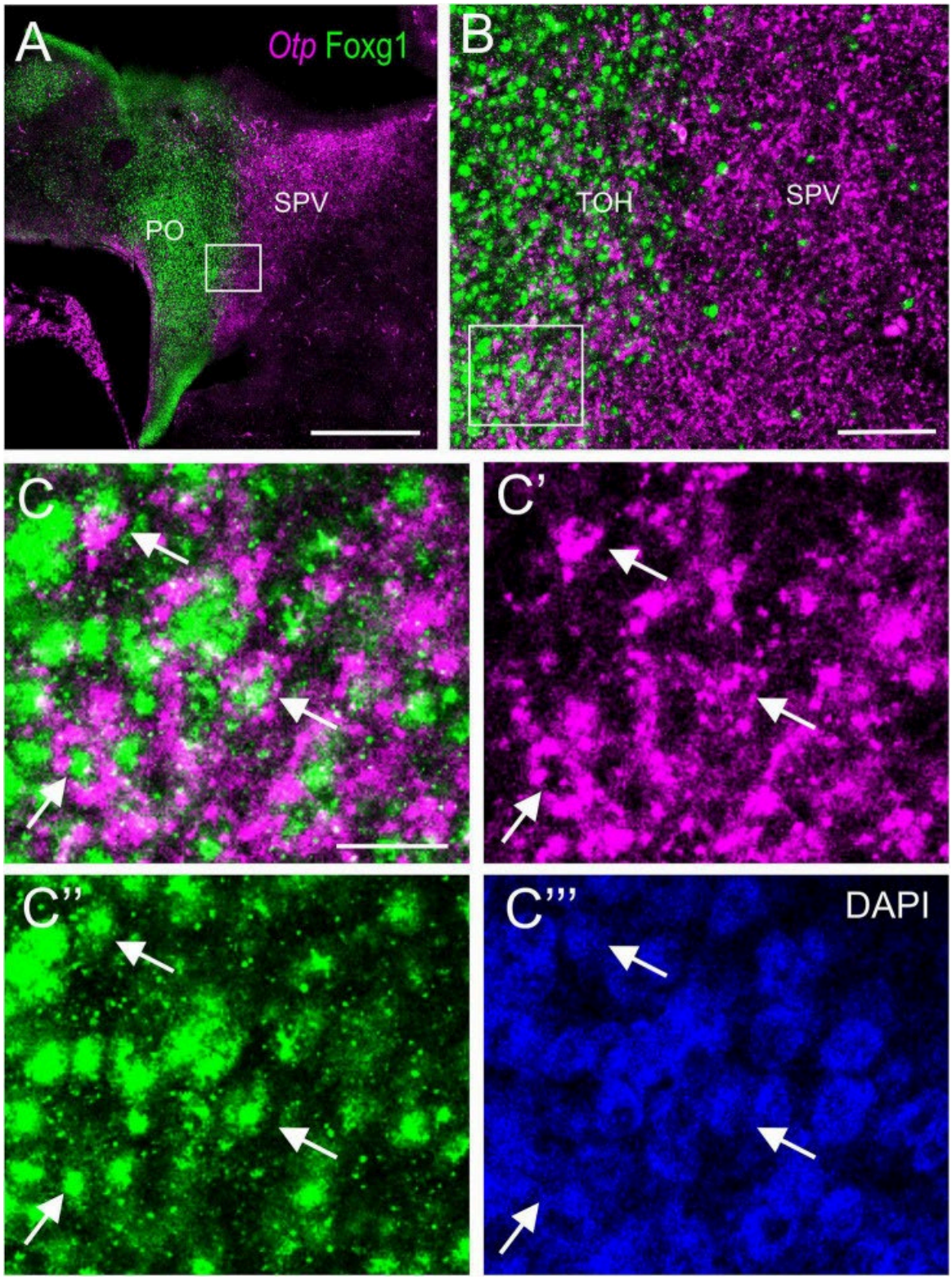


Figure 2. Fluorescent double labeling of Otp and Foxg1 cells in sagittal sections of the chicken embryonic forebrain at E8, with Otp in magenta (FISH) and Foxg1 in green (immunofluorescence). Both markers overlap in the TOH domain, located dorsally to the SPV core (A). In this image, the telencephalon is to the left. Detail of the area A shows coexpression of Otp/Foxg1 in cells of TOH (B). Details from the squared area in B are shown in C-C′′′ at higher magnification, with DAPI staining (blue) juxtaposed to magenta and green. Scale bars: (A) = 320 µm; (B) = 80 µm; (C) = 20 µm (applies to C–C‴). (Adapted from Metwalli et al. 2022)

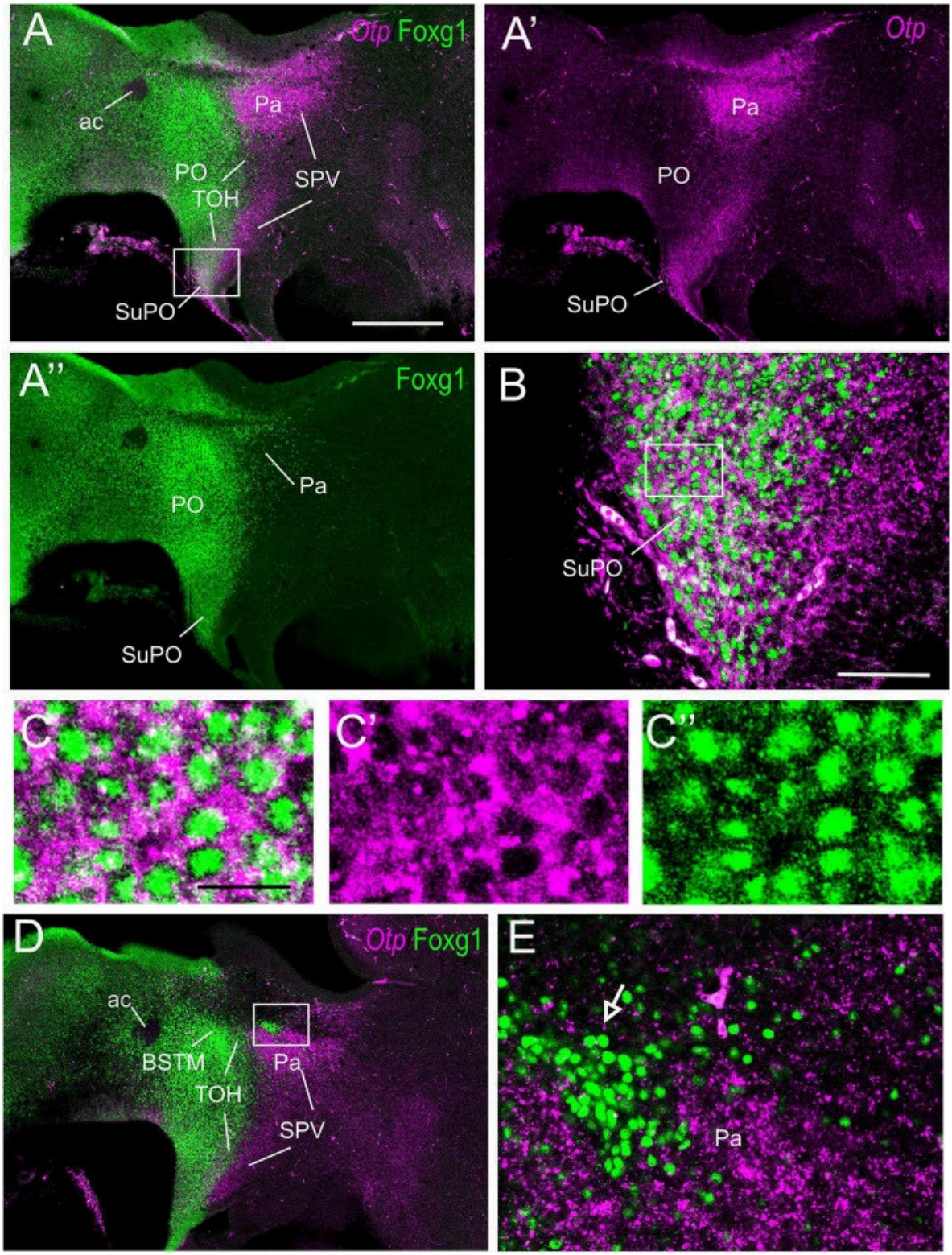


Figure 3. Fluorescent double labeling of Otp and Foxg1 cells in sagittal sections of chicken embryonic forebrain at E8, with Otp in magenta (FISH) and Foxg1 in green (immunofluorescence). In these images, the telencephalon is to the left. Both markers overlap in the TOH domain in A. Detail of the squared area from A shows coexpression of Otp/Foxg1 in cells of the subpreoptic region (B; higher magnitication in C-C´´). Detail of the area squared in (D) is displayed in E, showing a patch of Foxg1 cells (pointed with an arrow) inside the paraventricular nucleus (in the peduncular SPV core). Scale bars: (A) = 320 µm; (B) = 80 µm; (C) = 20 µm (applies to C–C‴). (Adapted from Metwalli et al. 2022)

The contribution of cells from the peduncular TOH domain to the BSTM and other parts of the medial extended amygdala is better understood when analyzing additional section planes, such the horizontal sections (Figure 4) and in the coronal sections (Figure 5). Our results demonstrate that the BSTM nucleus contains a subpallial dorsal part rich in single labelled Foxg1 cells, and a TOH ventral part rich in Otp/Foxg1 coexpressing cells (Figures 4A, B, details in Figure 4C-C''; and 5B, B', details in D). The ventral part of the BSTM with abundant expression of double-labelled cells corresponds to the subdivision previously named BSTM3 which is rich in glutamatergic cells but has low expression in subpallial marker genes (such Nkx2.1, Lxh6, and Lhx7/8; Abellán and Medina, 2008, 2009). This part of BSTM is continuous laterally with a cell corridor rich in double-labelled cells that reach the Me (Figures 4A,D,E; 5B, B'). Therefore, we postulate that the TOH domain is generating a continuous cell corridor that contributes to the formation of BSTM3 (medially) and to part of the Me (laterally). This cell corridor is also observed later in the embryonic stages (Fig. 5F).

Double-labelled cells originating in TOH, at terminal and peduncular levels, appear to migrate tangentially into the telencephalon, since many were observed to spread in the preoptic subpial surface and dorsolaterally in the zone that was previously considered to be part of the pallial extended amygdala (EA) (Figures 5A, C, E; arrows in 6B, C, with details in the Figure 6D-E'). A small portion of these cells was later observed in the arcopallium. In addition to double-labelled cells, the TOH portion of the medial extended amygdala also contains subpopulations of single Foxg1 and single Otp positive cells (Figure 5D). Scattered single Otp cells are also seen in subpallial parts of the medial extended amygdala (Figure 4B,D). Considering that these cells express the Otp marker only and not Foxg1, they could originate from the SPV core. Single Otp cells are also found in the preoptic area, as seen in more details in the horizontal sections, where a subgroup of Otp single labeled cells appear to spread dorsally (in topological terms) from the terminal part of the SPV core, and reach the terminal lamina following subpial pathway (Figure 6B, C). The subpial Otp single-labelled cells could be originating from the terminal part of SPV core, and likely represent a terminal part of the supraoptic nucleus which was previously identified in chickens in the ventral/posterior telencephalon based on its vasotocin containing neurons, and was designated as "ventral supraoptic nucleus" (Tennyson et al., 1985; Arnold-Aldea and Sterritt, 1996).

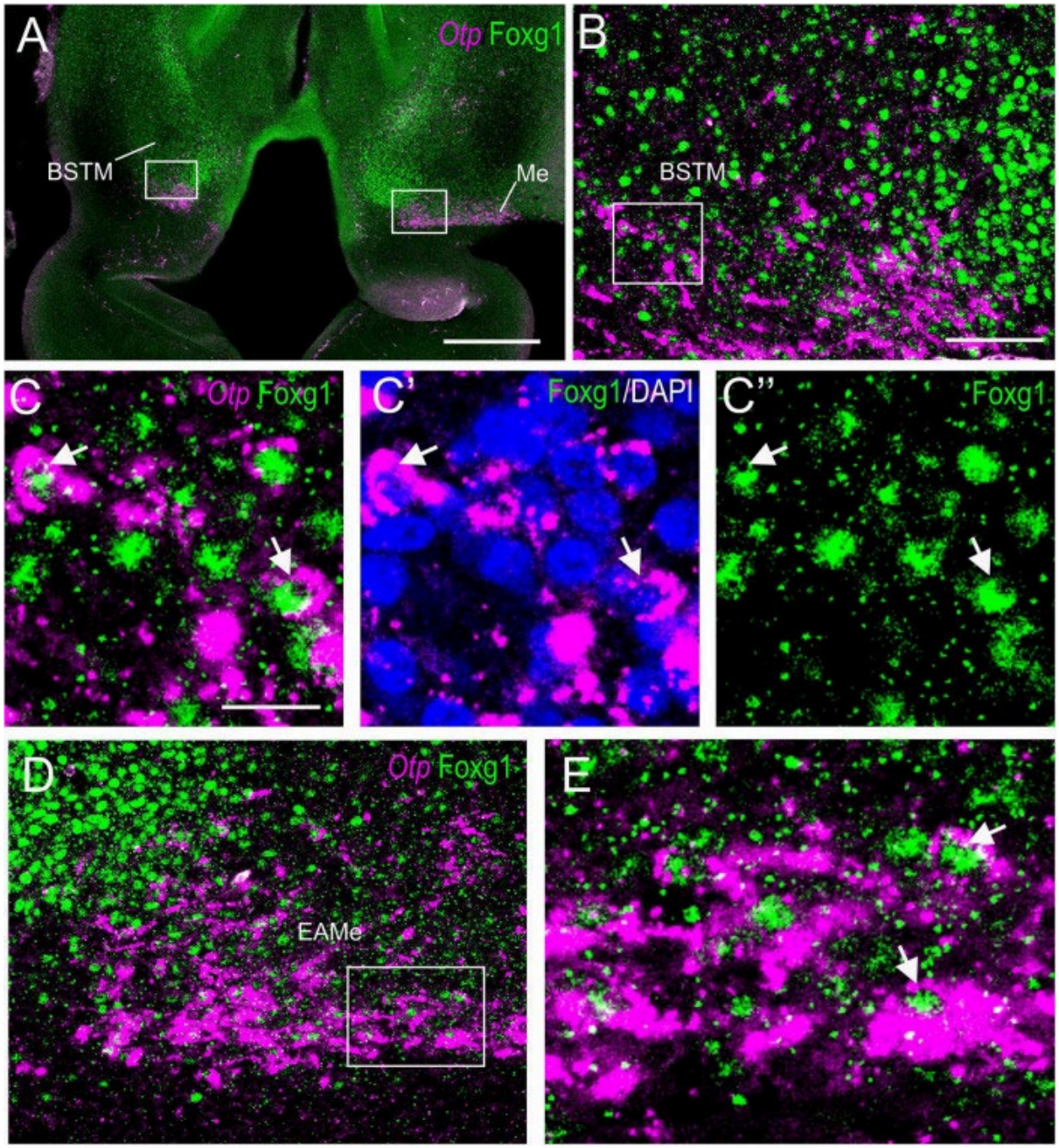


Figure 4. Fluorescent double labeling of Otp and Foxg1 cells in horizontal sections of chicken embryonic forebrain at E8, with Otp in magenta (FISH) and Foxg1 in green (immunofluorescence). In these images, the telencephalon is to the top. Both markers overlap in the TOH domain in A. (B) Detail of the area squared in (A) (left side) showing coexpression of Otp and Foxg1 in many cells of the ventral part of BSTM (using topological references according to the prosomeric model). Panels (C–C″) show the area squared at B at higher magnification (C: merged magenta/green channels; C′: magenta channel with DAPI; C″: green channel). Arrows point to examples of double-labeled cells. (D) Detail of the area squared in (A) (right side) showing coexpression of Otp and Foxg1 in many cells of the medial extended amygdala (a cell corridor that extends from BSTM to the Me). Panel (E) shows the area squared at higher magnification (example of a double-labeled cell pointed with an arrow). See text for more details. For abbreviations, see list. Scale bars: (A) = 320 µm; (B) = 80 µm (applies to B,D); (C) = 20 µm (applies to C–C″,E). (Adapted from Metwalli et al. 2022)

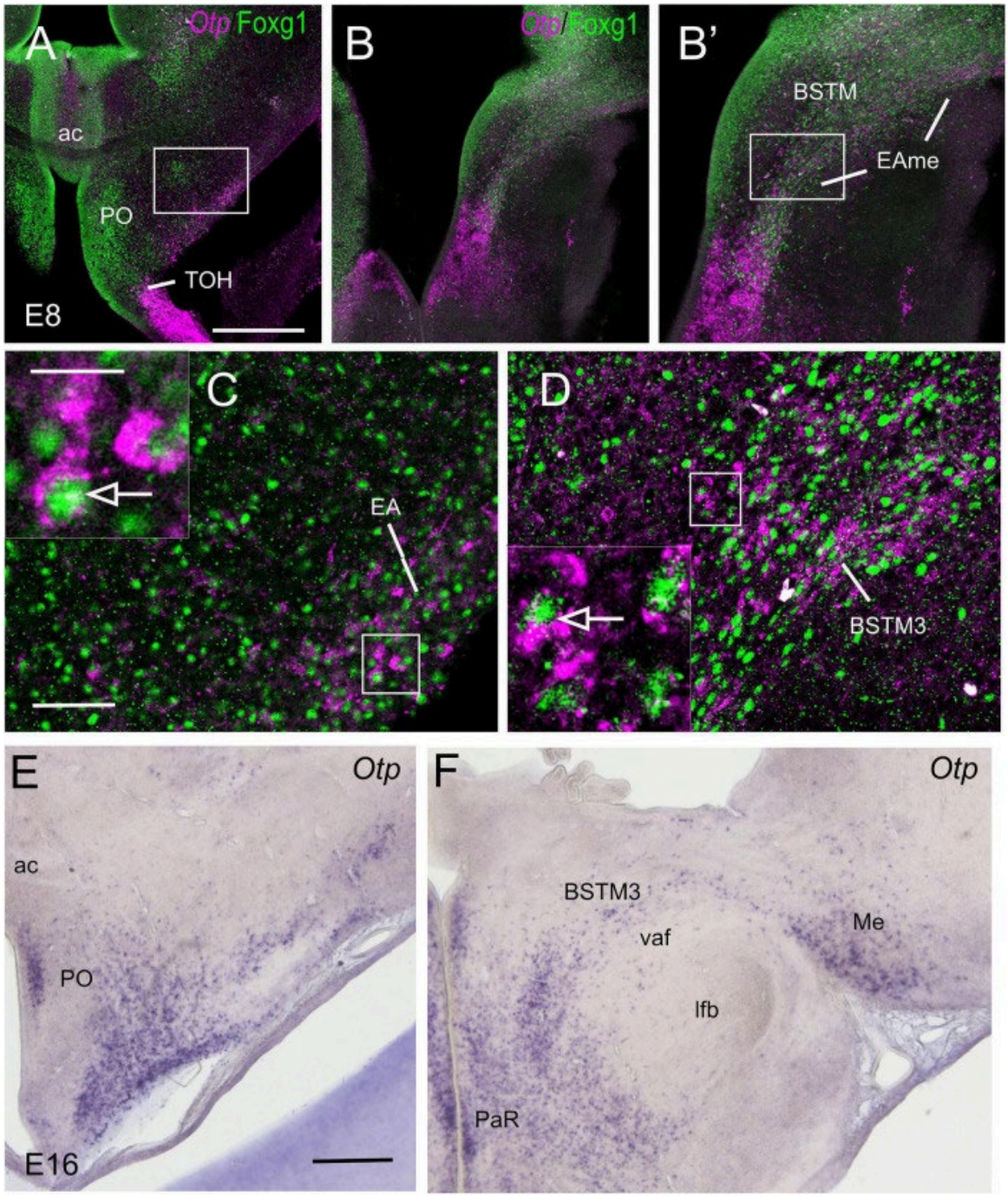


Figure 5. Single and double labeling of Otp and Fogx1 in chicken embryonic forebrain. (A,B) Frontal sections of the chicken embryonic forebrain (commissural and post-commissural levels), at E8, hybridized for Otp (magenta) and immunostained for Foxg1 (green). In these images, dorsal is to the top, and in B, C, E and F, medial is to the left. Panel (B′) is a detail of the cell corridor extending from BSTM to the Me (C). Detail of the area squared in (A) showing coexpression of Otp and Foxg1 in cells of the extended amygdala and parts of the preoptic area (PO) (insert shows a detail of a double-labelled cell). (D) Detail of the area squared in (B') showing coexpression of Otp and Foxg1 in cells of BSTM (BSTM3 subdivision; higher magnification detail of double-labelled cells pointed with an arrow in insert). (E,F) Frontal sections of the chicken embryonic forebrain, at E16, hybridized for Otp. Note the corridor of Otp cells extending from BSTM3 to the Me. Scale bars: (A) = 320 µm (applies to A,B); (C) = 80 µm (applies to C,D); (C) insert = 20 µm; (E) = 400 µm (applies to E,F). (Adapted from Metwalli et al. 2022)

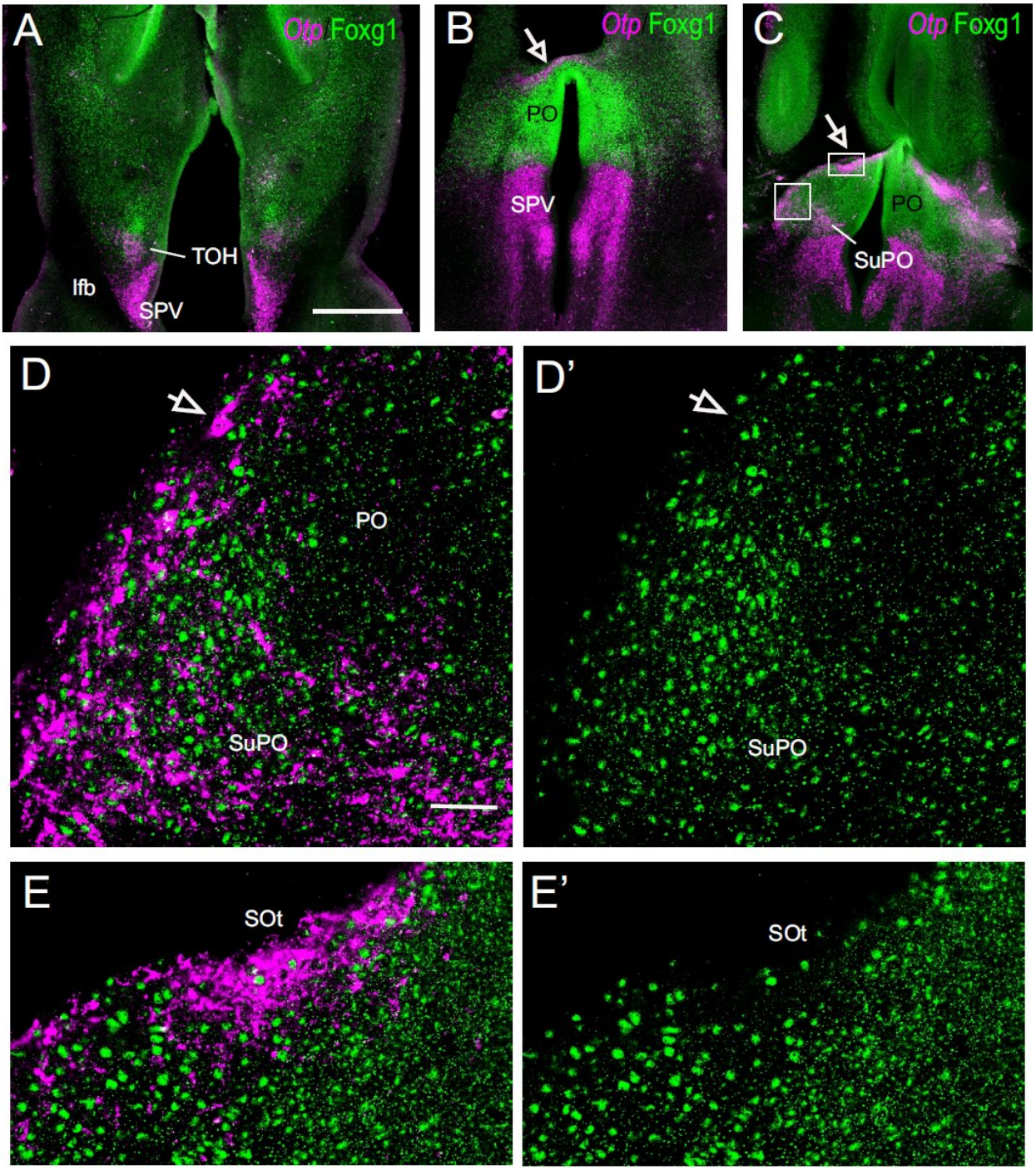
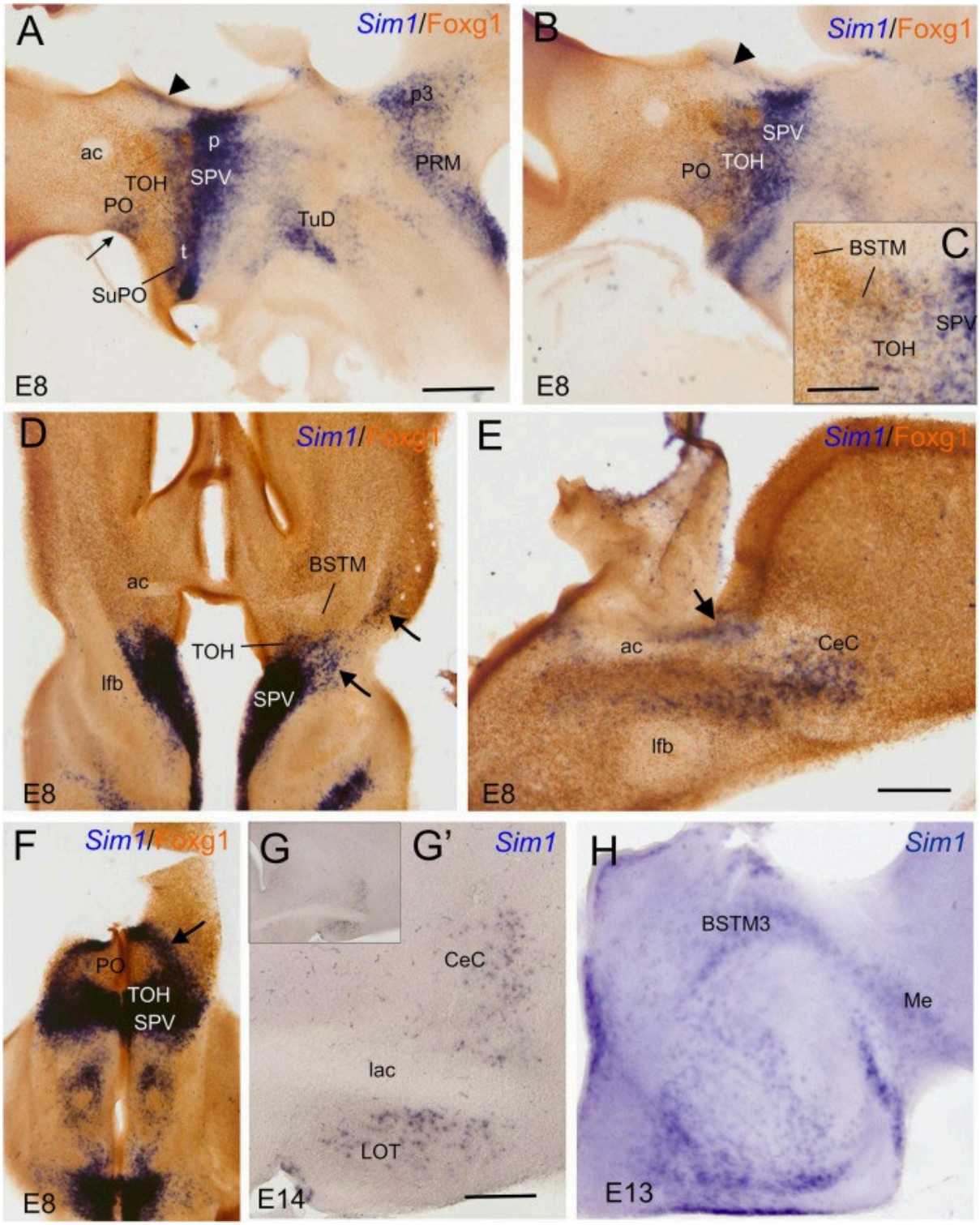


Figure 6. Fluorescent double labeling of Otp and Fogx1 in chickens. (A-C) Horizontal sections of the chicken embryonic forebrain, at E8, hybridized for Otp (magenta) and immunostained for Foxg1 (green). In these images, the telencephalon is to the top. Panel (A) is at the peduncular prosomeric level, while (B,C) are at terminal levels. Arrows in (B,C) point to a stripe of Otp cells in the PO. Panels (D–E') are details of the areas squared in (C) (D,E: merged channels; D',E': green channel). Panels (D,D') show double-labeled cells in the SuPO (in the terminal TOH domain), and some Otp single-labeled (arrow) and Otp/Foxg1 double-labeled cells that spread into the PO. Panels (E,E') show a prominent group of Otp single-labeled subpial cells, which seem to correspond to the terminal part of the supraoptic (SOt) nucleus. See text for more details. For abbreviations, see list. Scale bars: (A) = 320 µm (applies to A-C); (D) = 40 µm (applies to D,E').

Sim1 versus Fogx1

Sagittal, horizontal and coronal sections of the chicken forebrain at the stage E8 (Figure 7A, D, F) were double-labelled for Sim1 (chromogenic in situ hybridization) and Foxg1 (immunohistochemistry). Strong Sim1 signal was observed in the region where it coincides with the Otp expression as described previously – SPV domain from peduncular to terminal prosomeric levels, plus the newly identified domain, TOH. The latter was identified as an overlaying zone between Sim1 and Foxg1, similar to the previously described in Otp/Foxg1 staining. The TOH expands from the peduncular prosomeric zone (where it includes the BSTM3; Figure 7B, C) to the terminal prosomeric levels (where it contains the subpreotic region; Figure 7A). At the peduncular level, Sim1 cells spread into Me, the central extended amygdala and the arcopallium (Figure 7 D, E, G, G', H). Distinct Sim1 single-labelled cells were identified at the subpial surface of the subpallium (previously identified as the pallial extended amygdala). Finally, at the terminal levels, Sim1 cells are seen in the preoptic region of the subpallium (Figure 7F), with a dense group in subpial position following a similar corridor like the Otp cells. As expected, Foxg1 expression in all these named subpallial areas is ubiquitous. However, in chromogenic staining it is unclear whether any of the cells are double -labelled for Sim1 and Foxg1, and therefore double fluorescence staining was performed (Figures 8, 9).

Figure 7. Chromogenic double labelling of Sim1 and Foxg1 in sections of the chicken embryonic forebrain. (A–C) Sagittal, (D,F) horizontal, and (E) frontal sections of the chicken embryonic forebrain, at E8, hybridized for Sim1 (blue) and immunostained for Foxg1 (brown). In sagittal sections, the telencephalon is to the left, and in horizontal sections is to the top. In coronal sections, dorsal is to the top, and medial is to the left. In horizontal sections, D is above F (these sections are similar to those shown in Fig. 6A-C). Note the overlapping expression of both transcription factors in the TOH domain, just dorsal to the SPV core. Panel (C) shows a detail of the overlapping area covering part of BSTM (from a section a bit lateral to that seen in B). Arrowheads in (A,B) show a stream of Sim1 cells spreading dorsally from the SPV core. This stream reaches the BST (pointed with an arrow in E). A lateral stream of Sim1 cells (arrow in panel D) also reaches the capsular central amygdala and adjacent arcopallium (E). The arrows in (D,F) point to streams of Sim1 expressing cells spreading from the TOH and SPV domains to the capsular central amygdala (CeC) (D) or the preoptic region (PO) (F) . Panels (G–H) show details of frontal sections hybridized for Sim1 at the level of the central (G,G′) or the medial (H) extended amygdala in older embryos (E13 and E14). See text for more details. For abbreviations, see list. Scale bars: (A) = 400 μm (applies to A,B,D,F); (C,E) = 200 μm; (D) = 40 μm (applies to D,F). (G′) = 400 μm (applies to G′,H). (Adapted from Metwalli et al. 2022)


A
Sim1/Foxg1
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C
BSTM
SPV
TOH
E8
D
Sim1/Foxg1
BSTM
ac
TOH
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E8
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Sim1/Foxg1
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CeC
lfb
E8
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Sim1/Foxg1
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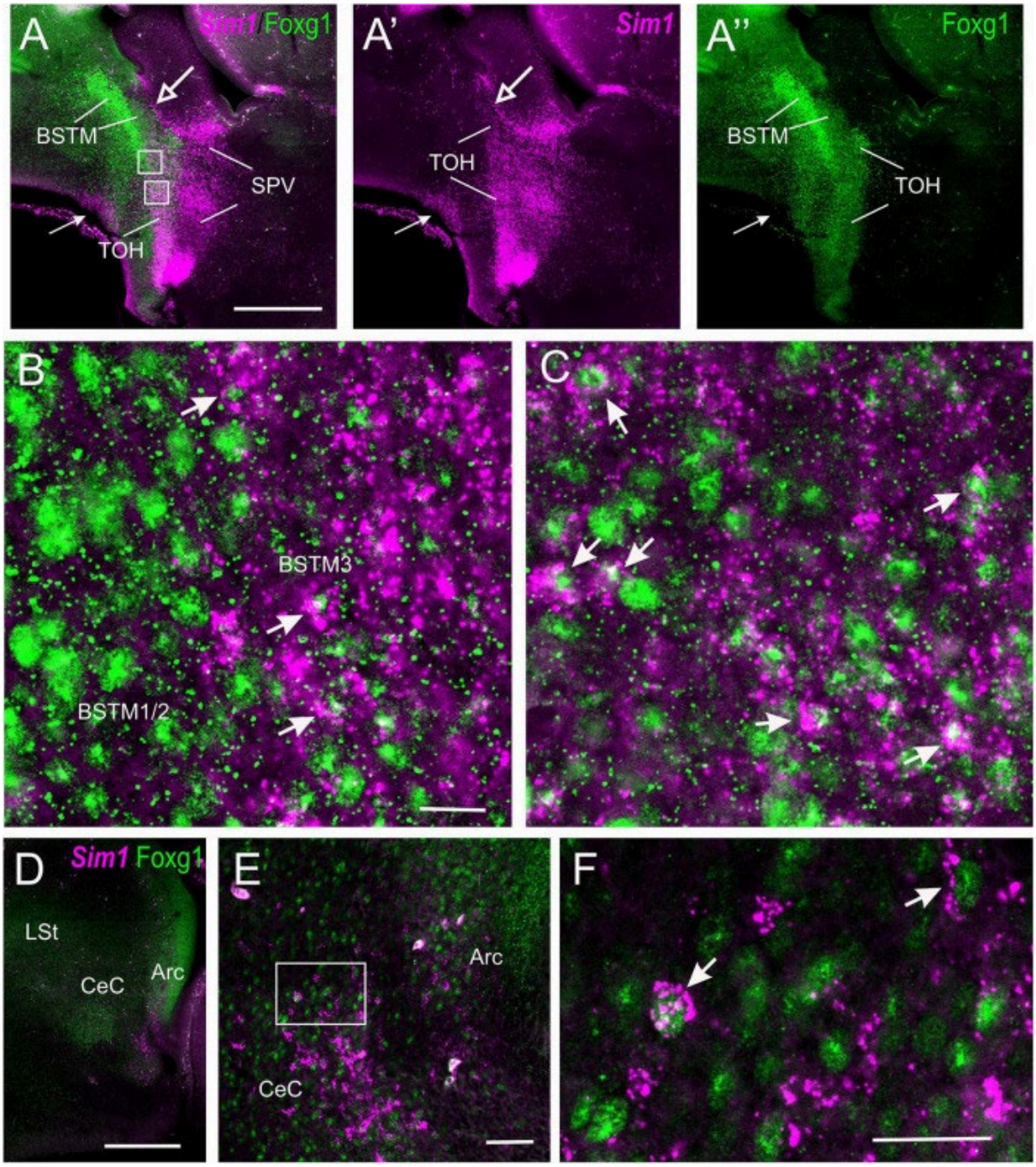
TOH
SPV
E8
G
G'
Sim1
CeC
lac
LOT
E14
H
Sim1
BSTM3
Me
E13

In order to study coexpression of Sim1 and Foxg1 in the TOH domain and its derivatives, chicken embryonic forebrains, at E8, were processed for double staining with indirect FISH for Sim1 and immunofluorescence for Foxg1 (Figures 8, 9). Our results demonstrate that the TOH domain is rich in double-labelled cells coexpressing the Sim1 and Foxg1 (Figure 8A - A'', details in B, C), where the Sim1 signal is seen in magenta (located in the cytoplasm of the cells) and the Foxg1 signal is seen in the green (localized in the cell nucleus). In the terminal prosomeric level, Sim1/Foxg1 double-labelled cells derived from the TOH domain populated the subpreoptic region. Moreover, a cell corridor that appeared to originate from the TOH and the SPV core was observed to reach the preoptic region in the telencephalic subpallium (PO). There, a cluster of subpial cells, some expressing both Sim/Foxg1 and some expressing only Sim1, was observed (arrow in Figure 8A).

Figure 8. Fluorescent Sim1 and Fogx1 in the sections of chicken embryonic forebrain. (A–A″,D) Sagittal sections of the chicken embryonic forebrain, at E8, hybridized for Sim1 (magenta) and immunostained for Foxg1 (green) (A shows merged channels, and A′,A″ show separate channels). In these images the telencephalon (and anterior) is to the left. Note the overlapping expression of both transcription factors in the TOH domain, just dorsal to the SPV core. Panels (B,C) show details of the squared areas in (A), showing coexpression of Sim1 and Foxg1 in many cells of the overlapping area, including BSTM3 (examples of double-labelled cells pointed with an arrow). The large arrow in (A,A′) points to a stripe of Sim1 cells, spreading from the SPV core to the vicinity of BSTM. The small arrow in (A–A″) points to Sim1 cells in a subpial position of the PO. Panel (D) shows a lateral section, at the level of the capsular central amygdala (CeC) and adjacent arcopallium (Arc). Panel (E) is a detail of these areas, and (F) shows a higher magnification detail of double-labelled cells in the capsular central amygdala (examples pointed with an arrow). This area also includes some Sim1 single-labelled cells. See text for more details. For abbreviations, see list. Scale bars: (A) = 320 μm (applies to A–A″); (B) = 40 μm (applies to B,C); (D) = 320 μm; (E) = 40 μm; (F) = 20 μm. (Adapted from Metwalli et al. 2022)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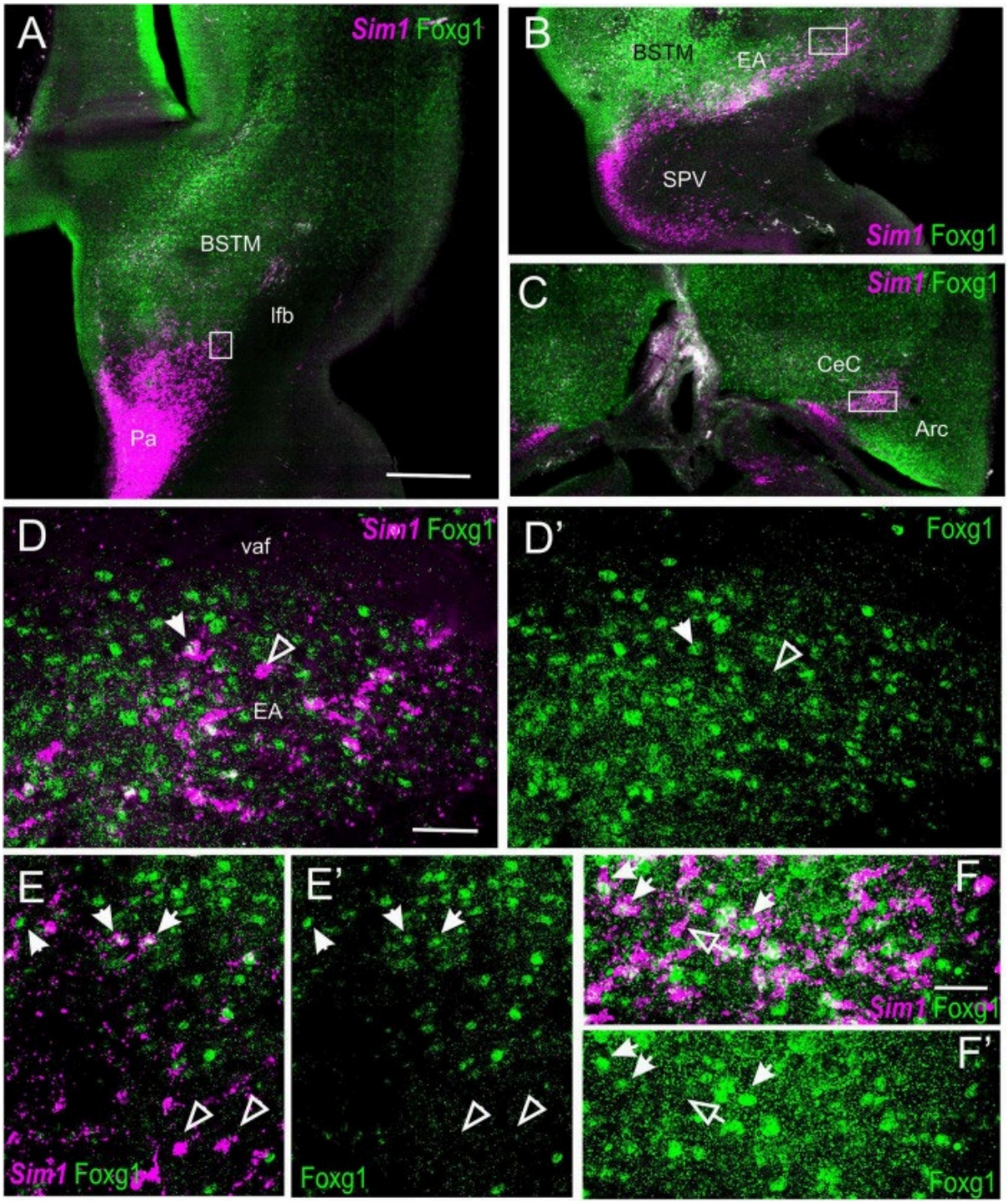
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Sim1 Foxg1
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Figure 9. Fluorescent Sim1 and Fogx1 in sections of the chicken embryonic forebrain. (A–C) Horizontal sections of the chicken embryonic forebrain, at E8, hybridized for Sim1 (magenta) and immunostained for Foxg1 (green) (left to right goes from bottom to top). In these images, the telencephalon is to the top. Panels (D, D′) are a detail of the area squared in (B), showing Sim1 single-labelled (empty arrow) and Sim1/Foxg1 double-labelled (filled arrow) cells in the medial extended amygdala. Panels (E, E′) are a detail of the area squared in (A), showing Sim1 single-labelled (empty arrow) and Sim1/Foxg1 double-labeled (filled arrow) cells in BSTM3 or ventral to it. Panel (F) is a detail of the capsular central amygdala from the section shown in (C). Sim1 single-labelled (empty arrow) and Sim1/Foxg1 double-labelled (filled arrows) cells are observed. See text for more details. For abbreviations, see list. Scale bars: (A) = 200 μm (applies to A–C); (D) = 40 μm (applies to D–E′); (F) = 20 μm (applies to F, F′). (Adapted from Metwalli et al. 2022)

At the level of the peduncular prosomere, a Sim1/Foxg1 coexpressing area was observed in the BSTM3 located ventrally to the subpallial BSTM (possibly including BSTM1/2), where Foxg1 single-labelled cells are abundantly present (Figure 8A, B). Moreover, we observed another layer of Sim1 single-labelled cells expanding from the peduncular SPV core to the BSTM (pointed with an arrow in the figure Figure 8A, A'). This demonstrates how different sources complement the cell composition of this nuclear complex.

Comparison of sagittal (Figure 8) with the horizontal sections (Figure 9), allows a better understanding of the contribution of TOH versus SPV core to the extended amygdala: it appears that both the TOH domain and the SPV core produce cells for the extended amygdala. TOH-derived double labelled cells populate the ventral parts of the medial extended amygdala, from medial to lateral levels (BSTM3 – Me), as seen in Figures 8A-B and 9A, B, and in details in Figure 9D, D'. Cells originating from the SPV core (therefore Sim1 single-labelled cells) appear to give rise to a distinct layer of the BSTM (Figures 8A and 9A, details of cells shown with an empty arrowhead in 9E), while another group of these cells is present in other portions of the amygdala (Figure 9D, D'). A distinct patch of Sim1 single-labelled cells is also seen in the arcopallium (Arc). In comparison to the Otp+ cells that populate the arcopallium, Sim1 cells are more abundant. Moreover, a small group of Sim1 cells populates the lateral parts (CeC) of the central extended amygdala (Figures 8D-F, 9C and in details in the Figure 9F). We also demonstrate that a few cells follow the medial path and reach the BSTL and another, bigger stream of cells follows the lateral path and eventually reaches the boundary between the arcopallium and the extended amygdala, overlaying an area that was previously described as the intercalated amygdala (Figure 9C). (Vicario et al., 2014, 2017). Cells from this area spread into the capsular central amygdala (CeC). Two subpopulations of Sim1 cells are observed in

the capsular central extended and intercalated amygdala: one group is formed by the Sim1/Foxg1 coexpressing cells (derived from TOH) and another group comprises Sim1 only cells (likely derived from SPV core) (Figure 9F). This pattern of distribution of the Sim1 single-labelled cells resembles the distribution of the previously described glutamatergic neurons in the same location (Abellán and Medina, 2009; see their Figure 16F showing VGLUT2 expressed in glutamatergic cells).

In order to better characterize these glutamatergic neurons and investigate their putative origin, a double-labelling of VGLUT2 and Foxg1 was performed (both in chromogenic and fluorescent staining, but only images of that latter are shown in Figure 10). Our results demonstrate that a subpopulation of the VGLUT2+ cells in the CeC in the chicken embryonic forebrains, at E18, were single labelled (no Foxg1, only VGLUT2) while another subpopulation (more numerous) was observed that consisted in double-labelled VGLUT2/Foxg1 cells (Figure 10B, B'). This indicates that there are at least two distinct groups of glutamatergic neurons in the capsular central extended amygdala. Knowing that the Foxg1 is a marker of cells of the telencephalic origin, the group of VGLUT2 single-labelled cells could originate from the Sim1 single-labelled cells produced in the SPV core domain. Regarding the VGLUT2/Foxg1 double-labelled cells, at least part of them may derive from the Sim1/Foxg1 cells that appear to originate in TOH.

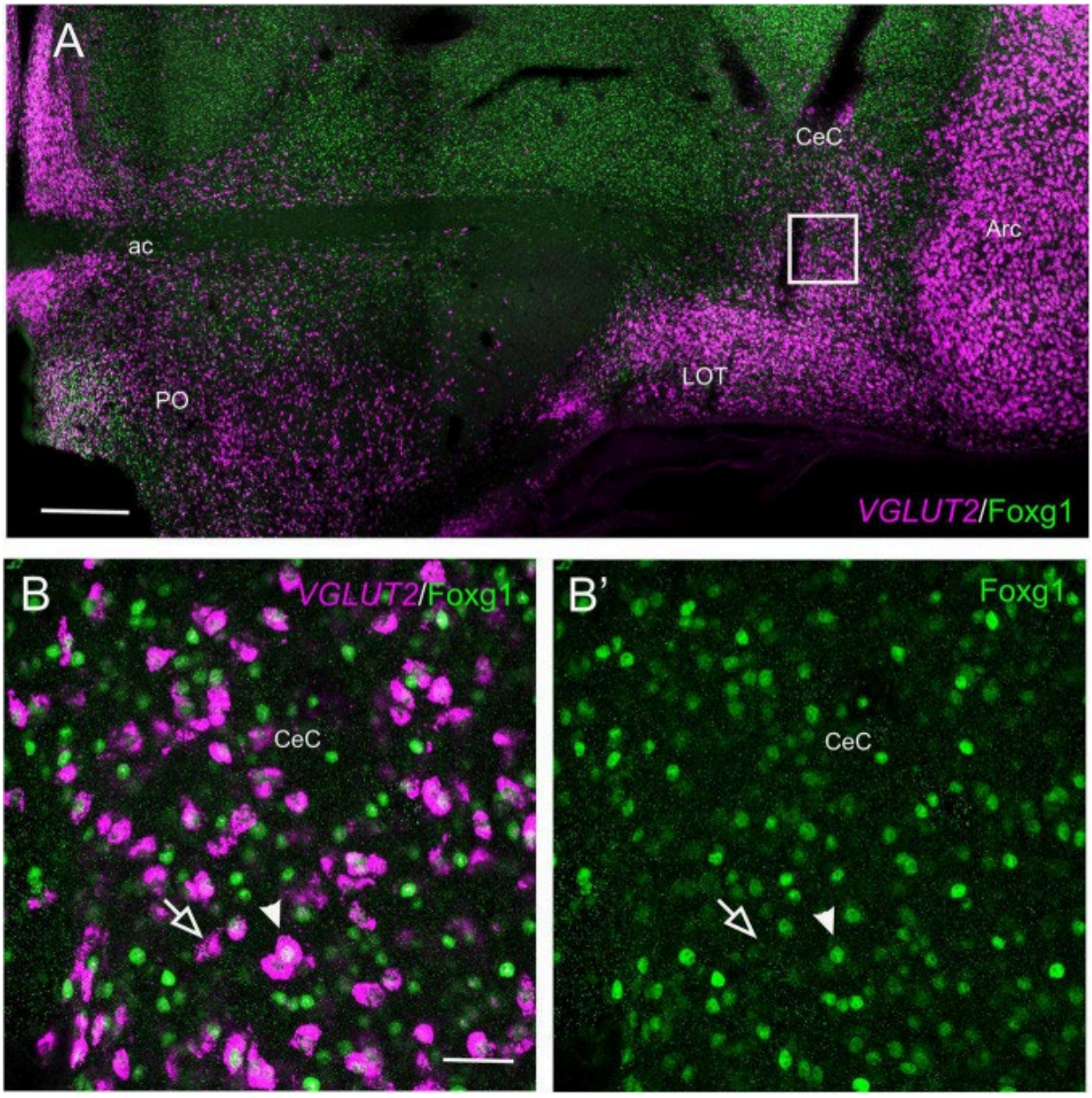


Figure 10. Fluorescent VGLUT2 and Foxg1 in frontal sections through the chicken central extended amygdala. (A) Frontal section of the chicken embryonic forebrain (commissural level), at E18, hybridized for VGLUT2 (magenta color) and immunostained for Foxg1 (green color). In this image, dorsal is to the top and medial to the left. In addition to those in the pallium, note the presence of a group of VGLUT2 cells in the capsular central amygdala. Panels (B,B′) are a detail of this area showing the presence of two types of VGLUT2 cells based on the coexpression of Foxg1 (B: merged channels; B′: green channel). The filled arrow points to an example of a VGLUT2/Foxg1 double-labelled cell, while the empty arrow points to an example of a VGLUT2 single-labelled cell. See text for more details. For abbreviations, see list. Scale bars: (A) = 400 µm; (B) = 40 µm (applies to B,B′). (Adapted from Metwalli et al. 2022)

4.2. Mapping of corticotropin-releasing factor, receptors and binding protein mRNA in the chicken telencephalon throughout ontogeny

Activation of HPA during the stress response obeys to similar rules in different vertebrates, including birds (Carsia et al., 1986; Vandenborne et al., 2005; Smulders, 2021), and this appears designed to help animals cope with adversity (Herman et al., 2016). In birds, it also involves activation of CRF neurons of the paraventricular hypothalamic nucleus, leading to release of ACTH by the pituitary and subsequent release of corticosterone by adrenal glands (Carsia et al., 1986; Vandenborne et al., 2005). The avian paraventricular hypothalamus also receives input from the extended amygdala, including the BST, where GABAergic neurons concentrate, and the latter receives input from the hippocampal formation (Atoji et al., 2006), suggesting the existence of a similar regulation of HPA by the telencephalon in birds. Moreover, CRF cell distribution and fiber systems in the brain also appear to be similar between birds and mammals (Józsa et al., 1984; Richard et al., 2004). Chicken CRF peptide is identical to human and rat CRF (Vandenborne et al., 2005), and the gene that encodes it is orthologous to CRF1 of mammals and other vertebrates (Cardoso et al., 2016, 2020). In addition, like in other vertebrates (except teleost fishes and placental mammals), a second type of CRF (CRF2) is found in chicken, which is expressed in the brain (as determined by qPCR) and, at least in vitro, is able to bind CRF receptors as follows: it shows high preference for and is a potent activator of CRFR2, and it has low affinity for CRFR1, being able to activate the latter only at high doses (Bu et al., 2019). Through activation of CRFR2, CRF2 is a potent stimulator of the production of thyroid stimulating hormone (TSH, beta subunit) in cultured pituitary cells, and may play a role in food intake regulation by way of the hypothalamo-pituitary-thyroid (HPT) axis (Bu et al., 2019; see also De Groef et al., 2003, 2005). Moreover, at high doses, CRF2 binds to CRFR1 and is able to stimulate ACTH production and activate the HPA (Bu et al., 2019). Concurrent activation of both HPA and HPT is observed following psychogenic stress in chicken, and in this case HPT activation may be needed to generate enough energy to cope with the stressful situation (Kadhim and Kuenzel, 2022). CRF appears to have dual actions on ACTH and TSH secretions in all non-mammalian vertebrates studied so far (including fishes, amphibians, and sauropsids) (De Groef et al., 2005, 2006), but this might be different in mammals, since one study in mouse found no mRNA expression of CRFR2 and very poor mRNA expression of CRFR1 in thyrotrope-like cells of the anterior pituitary (Westphal et al., 2009). Nevertheless, it is possible that CRF receptor expression is induced in these cells of the anterior pituitary at specific developmental and physiological

conditions, resembling the situation described in birds during hatching and in amphibians during metamorphosis (De Groef et al., 2005, 2006; Westphal et al., 2009).

Like CRF peptides, CRF receptors (1 and 2) and binding protein also are highly conserved in evolution (Yu et al.., 1996; De Groef et al., 2004; Lovejoy and de Lannoy, 2013; Cardoso et al., 2016, 2020; Wan et al., 2022). In contrast to mammals, only one variant (alpha) of CRFR2 is found in chicken (De Groef et al., 2004). Using in situ hybridization in chicken, CRFR1 was specifically found in ACTH producing pituitary cells, while CRFR2 was expressed in TSH producing cells (De Groef et al., 2003). Studies by way of qPCR showed that mRNAs of both receptors are expressed in several brain regions of chicken, such as telencephalon, hypothalamus, midbrain tectum, and hindbrain (De Groef et al., 2004), while CRFBP mRNA is abundant in the telencephalon and hypothalamus, poor in the midbrain, and poor to moderate in the hindbrain (Wan et al., 2022). However, information on the detailed expression of CRF receptors and binding protein in the central nervous system is missing in birds, and thus many aspects on the degree of conservation of the brain mechanisms regulating the stress response are still unknown. This information is crucial to understand how stress is regulated in different animals, and to improve animal welfare. The aim of this study (second thesis chapter) is to carry out a detailed mapping of mRNA expression of CRF receptors (*Crfr1* and *Crfr2*) and binding protein (*Crfbp*) in chicken telencephalon, compared to distribution of CRF mRNA (*Crf*) expressing cells. Since the stress response changes with age in different animals (Herman et al., 2016), including birds, passing through a hyporesponsive period around birth or hatching (Schapiro et al., 1962; Freeman and Manning, 1984), we aimed to analyze the dynamic expression patterns of CRF related peptides and receptors throughout embryonic and early posthatching development.

Chicken CRF mRNA (*Crf*)

We began studying the embryonic stage E8 (Hamburger and Hamilton stages 34-35 or HH34-35), during which we observed a moderate *Crf* signal in the primordia of the hippocampal formation (HF), situated in the medial pallium, primarily in its medial part where the medial parahippocampal area and dentate gyrus will form, as well as in the apical hyperpallium (HA), located in the dorsal pallium, in the caudal part of the nidopallium (N) in the ventral pallium, and in some parts of the basal magnocellular complex (BMC) containing corticopetal cells in the subpallium (Fig. 11A,B).

As the telencephalon matured and became gradually larger, the signal intensity increased in some areas and *Crf* expression became localized to specific subdivisions by E12-E14 (Fig. 11C-G'). In the pallium, we observed the highest expression in the lateral part of the apical hyperpallium extending to the interstitial nucleus of the hyperpallium (HA) (Fig. 11C-E), in specific regions of the intermediate and caudal nidopallium, such as the medial part of the intermediate nidopallium (NIm), the entopallial belt or shell (Es), and the auditory field L (Fig. 11E-G, detail in G'). We also observed light to moderate expression in the olfactory bulb (including mitral cells) and anterior olfactory area (OB, AO) (Fig. 11C,D), parts of the densocellular hyperpallium (HD), the medial part of the apical hyperpallium, several parts of the hippocampal formation (including dentate gyrus [DG], medial, lateral and caudolateral parahippocampal areas [APHm, APHl, APHcl]), the entorhinal cortex (Ent), parts of the mesopallium (M, mainly its medial intermediate part), some patches of the caudolateral nidopallium (NCL), and part of the arcopallium (Arc, including dorsal, core, amygdalopiriform, and amygdalohippocampal areas). In addition, we observed very low expression in the prepiriform and piriform cortices (PPir) and the nucleus basorostrallis.

In the subpallium of the E12-E14 chicken, we observed strong expression of *Crf* in the medial striatum from rostral to intermediate levels (including the nucleus accumbens) (MSt), as well as in the large perikarya of the basal magnocellular complex (BMC), which spread over the medial forebrain bundle/ventral pallidum and the medial aspect of the lateral forebrain bundle to reach the intrapeduncular nucleus and medial aspect of the globus pallidus (Fig. 11F, detail in F'). At E14, part of the central extended amygdala also expressed low to moderate levels of *Crf*, mostly in the lateral BST (BSTL; Fig. 11F',G'), and scattered cells of the medial BST (BSTM) and medial preoptic region (PO) expressed moderate levels of *Crf* at this embryonic age (Fig. 11G,G'). In the septum, we observed strong expression in the dorsal part of the nucleus of the hippocampal commissure (HiC) and adjacent part of the septofimbrial nucleus, while the medial and latero-intermediate nuclei (LSI) showed light signal (Fig. 11G').

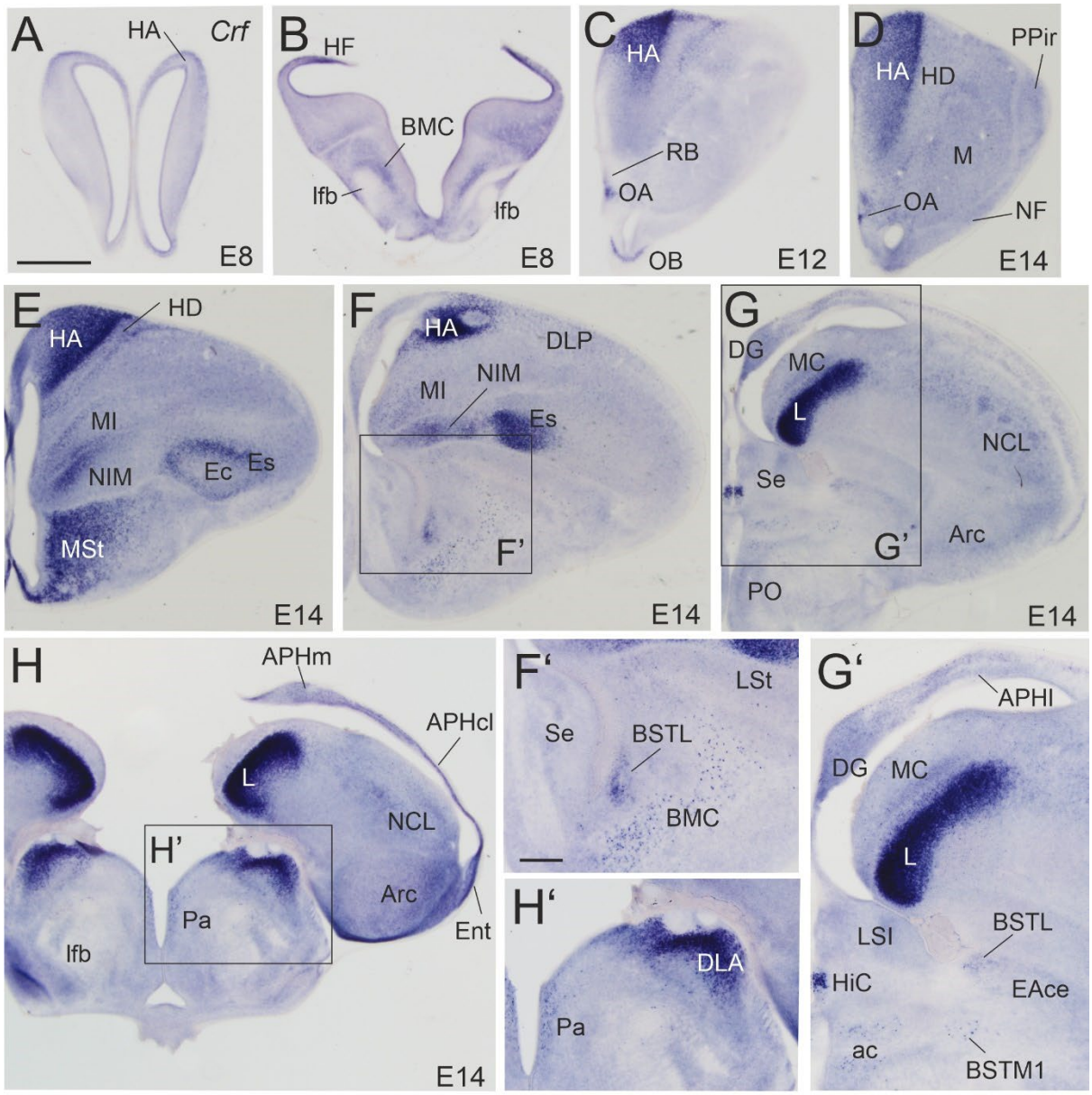


Figure 11. Images of frontal sections through the embryonic telencephalon and anterior hypothalamus of chicken (E8 and E14), from anterior to posterior levels, showing hybridization signal for chicken *Crf*. In these images, dorsal is to the top and in C-G, F'-G', medial to the left. Note the strong signal at E14 in visual and auditory pallial areas (hyperpallium, entopallium, field L). At this age, expression is also seen in the parvocellular paraventricular hypothalamic nucleus, as well as in several nuclei of the thalamus. A remarkable expression difference between MSt (rostrointermediate levels) and LST can be obsserved in E-G. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to A-H); F' = 200 µm (applies to F'-H'). (Adapted from Metwalli et al., 2023)

By E18 (Fig. 12), *Crf* expression pattern in the telencephalon remained quite similar to that seen at E14, with little changes, such as the high increase in *Crf* signal in the BSTL and its lateral extension into the perioval zone of the central extended amygdala (Fig. 12C-E, details in C' and E'), and in the septofimbrial nucleus (SFi; Fig. 12E'). Signal continued to be quite strong in the lateral part of the apical hyperpallium (at E18 became particularly strong in the interstitial nucleus of the apical hyperpallium or IHA; Fig.12A, B, detail in A'), field L of the caudal nidopallium (L; Fig. 12C-E), rostral (accumbens) parts of the medial striatum (MSt; Fig. 12B) and adjacent olfactory tubercle, and the large perikarya of the basal magnocellular complex (BMC; Fig. 12C, detail in C'). Light or moderate signal was present in other pallial and subpallial areas. In the pallium, these included the hippocampal formation (including the dentate gyrus [DG] and the parahippocampal areas [APH]; Fig. 12C,D), medial apical and densocellular parts of the hyperpallium (Fig. 12A), the caudomedial mesopallium (M; Fig. 12C), medial part of the intermediate nidopallium (NI; Fig. 12B), parts of the arcopallium (Arc, Fig. 12E), the prepiriform and piriform cortices (PPir, Pir; Fig. 12A,B), the anterior olfactory area and the olfactory bulb (OB; Fig. 12A). In the subpallium, scattered cells expressing Crf were seen at intermediate/caudal levels of the medial and lateral striatum (MSt, LSt), globus pallidus (GP), intrapeduncular nucleus (INP) (Fig. 12C, detail in C'). The medial aspects of GP and INP also included large perikarya expressing Crf, belonging to BMC (Fig. 12C'). More caudally, scattered *Crf* expressing cells were seen in lateral parts of the central extended amygdala (including perintrapeduncular and capsular areas), the diagonal band nucleus and septopallidal area (PaS), part of the septocommissural area (including the caudocentral septal nucleus or CCS), the BSTM and the preoptic region (Fig. 12D,E,E').

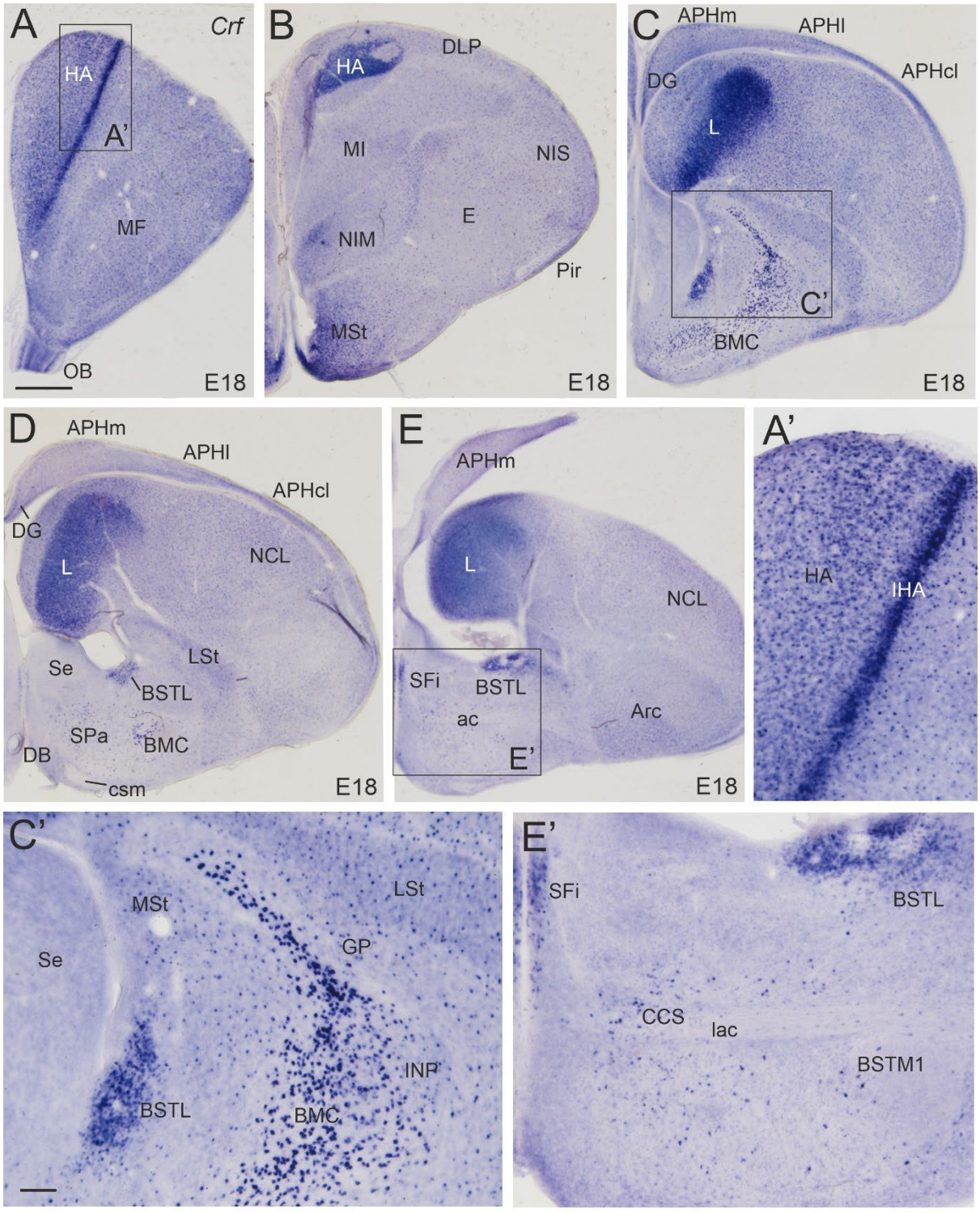


Figure 12. Images of frontal sections through the embryonic telencephalon of chicken (E18), from anterior to posterior levels, showing hybridization signal for chicken *Crf* (details in A', C' and E'). In these images, dorsal is to the top and medial to the left. Note the strong expression in visual and auditory areas of the pallium, in dopaminoreceptive nuclei of the striatum (including the rostromedial striatum and adjacent olfactory tubercle), in corticopetal cells of the subpallium (BMC) and in part of the extended amygdala (BSTL). For abbreviations see list. Scale bars: A = 1mm (applies to A-E); C' = 200 µm (applies to A', C', E'). (Adapted from Metwalli et al., 2023)

At hatching (P0), the *Crf* expression pattern remained quite similar to that seen at E18, although the intensity of the expression became more homogeneous between areas, with a tendency to be light to moderate. The only sites where the signal was high were the IHA in the hyperpallium (Fig. 13A), field L in the caudal nidopallium (Fig. 13C,D), part of the piriform cortex (Pir; Fig. 13C), the septofimbrial nucleus (SFi, just dorsal to HiC; Fig. 13C, detail in C'), the rostromedial striatum (MSt, including nucleus accumbens; Fig. 3A), the BSTL (Fig. 3C, detail in C'), the perikarya of the basal magnocellular complex (BMC; Fig. 13B), and the medial preoptic region (PO; Fig. 13C). At P7, the pattern remained similar, and most of the same sites continued to show higher levels of *Crf* expression than the surrounding areas (Figs. 14B,C,E; 15B-D). In addition, the olfactory bulb and the anterior olfactory area also showed high expression at P7 (Fig. 14A). In the olfactory bulb, many cells of the mitral cell layer and some of the granular cell layer expressed high levels of *Crf*. Except the olfactory bulb, olfactorecipient areas, hyperpallium, medial intermediate nidopallium and field L, in the rest of the pallium, *Crf* expressing cells were less densely grouped at P7 compared to previous ages (Figs. 14, 15). In the hippocampal formation, only few, scattered cells were observed in the medial layer of the V-field (containing the dentate gyrus; DG) and the medial and lateral parts of the parahippocampal area (APHm, APHl; Fig. 15A). In the subpallium, the distribution and density of *Crf* expressing cells was quite similar between P0 and P7. Expression continued to be quite high in many cells of SFi and adjacent dorsal aspect of HiC, BSTL and BMC (Fig. 15B-D). Some cells expressing *Crf* were also present in the septopallidal area, striatum, globus pallidus and lateral parts of the central extended amygdala, where a distinct group of *Crf* expressing cells became visible in the medial aspect of the capsular central amygdala (Fig. 15C). Outside the telencephalon, we would like to remark the presence of cells expressing moderate levels of *Crf* in the parvocellular part of the paraventricular hypothalamic nucleus, on the top of the hypothalamo-pituitary-adrenal axis, from E14 (Fig. 11H, detail in H'). Compared to E14, the expression levels in these cells appeared to be higher at E18 and hatching (P0; Fig. 13D, detail in D'), and even higher at P7 (Fig. 15E), at least based on hybridization signal. Many *Crf* expressing cells were also seen in visual, auditory and somatomotor parts of the thalamus from E14 (Fig. 11H, detail in H'). In particular, strong expression was seen in many cells of the anterior dorsolateral/dorsointermediate nuclei (DLA, DIA). In addition, some *Crf* expressing cells were present in the dorsointermediate ventral anterior nucleus (DIVA, named dorsal somatosensory nucleus by Puelles etal., 2019) and ventrointermediate nucleus (VIA, named dorsal motor nucleus by Puelles et al., 2019), suprarotundus (or epirotundus) and subrotundus nuclei, medial pole of nucleus rotundus, and periovoidal region or shell (i.e.

around nucleus ovoidalis, also named as the avian medial geniculate nucleus by Puelles et al., 2019). In the dorsal midbrain, visual and auditory centers (optic tectum and torus semicircularis) also showed *Crf* expression at least from E14.

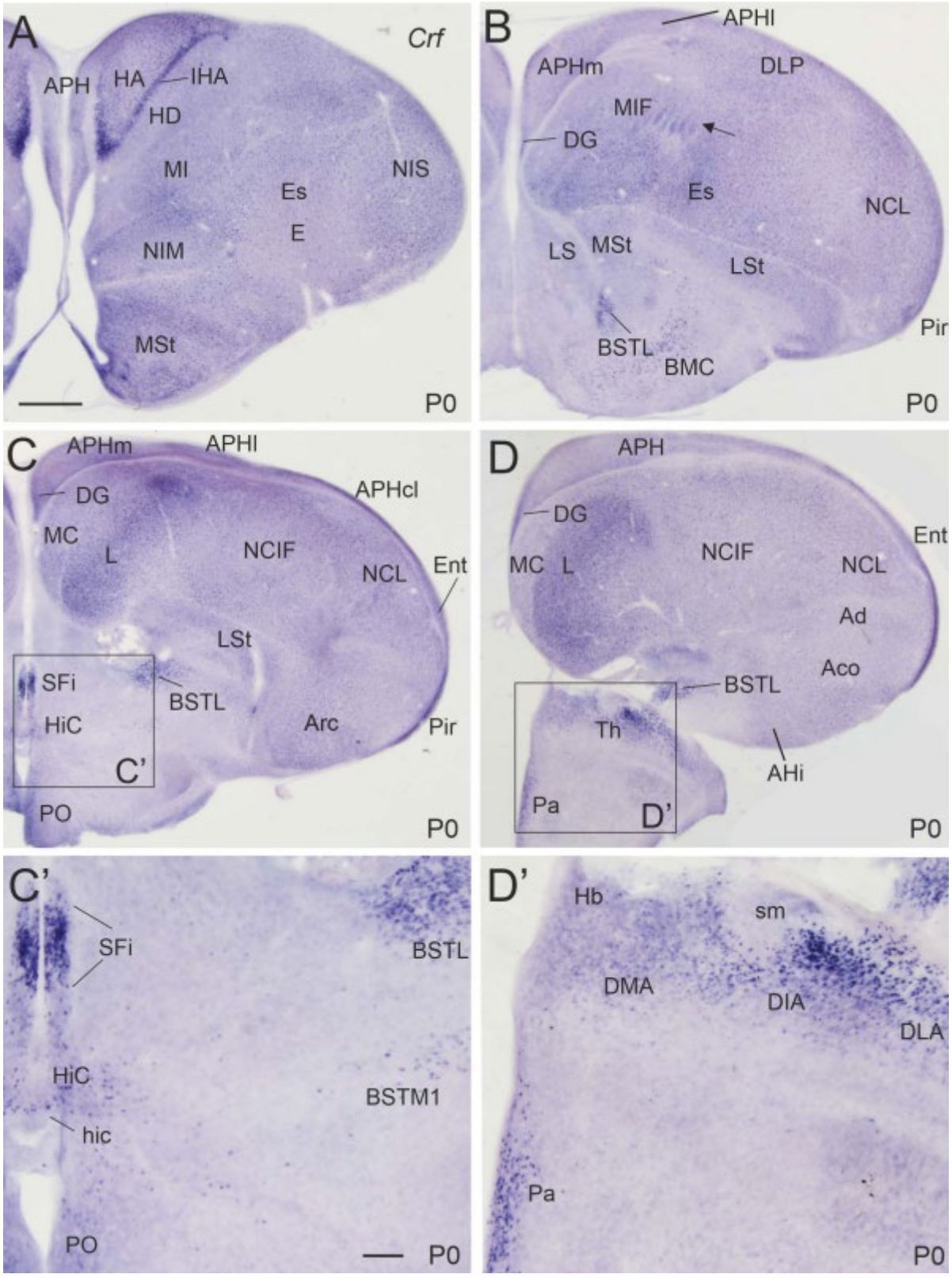


Figure 13. Images of frontal sections through the telencephalon (A-D, C') and anterior hypothalamus (D, D') of chicken at hatching (P0), from anterior to posterior levels, showing hybridization signal for chicken Crf (details in C' and D'). In these images, dorsal is to the top and medial to the left. For abbreviations see list. Scale bars: A = 1mm (applies to A-D); C' = 200 μm (applies to C', D'). (Adapted from Metwalli et al., 2023)

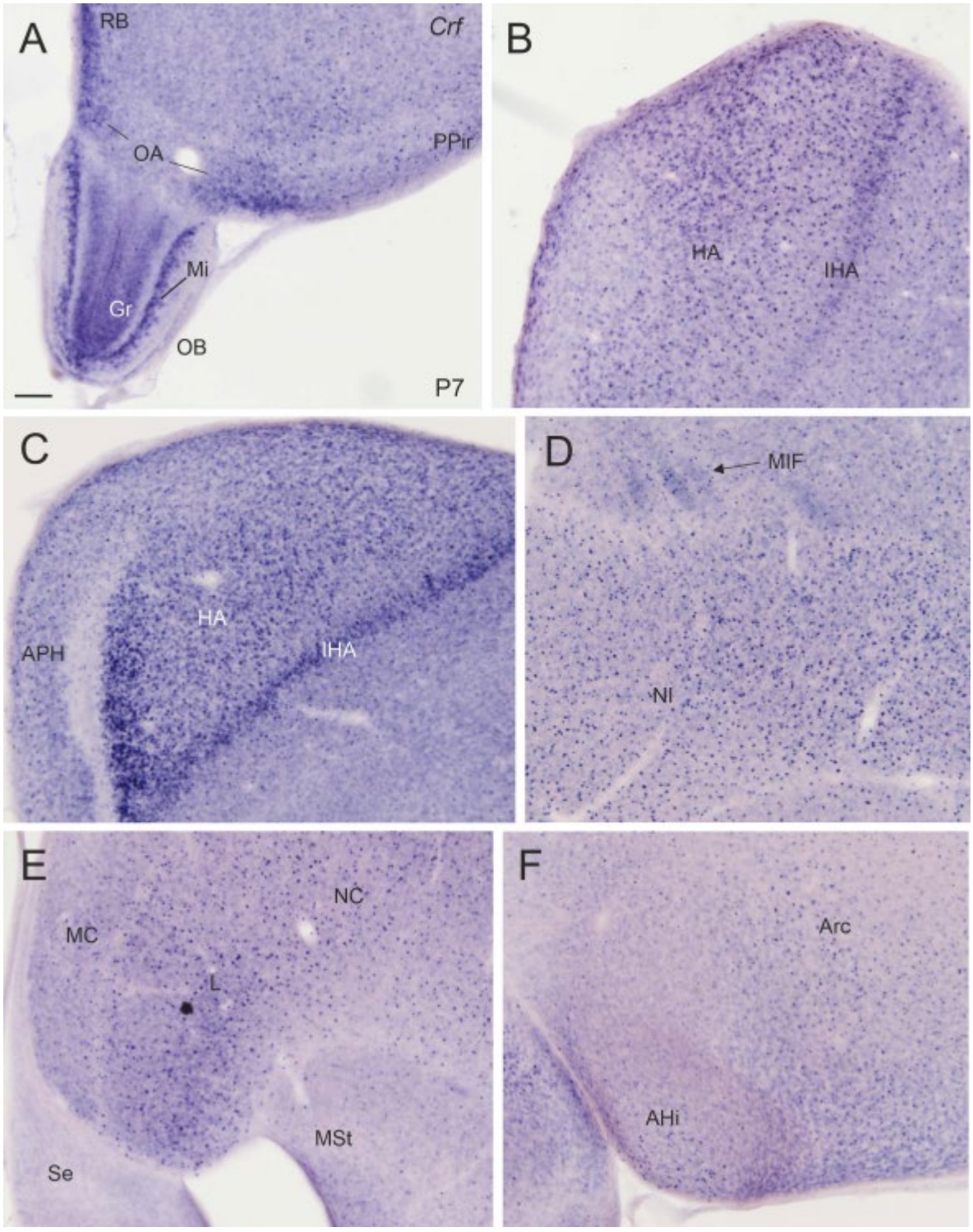


Figure 14. Details of frontal sections through the telencephalon of chicken at P7, showing hybridization signal for chicken *Crf*. In these images, dorsal is to the top and medial to the left. A is at the level of the olfactory bulb and anterior olfactory area; B and C show details of the hyperpallium, at different anteroposterior levels (C is more posterior and also shows part of the parahippocampal formation); D is a detail of the intermediate nidopallium, and the patches of the mesopallial island field near the mesopallium-nidopallium boundary (arrows); E shows the caudomedial nidopallium and mesopallium; F is a detail of the arcopallium. For abbreviations see list. Scale bars: A = 200 μm (applies to all). (Adapted from Metwalli et al., 2023)

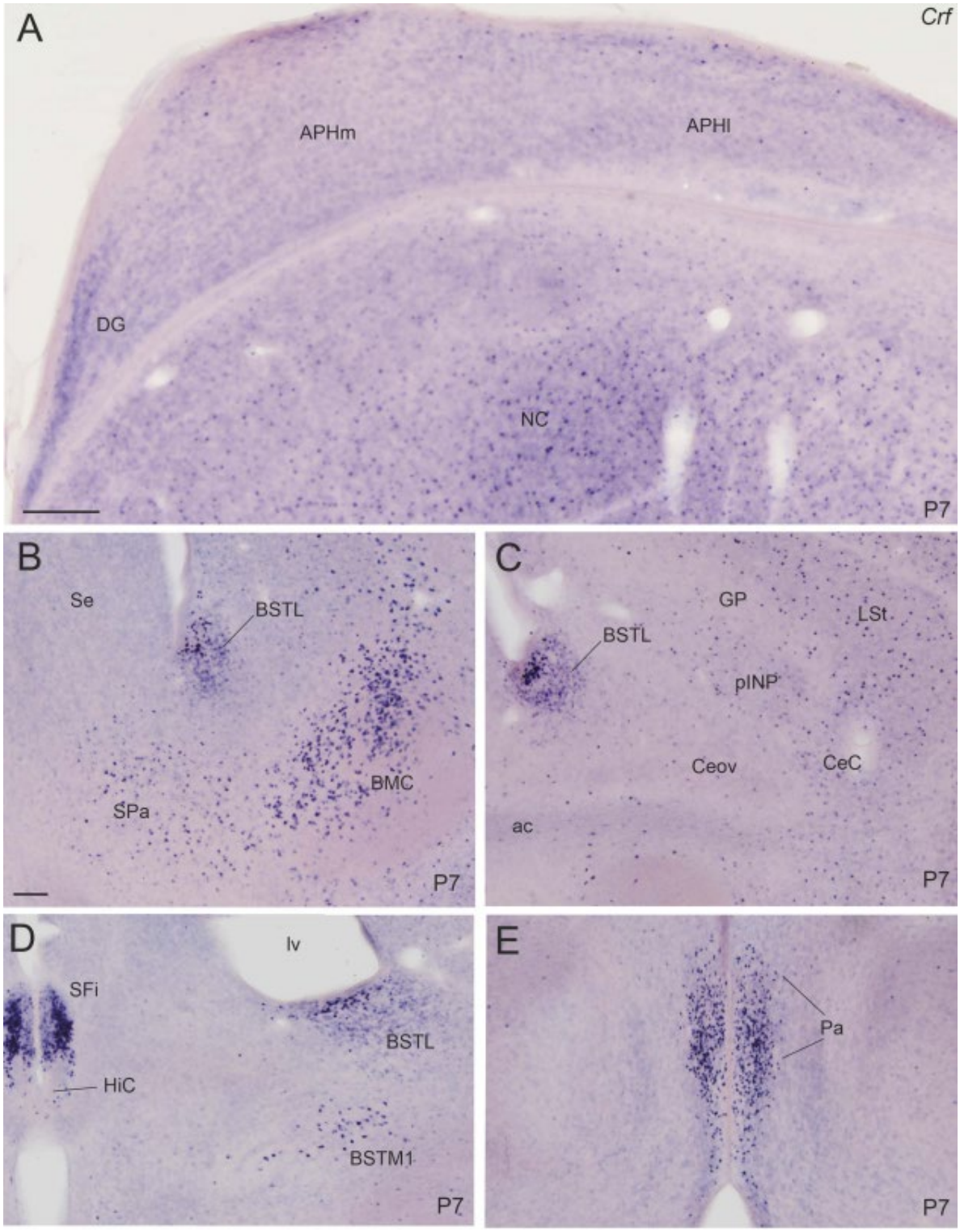


Figure 15. Details of frontal sections through the telencephalon (A-D) and anterior hypothalamus (E) of chicken at P7, showing hybridization signal for chicken *Crf*. In these images, dorsal is to the top and medial to the left (except in E, which is midline is in the middle). A is at the level of the hippocampal formation; B-D show details of the subpallium, from anterior to posterior levels, showing the basal magnocellular complex (BMC), several nuclei of the extended amygdala (including BSTL, BSTM, pINP and CeC); D also shows the septum, with strong signal in HiC/SFi nuclei. E shows a detail of the parvocellular part of the paraventricular hypothalamic nucleus. For abbreviations see list. Scale bars: A = 200 μm (applies to all). (Adapted from Metwalli et al., 2023)

Chicken CRF receptor 1 mRNA (*Crfr1)*

At E8, certain areas of the pallium exhibited strong or very strong expression of *Crfr1*, including the hippocampal formation primordium, various olfactorecipent areas located on the ventral pallium's surface (such as the piriform cortex primordium), the intermediate and caudal nidopallial levels, and the arcopallium (Fig. 16A-C). The dorsolateral pallium showed only moderate *Crfr1* expression at this stage. In contrast, *Crfr1* expression in the subpallium was limited, with the exception of two small groups of subpial cells found in the septofimbrial nucleus (medially; Fig. 16A) and the olfactory tubercle (laterally, indicated by arrow in Fig. 16A), as well as a few scattered cells in the lateral striatum, and the preoptic region (PO) (Fig. 16B). The extended amygdala cell corridor extending from the telencephalon-opto-hypothalamic domain (TOH) to the pallial amygdala, running along the subpial surface of the subpallium and encompassing the nucleus of the lateral olfactory tract (LOT), also showed light expression (Fig. 16B).

At E14, the telencephalon was more developed and it was possible to identify better the areas expressing the receptor mRNA (Fig. 16D-I). *Crfr1* expression continued to be moderate to high in most subdivisions of the pallium, except the mesopallium, but a clear pattern emerged with areas of high expression and others with light or no expression. Both the hippocampal complex (including the hippocampal formation and the entorhinal cortex) and the arcopallium showed the highest and most extensive expression levels (Fig. 61G-I). In the hippocampal complex, high expression covered all areas (DG, APH, Ent) and layers, along all mediolateral and anteroposterior levels (Fig. 16E-I). In the arcopallium, moderate to strong expression was seen in many areas, increasing from anterior to posterior levels (Fig. 16G-I), and the strongest signal was observed in the dorsal (Ad), amygdalopiriform (APir), and amygdalohippocampal (AHi) areas (Fig. 16I). The dorsolateral pallium (DLP) (Fig. 16F) and all olfactorecipient areas of the ventral pallium (anterior olfactory area [AO; Fig. 1], prepiriform cortex [PPir; Fig. 16D, F] and piriform cortex [Pir; Fig. 16G,H]) showed moderate to high expression. In the hyperpallium and nidopallium, expression was high in some areas and low in others, as follows. In the hyperpallium, moderate to high expression was observed medially in the apical hyperpallium and in the IHA, but expression was low in the lateral apical and densocellular hyperpallium (Fig. 16D,E). In the nidopallium (N), signal was moderate to strong in the frontal, part of the intermediate, and part of the caudal nidopallium (Fig. 16D-I). At frontal levels (NF), expression was moderate to high in the frontolateral nidopallium (NFL) and in nucleus basorostralis (Bas) (Fig. 16D,E). At intermediate levels (NI), expression was moderate in the superficial stratum

(NIS), but the entopallium and the medial area only contained light or negligible expression (Fig. 16E,F). In the caudal nidopallium (NC), moderate expression was observed in the caudolateral area (NCL), the dorsal nidopallium, and the periphery of field L in the caudomedial area (Fig. 16G,H).

Regarding the mesopallium, this pallial subdivision was quite poor in *Crfr1* expression, except for its frontomedial, retrobulbar pole (RB, Fig. 16D), and the core nucleus of the ventral mesopallium (MVco, Fig. 16E). In contrast to the pallium, at E14 the subpallium was almost free of *Crfr1* expression, except part of nucleus accumbens (Acb, Fig. 16E), part of the septum (laterointermediate septum [LSI] and the part of the septofimbrial nucleus [SFi] adjacent to HiC; Fig. 16G), the medial amygdala (Me; Fig. 16H), and the medial preoptic nucleus (Fig. 16G), which showed moderate levels of expression. Ventral to the subpallium, moderate expression was also present in the subpreoptic region (SuPO) of the telencephalon-opto-hypothalamic domain or TOH (Fig. 16G). The cell corridor extending between the arcopallium and the TOH, which includes the nucleus of the lateral olfactory tract (LOT), also contained moderate expression of *Crfr1* at E14 (Fig. 16G).

At E18, *Crfr1* expression intensity increased in the pallium, and it spread to cover more areas. At this age, all areas of the hippocampal complex, apical hyperpallium, nidopallium, and arcopallium showed moderate to high levels of *Crfr1* (Fig. 17A-F). In the hyperpallium, the highest expression was seen in the apical hyperpallium and IHA (Fig. 17A), while in the nidopallium, the highest signal was observed in the frontolateral (NFL; Fig. 17A) and medial intermediate (NIM; Fig. 17B,C) areas, as well as in field L, the patches of the island field (NCIF), and the caudolateral part (NCL) of the caudal nidopallium (Fig. 17E,F). In comparison, most of the densocellular hyperpallium (HD, except its superficial part) and most parts of the mesopallium (M, except MVco) showed light expression (Fig. 17A-C). Close to the pial surface of the ventral pallium, the piriform cortex also showed strong expression of *Crfr1* (Fig. 17D,E). In the subpallium, expression remained relatively low, although some areas increased a bit their signal, such as the striatal capsule (StC; Figs. 17D, 18A), the accumbens shell (AcS; Fig. 17B,C), the globus pallidus (GP; Fig. 18A), the intrapeduncular nucleus (INP; Fig. 18A), part of the central extended amygdala, including the oval nucleus and peri/post-intrapeduncular island field (Ceov, pINP; Figs. 17E,F; 18B), the septum (including the lateral septum [LS] and the septofimbrial nucleus) (Figs. 17E; 18A), and the medial preoptic region (PO; Fig. 17F, 18C). At E18, scattered *Crfr1* expressing cells were also seen in the medial BST of the medial

extended amygdala (Fig. 17F, detail in F'), and in other striatal and ventral pallidal parts of the basal ganglia not mentioned before (Fig. 18A).

The expression pattern of *Crfr1* remained similar at hatching. One week after (P7), the pattern remained the same, but the signal intensity increased in both the pallium and the subpallium (Figs. 19, 20). In the pallium, expression increased in the mesopallium, while keeping a lower intensity compared to other pallial subdivisions, such as the hyperpallium and the nidopallium (Fig. 19B,C). In the subpallium, expression was a bit stronger in the septum (Fig. 19D), parts of the extended amygdala (Figs. 19C,C'; 20), and the preoptic region (Figs. 19D; 20). In the central extended amygdala, moderate expression was visible in the oval nucleus (Ceov), part of the peri/post-intrapeduncular island field (pINP) (Figs. 19C, detail in C'; 19D, detail in 20), and the capsular central amygdala (Fig. 20). In the septum, moderate to high signal was seen in the laterointermediate nucleus (LSI), septofimbrial nucleus (SFi), and nucleus of the hippocampal commissure (HiC) (Figs. 19D, detail in 20). Scattered cells expressing *Crfr1* were also observed in nuclei of the septopreoptic territory, including the septocommissural nucleus or CoS (Fig. 20). In the preoptic region, high expression was observed in the medial preoptic nucleus, although scattered cells expressing *Crfr1* were also seen in the medial preoptic area (Fig. 20). Finally, the subpreoptic region (SuPO, in TOH) and the subpial cell corridor extending from TOH to the amygdala contained abundant cells expressing *Crfr1* (arrows in Fig. 20). Several cell populations were observed along this subpial corridor, including a migrated group of the supraoptic nucleus (SO), with *Crfr1* expression (Fig. 20).

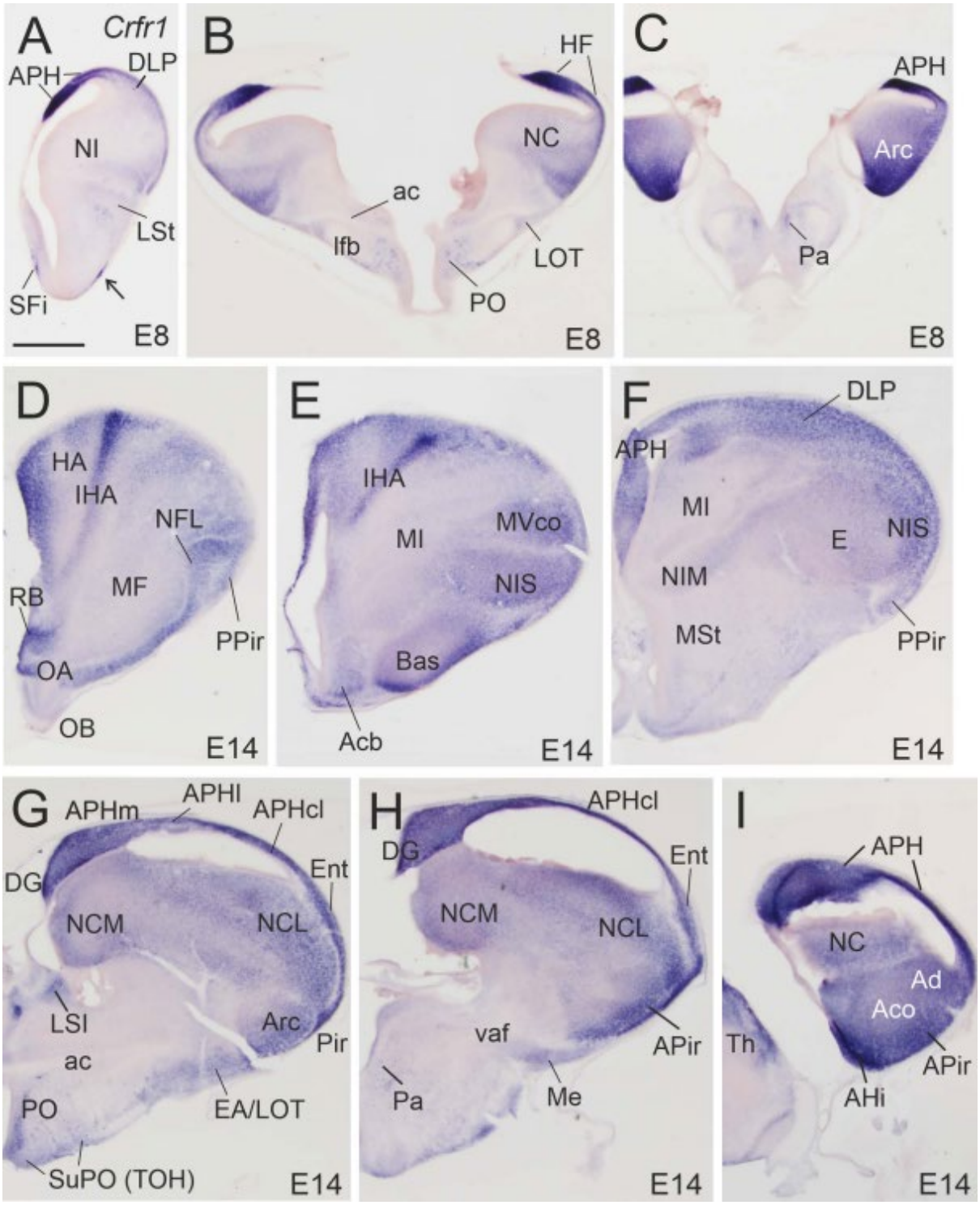


Figure 16. Images of frontal sections through the embryonic telencephalon and anterior hypothalamus of chicken (E8 and E14), from anterior to posterior levels, showing hybridization signal for chicken *Crfr1*. In these images, dorsal is to the top; in addition, in D-I medial to the left. Note the strong signal from E8 in the hippocampal formation and in pallial areas involved in high-order association (MVCo, NCL) and sensorimotor integration (HA, Arc). From E8, expression is also seen in the septum (including SFi), in a subpial patch of the olfactory tubercle (pointed with an arrow), and in the paraventricular hypothalamic nucleus. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

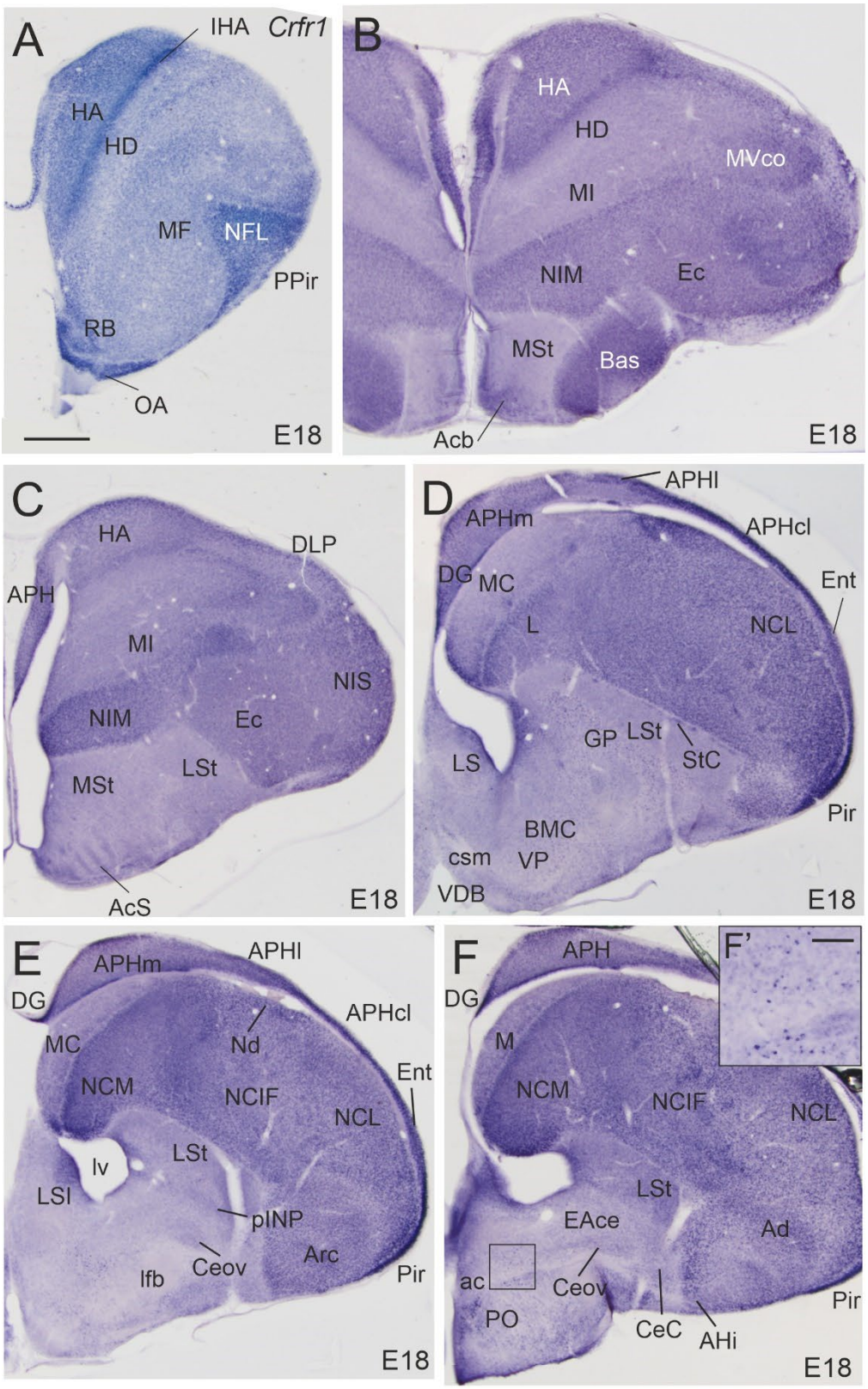


Figure 17. Images of frontal sections through the embryonic telencephalon and anterior hypothalamus of chicken (E18), from anterior to posterior levels, showing hybridization signal for chicken *Crfr1*. In these images, dorsal is to the top and medial to the left. Note the strong signal in the pallium, but low expression in the subpallium, except parts of the striatum, septum, and medial preoptic region. The detail in F' shows scattered cells in BSTM. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to A-F); F' = 200 µm. (Adapted from Metwalli et al., 2023)

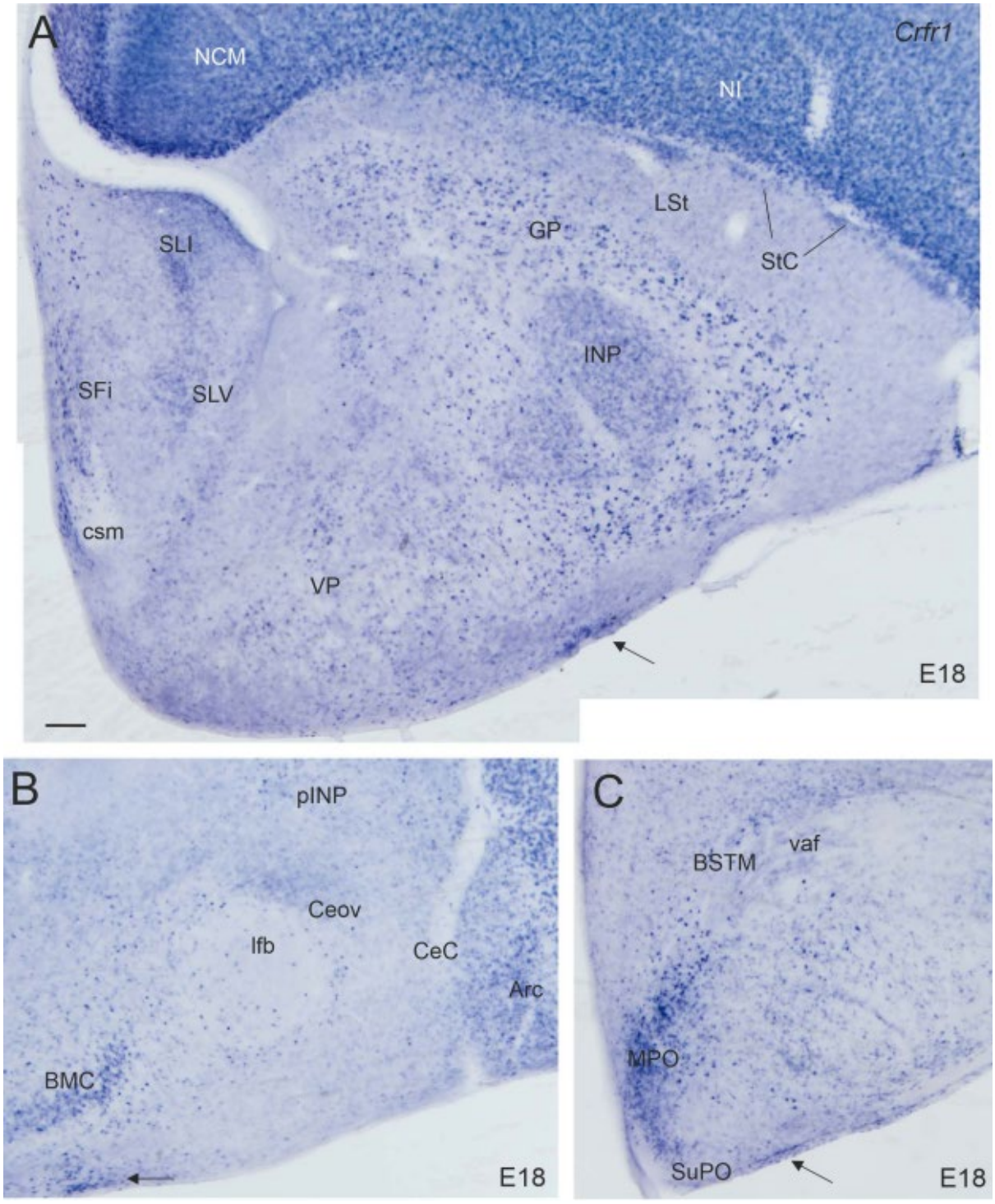


Figure 18. Details of *Crfr1* in the subpallium of the embryonic telencephalon at E18. In these images, dorsal is to the top and medial to the left. Compared to the pallium, expression in the subpallium is generally low, with a few exceptions. Note the signal in the striatal capsule (StC), globus pallidus, INP, parts of the septum, and medial preoptic region. The arrows in A-C) point to subpial expression that appears to extend from the subpreoptic region (SuPO, in the TOH domain; C), reaching the lateral subpallial surface, where the olfactory tubercle locates (A). Low signal is also seen in parts of the extended amygdala (Ceov, BSTM). See more details in text. For abbreviations see list. Scale bars: A = 200 µm (applies to all). (Adapted from Metwalli et al., 2023)

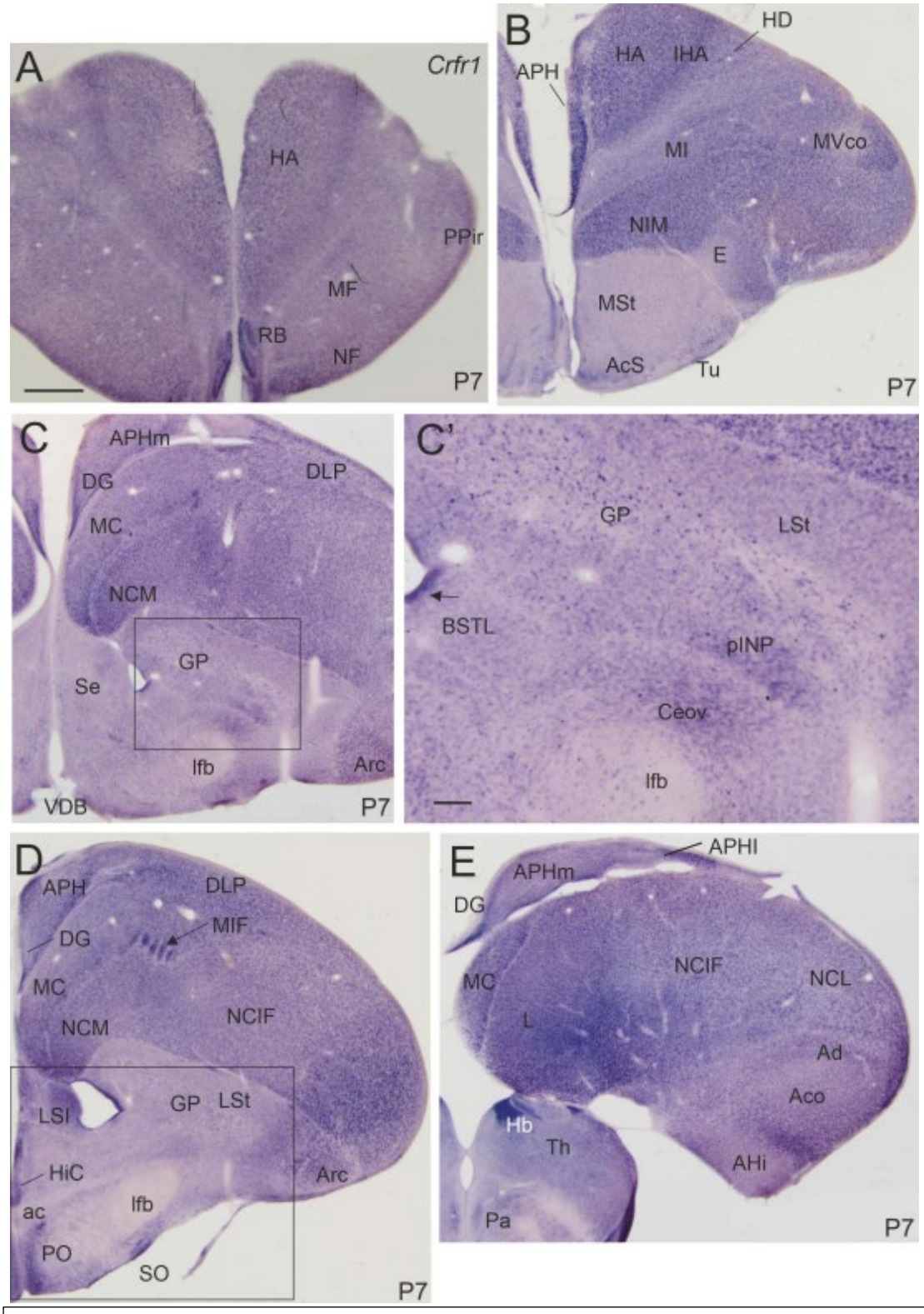


Figure 19. Images of frontal sections through the telencephalon and anterior hypothalamus of chicken at P7, from anterior to posterior levels, showing hybridization signal for chicken *Crfr1*. In these images, dorsal is to the top and medial to the left. C' shows a detail of the area squared in C. Note the strong signal in the pallium, but low to moderate expression in the subpallium. In the subpallium, moderate signal is observed in part of the striatum, septum, central extended amygdala (Ceov, pINP) and medial preoptic region. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to A-C, D, E); C' = 200 µm. (Adapted from Metwalli et al., 2023)

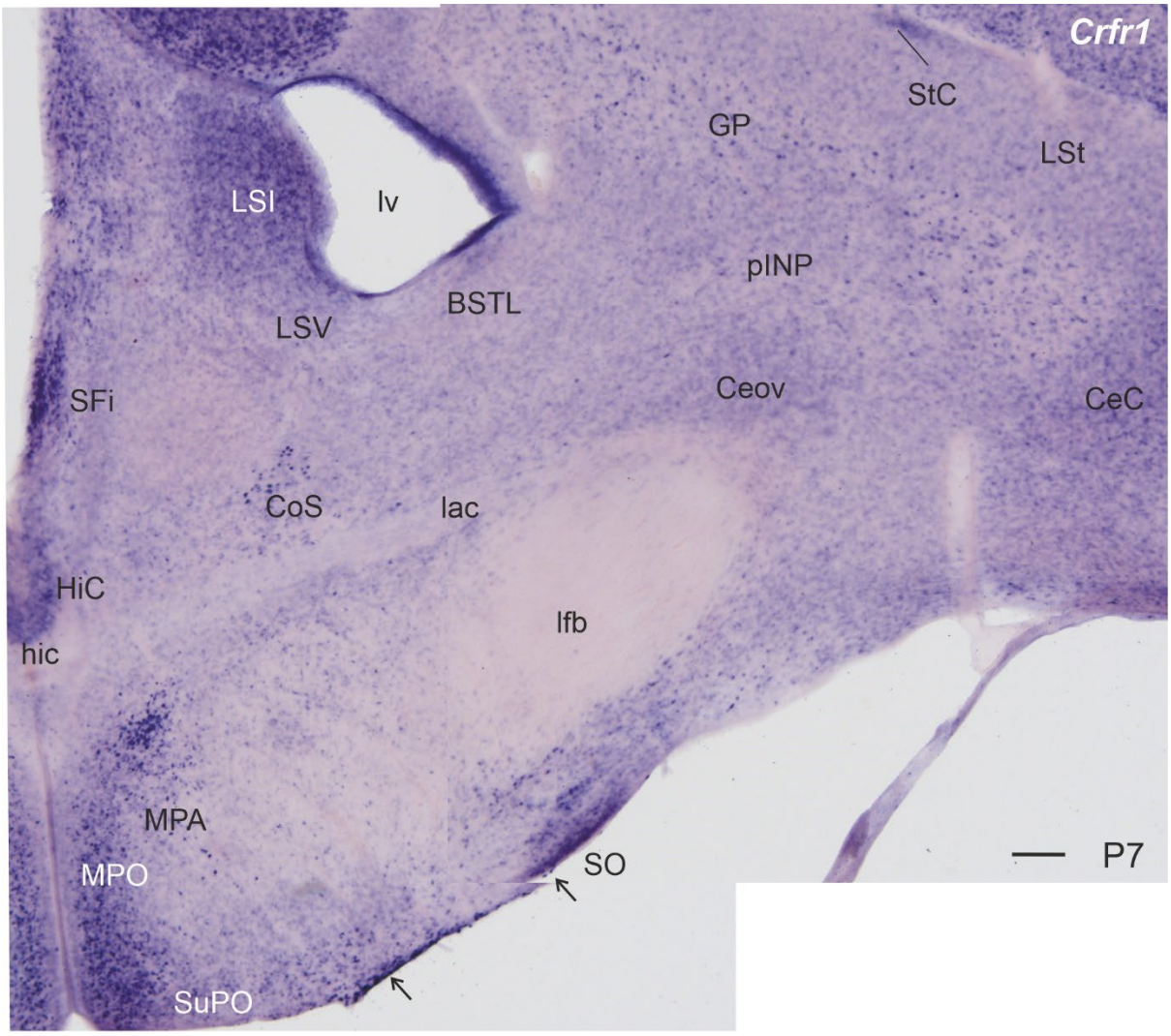


Figure 20. Detail of *Crfr1* in the subpallium of chicken at P7 (photomontage). In this image of a frontal section, dorsal is to the top and medial to the left. Note the signal in the striatal capsule (StC), globus pallidus, parts of the extended amygdala (pINP, Ceov, CeC) and septum (LSI, HiC, SFi), and medial preoptic region. Expression is also seen in a subpial area (arrows) that appears to extend dorsolaterally from the subpreoptic region (SuPO, in the TOH domain), reaching the lateral surface of the subpallium. Part of the supraoptic nucleus (SO) locates in the middle of this Crfr1 expressing subpial area. Low signal is also seen in parts of the extended amygdala (Ceov, BSTM). See more details in text. For abbreviations see list. Scale bars: A = 200 µm. (Adapted from Metwalli et al., 2023)

Chicken CRF receptor 2 mRNA (*Crfr2*)

Throughout development, *Crfr2* showed an expression pattern remarkably different from that of *Crfr1*. At E8, *Crfr2* signal was very high in the developing mantle of the mesopallium (lateral pallium), in medial and intermediate parts of the hippocampal formation, and in nucleus basorostralis of the nidopallium (Fig. 21A). Light to moderate expression was also seen in the lateralmost part of the developing hippocampal formation, the mantle of the dorsal hyperpallium and dorsolateral pallium, the medial part of the intermediate nidopallium, and in a superficial layer of the caudal ventral/ventrocaudal pallium (Fig. 21A,B). The rest of the pallium was basically free of expression. At E8, the subpallium was free of *Crfr2* expression, while signal was visible in the medial part of the subpreoptic region, in TOH (Fig. 21B).

At E14, *Crfr2* expression remained high in most parts of the mesopallium, being particularly strong in MVco (Fig. 21C,D). At this age, *Crfr2* was also high in the densocellular hyperpallium (HD; Fig. 21C), while the dorsolateral pallium and apical hyperpallium showed moderate expression only in part of the area (Fig. 21C,D). The nidopallium and arcopallium showed only light or no expression (Fig. 21C-F), the only exception being the nucleus basorostralis, which maintained high expression at E14 (Fig. 21D). In the rest of the nidopallium, light expression was visible in part of the medial intermediate nidopallium (just medial to the entopallium) (Fig. 21D), and part of the caudolateral nidopallium (Fig. 21E). In the arcopallium, light expression was present in the dorsal arcopallium and the amygdalohippocampal area (Fig. 21F). In the hippocampal formation, expression was light or moderate in several areas from rostromedial to caudolateral, including the primordia of the dentate gyrus and part of the medial, lateral and caudolateral parahippocampal areas (Fig. 21D-F). The entorhinal cortex (Fig. 21E) and the anterior olfactory area (Fig. 21C) also displayed moderate expression. Regarding the subpallium, very light expression was seen in the lateral striatum (Fig. 21E), and lateral and ventrointermediate parts of the septum (Fig. 21E). More ventrally, in the subpreoptic region, signal was seen in a superficial area that appear to correspond to the supraoptic nucleus (Fig. 21F).

At E18, the expression pattern of *Crfr2* remained quite similar to that seen at E14 (Fig. 22). Expression was generally high in the mesopallium, but now it was possible to recognize differences in signal intensity between subdivisions within this pallial division (Fig. 22A-E). The highest intensity was seen in the ventral mesopallium, especially in its known associative centers of the ventral mesopallium at frontal (MF; Fig. 12A), intermediate (MVCo-also called

MVL- and MVM; Fig. 22B,C), and caudal levels (MC, Fig. 22D,E), including the mesopallial island field (MIF) adjacent to the boundary with the nidopallium (Fig. 22C,D). The dorsal mesopallium (MD) contained areas of high or moderate expression and areas of light expression (Fig. 22B,C). In the hyperpallium, expression was high in the densocellular subdivision (HD), but signal in the apical hyperpallium (HA) ranged from moderate at rostral levels (Fig. 22A) to low or negligible at intermediate or caudal levels (Fig. 22B,C). At this age, *Crfr2* expression in the hippocampal formation, dorsolateral pallium, nidopallium and arcopallium was light (for example, in DG and NCL; Fig. 22E) or negligible in most areas. Light expression was also observed in the olfactory bulb, anterior olfactory area (Fig. 22A), prepiriform cortex (Fig. 22A), and moderate to high expression was seen in the piriform cortex (Fig. 22C-E), and the entorhinal cortex (Fig. 22E). In the subpallium, signal was low to moderate in the lateral striatum, lateral part of the medial striatum (Fig. 22B,C), part of the olfactory tubercle (Fig. 22C), and lateral part of the peri/post-intrapeduncular island field (Fig. 22D), and extremely light in the BSTM and the medial part of the preoptic region (Fig. 22D,E). In the septum, light expression was visible in the lateral and ventrointermediate subnuclei (Fig. 22C,D), and in the nucleus of the hippocampal commissure (Fig. 22E).

At P0 (Fig. 23), the pattern remained similar to that at E18, but signal intensity increased in the dentate gyrus of the hippocampal formation, to reach levels comparable to those in the mesopallium (Fig. 23D-F, detail in F''). At this age, expression was also intense in the piriform cortex (Fig. 23F, detail in F'). In the subpallium, in addition to other areas mentioned above for E18, light signal was now visible in other parts of the medial striatum, including patches related to the shell of nucleus accumbens (AcS; Fig. 23C). In addition, light to moderate signal was seen not only in the lateral striatum (LSt), but also in the adjacent striatal capsule (StC, Fig. 23C,D). By P7, the intensity of *Crfr2* expression generally decreased, while the pattern was similar to that seen at hatching and near hatching ages (Fig. 24).

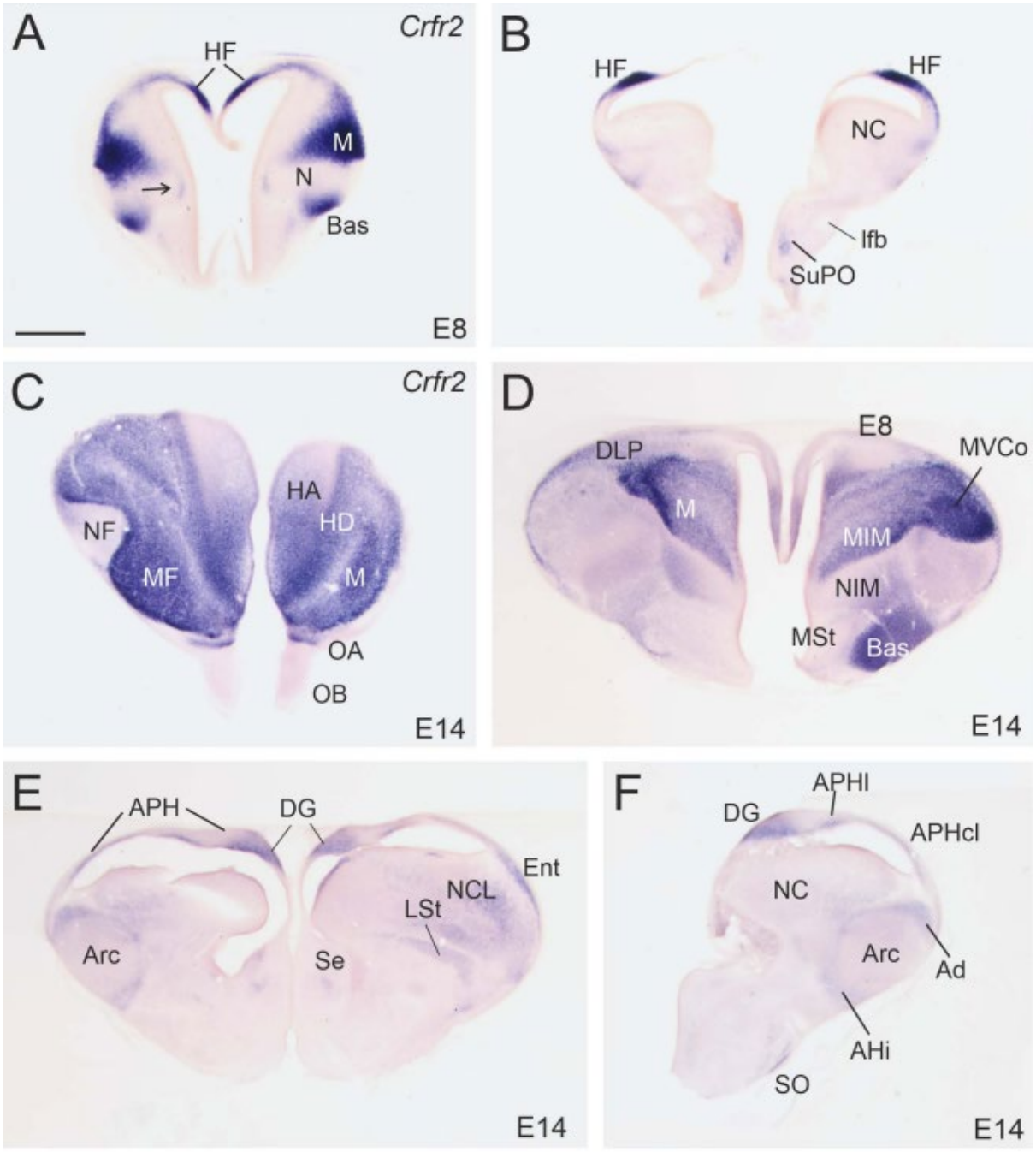


Figure 21. Images of frontal sections through the embryonic telencephalon and anterior hypothalamus of chicken (E8 and E14), from anterior to posterior levels, showing hybridization signal for chicken *Crfr2*. In these images, dorsal is to the top; in F, medial to the left. Note the strong signal from E8 in the hippocampal formation, the mesopallium (M), and in nucleus basorostralis (Bas). Expression in the hippocampal formation becomes less intense at E14. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

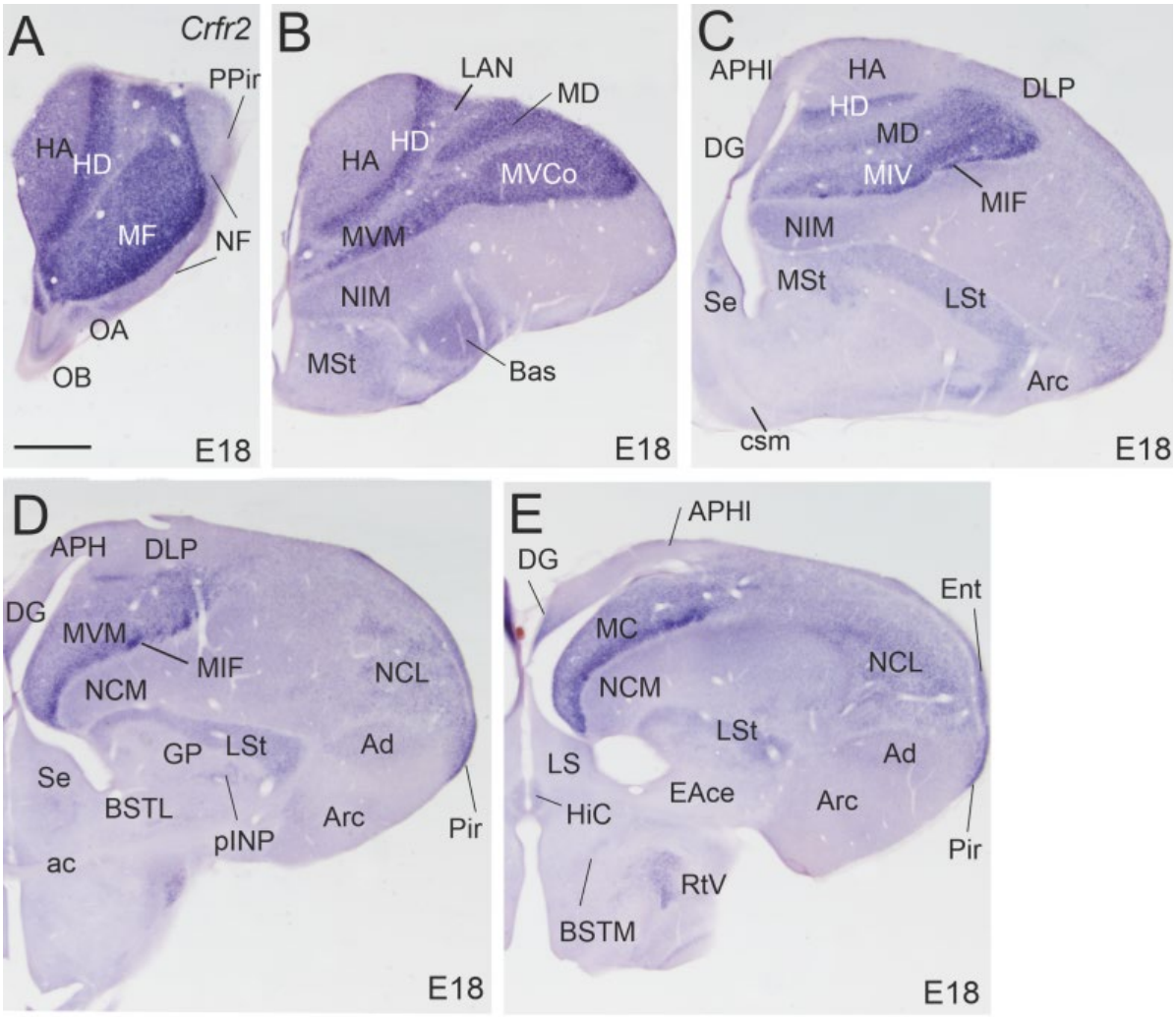


Figure 22. Images of frontal sections through the embryonic telencephalon and anterior hypothalamus of chicken (E18), from anterior to posterior levels, showing hybridization signal for chicken *Crfr2*. In these images, dorsal is to the top and medial to the left. Note the strong signal in the mesopallium (M) and densocellular hypepallium (HD). The olfactorecipient piriform cortex (Pir) also shows expression. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

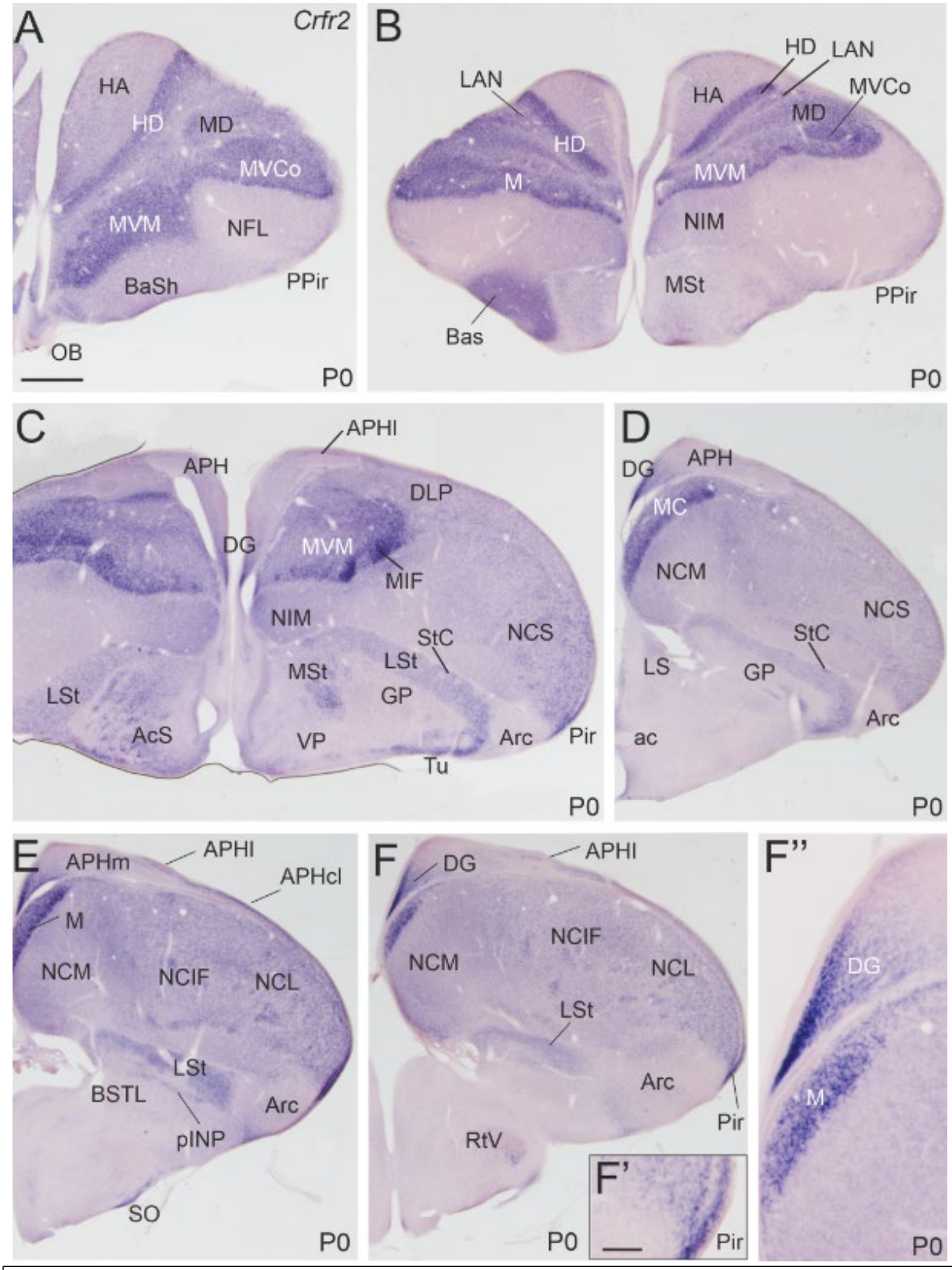


Figure 23. Images of frontal sections through the telencephalon and anterior hypothalamus of chicken at hatching (P0), from anterior to posterior levels, showing hybridization signal for chicken *Crfr2*. In these images, dorsal is to the top and in A, D-F medial to the left. Note the strong signal in the mesopallium (M) and densocellular hyperpallium (HD). F' shows a detail of the intense signal in the piriform cortex (Pir), while F'' shows a detail of the dentate gyrus (DG) of the hippocampal formation. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to A-F). F' = 200µm (applies to F', F''). (Adapted from Metwalli et al., 2023)

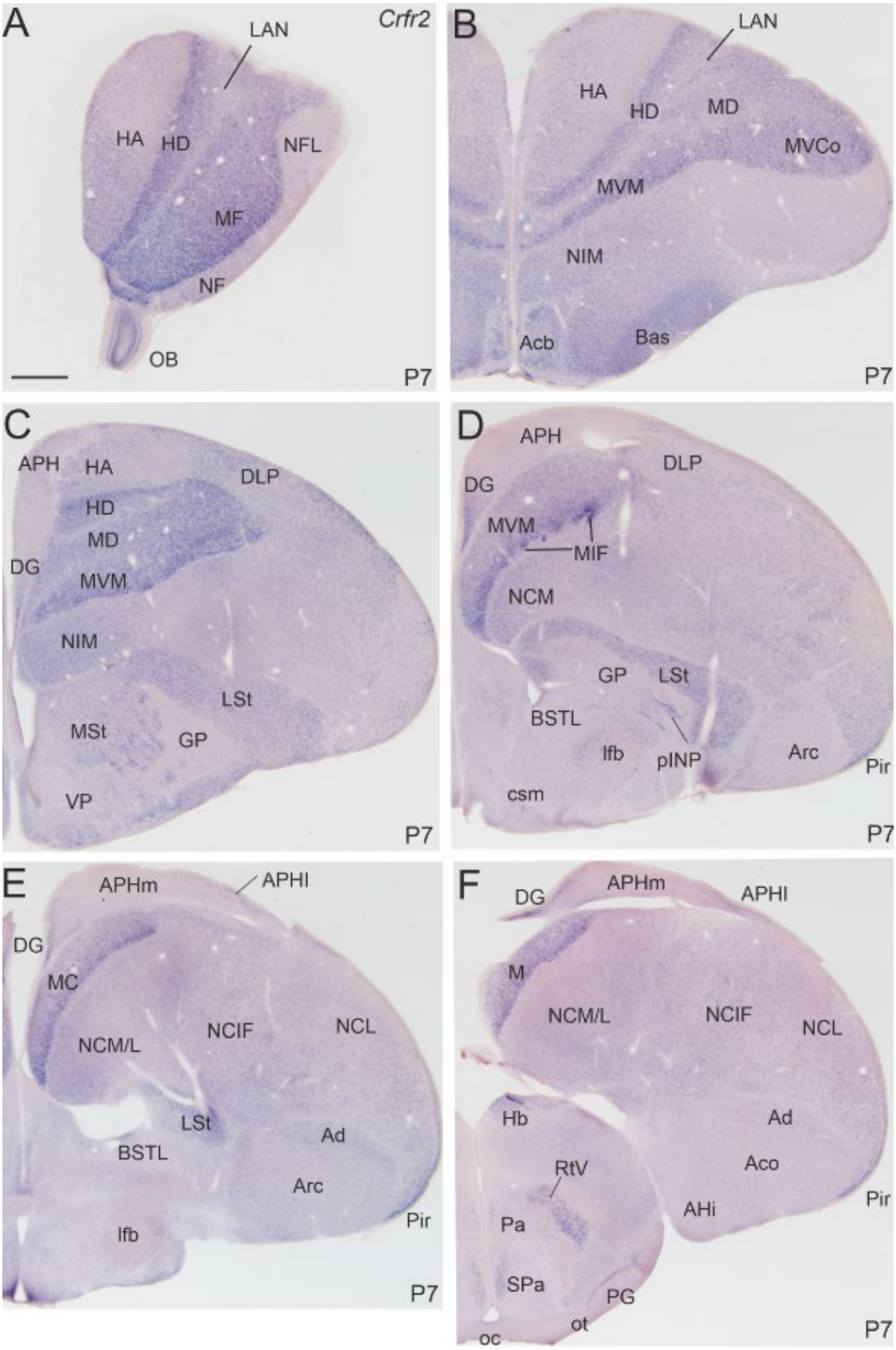


Figure 24. Images of frontal sections through the telencephalon and anterior hypothalamus of chicken at P7, from anterior to posterior levels, showing hybridization signal for chicken *Crfr2*. In these images, dorsal is to the top and medial to the left. The expression pattern is similar to that seen at P0. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

Chicken CRF binding protein mRNA (*Crfbp*)

During embryonic and early posthatching development, the expression pattern of *Crfbp* was very different to those of both CRF receptors (Figs. 25-28). At E8, the only telencephalic region with expression was the developing striatal capsule and adjacent lateral striatum (LSt), which displayed moderate signal of the CRF binding protein mRNA (Fig. 25A,B).

Signal in these areas (StC and LSt) became remarkably high by E14 (Fig. 25C-E), decreasing at caudal levels (Fig. 25F). At E14, high expression was also observed in part of the accumbens shell (AcS) and in the olfactory tubercle (Tu; Fig. 25D). In addition, *Crfbp* was moderately expressed in part of the peri/post-intrapeduncular island field (pINP) and in part of BSTL (Fig. 25F). Minor subsets of cells were also present in the medial amygdala and BSTM (Fig. 25F). In the pallium, *Crfbp* expression was light or moderate and mostly present only in restricted parts of the medial pallium, nidopallium and arcopallium. In the nidopallium, most subpopulations of *Crfbp* expressing cells were located in its sensory nuclei including nucleus basorostralis (Bas; Fig. 15C), the entopallium and entopallial belt (E, Es; Fig. 25D,E), and field L in the caudomedial nidopallium (NCM; Fig. 25F). Expression was also observed in the subnidopallium, ventral to caudal nidopallium (SuN; Fig. 25F). In the medial pallium, minor subpopulations of *Crfbp* expressing cells were observed in the dentate gyrus (DG) and lateral parahippocampal area (APHl), and signal in the latter increased towards caudal levels (Fig. 25E-G). In the arcopallium, most of the *Crfbp* expressing cells were found in the medial part of the core subdivision (ACoM; Fig. 25G). The core nucleus of the mesopallium (MVco) and the hyperpallium (H) also contained minor subsets of *Crfbp* expressing cells (Fig. 25C,D), but the rest of the pallium remained free of expression or contained extremely few expressing cells.

At E18 (Fig. 26A-C) and at P0 (Fig. 26D-H), the expression increased a bit in the pallium, but decreased in the supallium, while maintaining a similar pattern to that described at E14. In the hyperpallium, a moderate number of *Cfrbp* expressing cells were observed in the apical hyperpallium (HA), with a trend to be more abundant in lateral levels (Fig. 26A, D, E). In the nidopallium, in addition to the cells of the sensory nuclei (nucleus basorostralis, entopallium, field L), expression was also observed in a subset of cells of the island field of the caudal nidopallium (NCIF; Fig. 26B,G,H). In the supallium, *Crfbp* expression was similar to that at E14, but decreased in the lateral striatum, where expression became restricted to its caudal pole (Fig. 26G). In the septal region, signal was observed in the diagonal band nucleus (vertical part;

DBV), around the cortico-septo-mesencephalic tract (csm), and in the pallidoseptal area (PaS; Fig. 26B,G).

By P7 (Fig. 27, details in Fig. 28), the pattern of expression remained quite similar to that at hatching, but now the expression was a bit higher in the medial pallium, arcopallium, and the basal ganglia of the subpallium. In the medial pallium, in addition to the expression in the dentate gyrus and lateral parahippocampal area, very few and scattered *Crfbp* expressing cells were seen in the medial parahippocampal area (APHm; Fig. 28A). In the arcopallium, *Crfbp* expressing cells were seen in several subdivisions, such as the dorsal part (Ad), the core nucleus (Aco), the amygdalopiriform (APir) and the amygdalohippocampal (AHi) subdivisions (Figs. 27F; 28B). In the basal ganglia, in addition to the cells found in the lateral striatum and its capsule (LST, StC), expressing cells were also seen in the medial striatum (MSt) at intermediate/caudal levels, and very few cells were seen in the pallidum (Fig. 28C,D). As before, in the extended amygdala, expressing cells were present in the peri/post-intrapeduncular island field (pINP; Fig. 27D,E).

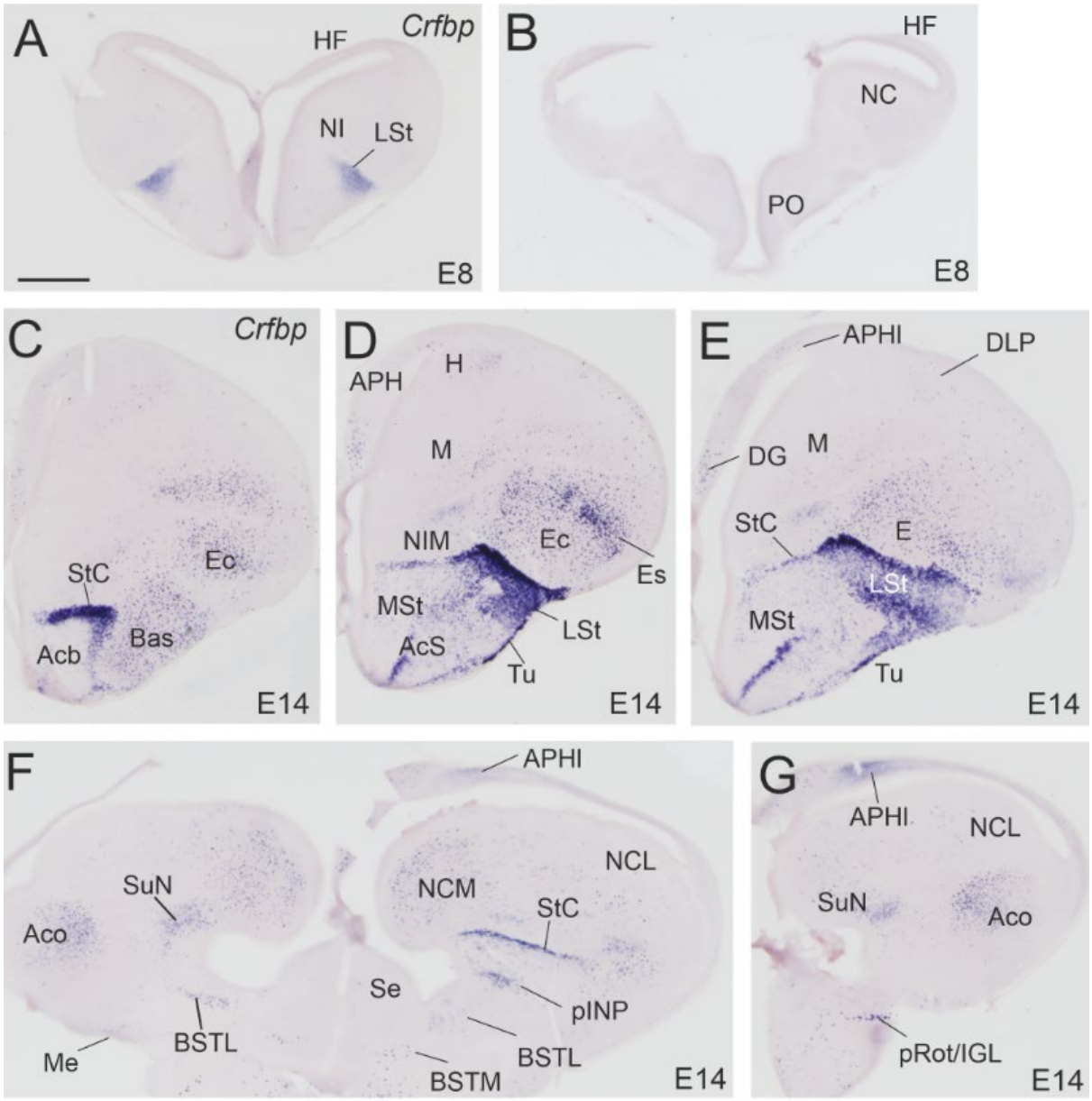


Figure 25. Images of frontal sections through the embryonic telencephalon and anterior hypothalamus of chicken (E8 and E14), from anterior to posterior levels, showing hybridization signal for chicken *Crfrbp*. In these images, dorsal is to the top; in C-E and G, medial to the left. Note the moderate signal in part of the striatum from E8, which becomes intense at E14, encompassing the striatal capsule and adjacent lateral striatum, as well as part of the central extended amygdala (pINP). Most of the pallidum shows light or no expression at these embryonic ages, except for the posterior levels of APHl in the hippocampal formation. In the thalamus, note the strong expression in some retinorecipient areas, as the intergeniculate leaflet (IGL) and the perirotundic belt (pRot). See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

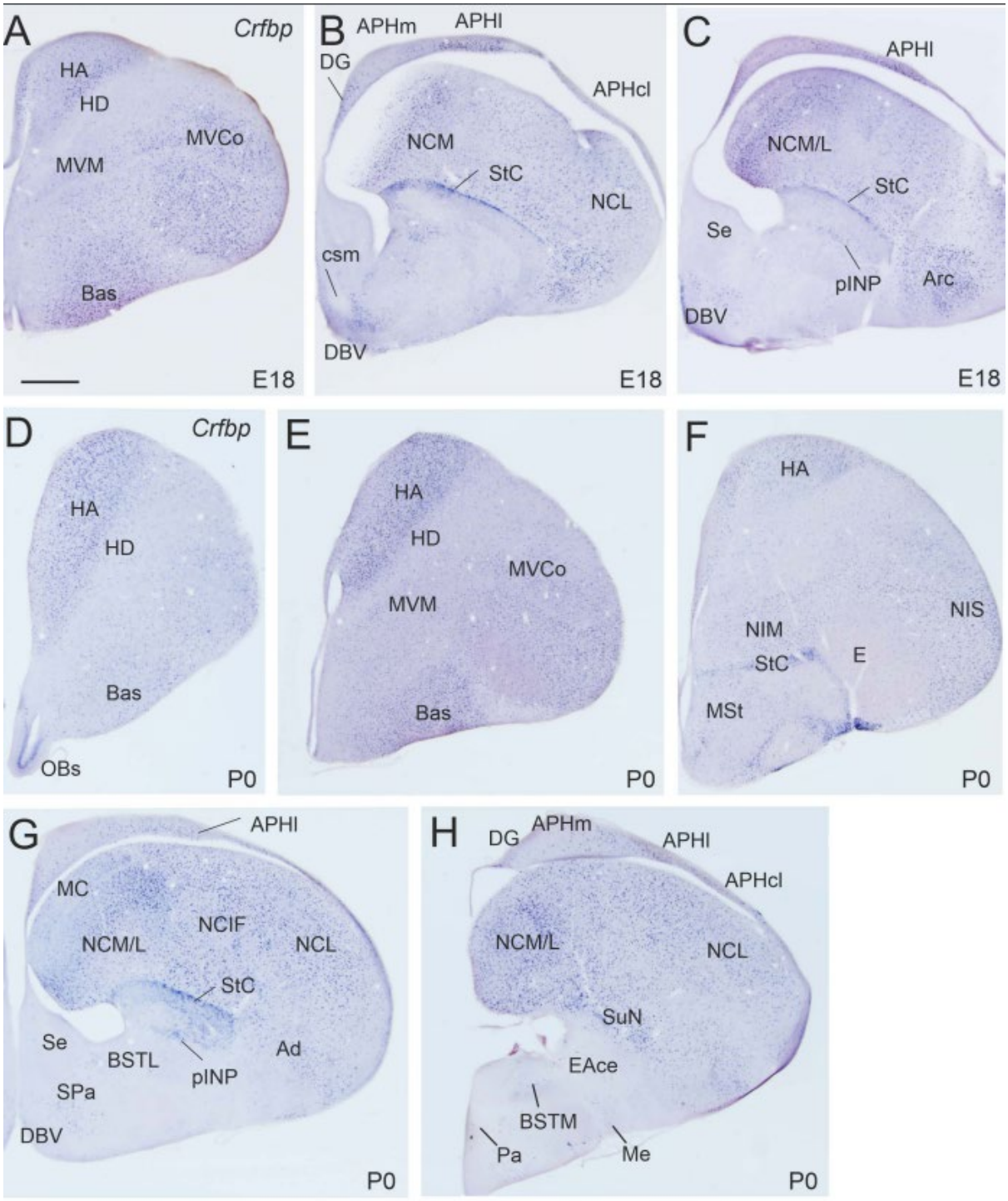
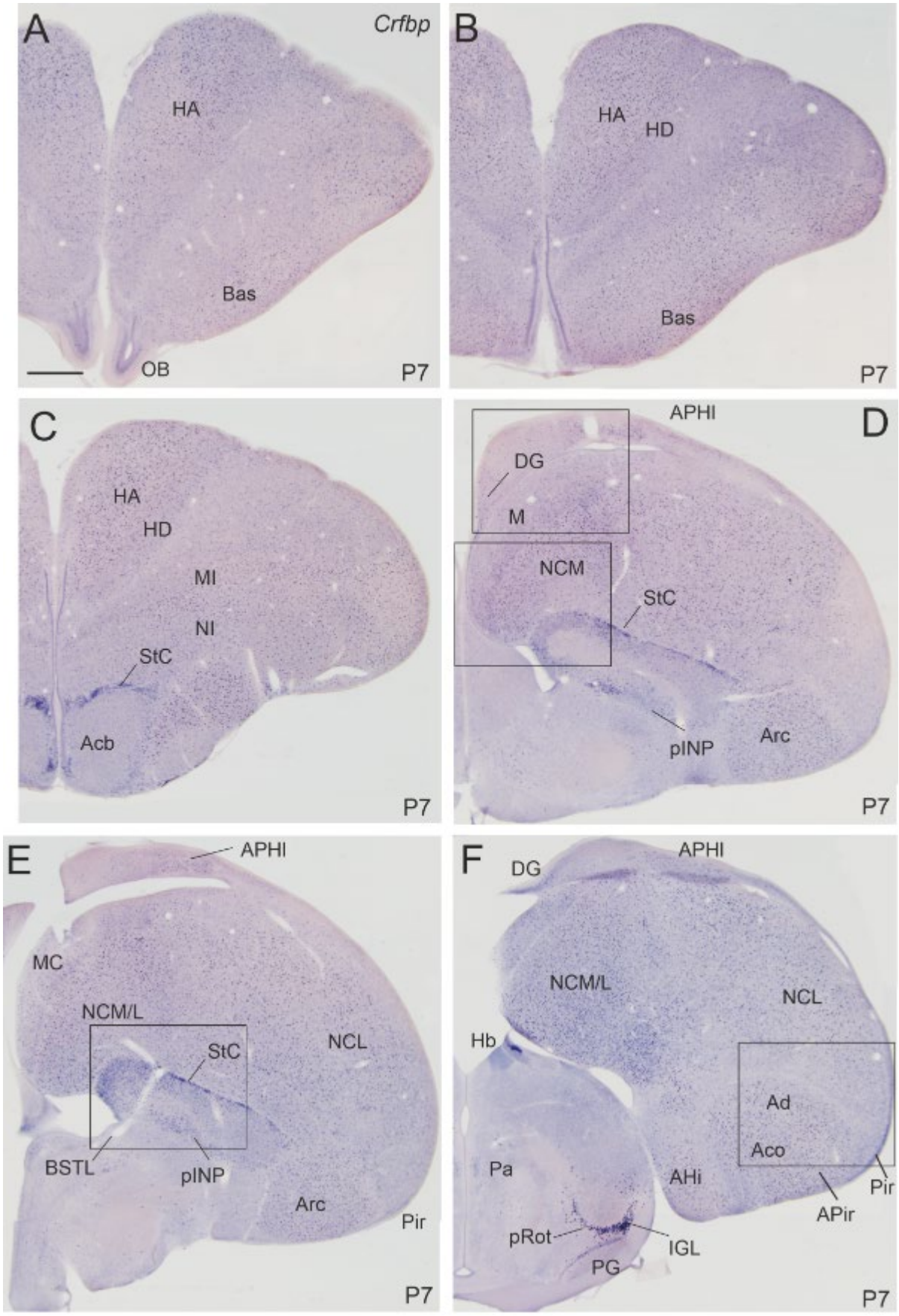


Figure 26. Images of frontal sections through the telencephalon and anterior hypothalamus of chicken (E18 and P0), from anterior to posterior levels, showing hybridization signal for chicken *Crfrbp*. In these images, dorsal is to the top and medial to the left. Expression in the subpallium is less intense compared to E14, but is seen in similar areas. Regarding the pallium, at E18 and P0 expression is moderate in some parts of the pallium, including parts of the hippocampal formation (APHl), hyperpallium (HA), mesopallium (MVco), and nidopallium (NCM, NCL). See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)


A
Crfbp
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P7

Figure 27. Images of frontal sections through the telencephalon and anterior hypothalamus of chicken at P7, from anterior to posterior levels, showing hybridization signal for chicken *Crfrbp*. In these images, dorsal is to the top and medial to the left. Expression pattern in the subpallium is similar to that seen at hatching, while in the pallium is more spread. Details of the squared areas in D-F are shown in Figure 28. See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

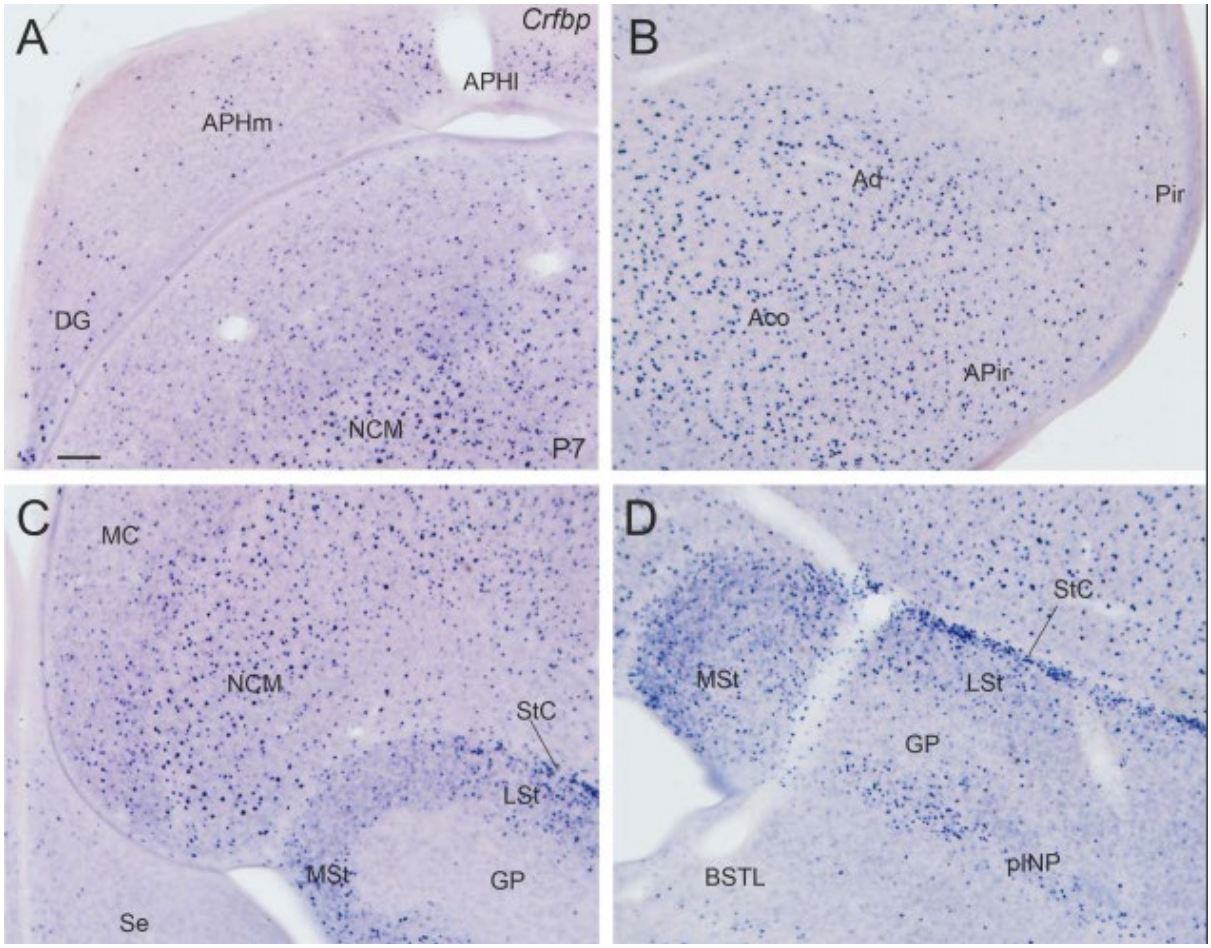

Figure 28. Details of the expression of *Crfbp* in the telencephalon of chicken at P7, from the squared areas in some sections shown in Figure 17. In these images, dorsal is to the top and medial to the left. A shows a detail of the hippocampal formation (from the section in Fig. 17D); B shows a detail in the arcopallium (from the section in Fig. 17F); C shows a detail in the caudomedial nidopallium (from the section in Fig. 17D); D shows a detail in the subpallium (showing StC, LSt, and pINP), from the section in Fig. 17E). See more details in text. For abbreviations see list. Scale bars: A = 1mm (applies to all). (Adapted from Metwalli et al., 2023)

5. Discussion

In this thesis we focused on two major aspects of the brain relevant for regulating emotions and stress responsivity in chicken. The first one (section 4.1) is the developmental origin of the glutamatergic neurons in the extended amygdala, involved in the excitatory/inhibitory regulatory networks, which unbalance is behind some neurodevelopmental disorders in humans. The second (section 4.2) is the development of stress-related corticotropin releasing factor (CRF) receptors and peptides during embryonic and post hatching developmental periods. Regarding the first aim, we found a new domain in the ventralmost part of the telencephalon, the TOH, that appears to produce glutamatergic cells for the extended amygdala. This opens new venues for future studies to analyse the relationship of these cells with those that originate in the subpallium (which are GABAergic) and the pallium (glutamatergic). Regarding the second aim, we found that CRF systems start develop in many areas of the pallium from early embryonic stages. These include the presence of CRF in sensory visual and auditory areas, and the presence of CRF receptors in high-order association and sensorimotor integration of the pallium. The results can help to understand the developmental and long-term effects of light and noise exposure during prehatching in chicken.

5.1. Main findings of section 4.1: A new telencephalic domain

Our results show the existence of a TOH domain, with coexpression of Foxg1 and Otp, in chicken. This domain was also previously found in mouse (Morales et al., 2021) and has also been found in a lacertid lizard (Metwalli et al., 2022). Thus, the TOH was likely present in the forebrain of stem amniotes. Moreover, an area of Foxg1/Otp overlap resembling TOH has been found in zebrafish (Affaticati et al., 2015), suggesting that it may be a common division in the brain of jawed vertebrates. Like in the mouse, the TOH division of chicken also expresses Sim1, and our results show high level of cellular coexpression Foxg1 and Sim1 in this domain. Given the high abundance of Otp/Foxg1 and Sim1/Foxg1 cells, it is likely that many cells in TOH coexpress Otp, Sim1 and Foxg1. However, considering the observed differences on the presence of Foxg1/Otp versus Foxg1/Sim1 cells in parts of the extended amygdala (as discussed below), it seems that there are at least two different cell populations: 1) cells coexpressing Otp, Sim1 and Foxg1; 2) cells with Sim1 and Foxg1, but without Otp. The first group seems the most abundant (but more studies are needed to quantify) in TOH, including the subpreoptic area and a ventral subdivision of the medial extended amygdala. The second group seems to produce a distinct population that tangentially migrates to the central extended

amygdala. Groups of Foxg1/Otp and Foxg1/Sim1 cells also spread tangentially into the preoptic area, but it is unclear if they are the same or different cell populations.

As discussed previously (Morales et al., 2021), the TOH appears to correspond to the dorsal subdivision of classical SPV (Puelles et al., 2012; Díaz et al., 2015; Ferran et al., 2015). The SPV, characterized as a domain of the alar hypothalamus expressing Otp but free of Dlx2/5 and Nkx2.1, and mostly free of Islet1, has been found in different amniotes and anamniotes

(Bardet et al., 2008; Morales-Delgado et al., 2011; Moreno et al., 2012; Domínguez et al., 2013; 2015; Herget et al., 2014; Santos-Durán et al., 2016; López et al., 2022). However, based on its expression of the telencephalic Foxg1 in the ventricular zone and mantle, we previously proposed that the TOH might be part of the telencephalon (Morales et al., 2021). This led us to use the term SPV core to refer to the central and ventral parts of classical SPV (Morales et al., 2021). In mouse and chicken, the dorsal subdivision of SPV (apparently corresponding to our TOH) also differs from the SPV core because the former does not express Brn2 (Michaud et al., 1998; Caqueret et al., 2005). In amphibians, the ventral part (in topological terms) of SPV expresses the transcription factors Nkx2.2 and Lhx5 (Domínguez et al., 2013). The relation between these two sectors of amphibian SPV with the TOH and SPV core of amniotes requires further investigation, adding Foxg1 as one of the markers. Lhx5 has also been found in the SPV of mouse and chicken (Abellán et al., 2010), and colocalization between Otp and Lhx5 has been shown in mouse (García-Moreno et al., 2010). However, at least in mouse and zebra finch, Lhx5 seems to cover a large part (if not all) of SPV, and is abundantly found in cells of the TOH- derived part of the medial extended amygdala (Abellán et al., 2010; García-Moreno et al., 2010; Vicario et al., 2017).

In mouse, the SPV core gives rise to the main portions of the paraventricular and supraoptic hypothalamic nuclei (Puelles et al., 2012; Morales et al., 2021). Based on previous studies (Arnold-Aldea and Sterritt, 1996; Caqueret et al., 2005; Bardet et al., 2008), combined with our results, this appears to be similar in chicken. In addition, our results in chicken suggest that it also produces minor subpopulations of Otp and Sim1 cells, not coexpressing Foxg1, that migrate tangentially to the preoptic area, extended amygdala and pallial amygdala/arcopallium (only the Sim1 cells appear to reach the arcopallium). The ventrodorsal dispersion of SPV-derived cells (posterior to anterior in topographic terms) was previously suggested in chicken based on analysis of the distribution of vasotocin/mesotocin immunoreactive cells during development (Arnold-Aldea and Sterritt, 1996). From their site of origin in the anterior (alar)

hypothalamus, Arnold-Aldea and Sterritt (1996) described two migration pathways for the vasotocin/mesotocin cells: (1) a dorsolateral pathway, which produces the main supraoptic nucleus (named 'dorsolateral anterior nucleus, magnocellular component'), by radial migration based on comparison with vimentin, and cells that spread into the BST and the external supraoptic nucleus; and (2) a ventrolateral pathway, that appears to produce the ventral division of the supraoptic nucleus. Our results partially agree with this suggestion, but clarifying that the dorsolateral pathway occurs within the peduncular prosomere, while the ventrolateral pathway occurs within the terminal prosomere. In both cases, cells first migrate radially to the subpial surface, and then some appear to take a tangent to continue dorsally (such as those of the external and ventral supraoptic nuclei). Moreover, based on double- labeling of Otp/Foxg1 and Sim1/Foxg1, it appears that cells that produce the different parts of the supraoptic nucleus do not coexpress Foxg1 and might specifically originate in the peduncular and terminal parts of SPV core. In addition, the TOH domain gives rise to double-labeled Otp/Foxg1 and Sim1/Foxg1 cells of the extended amygdala and subpreoptic/preoptic areas (some radially, some tangentially, as discussed below). In chicken, TOH- and SPV core-derived cells also appear to reach the arcopallium. The presence of these various migration routes from TOH and SPV core toward the preoptic area, extended amygdala and pallial amygdala agrees with previous descriptions in mouse based on Otp/Foxg1 double labeling (Morales et al., 2021, 2022), as well as with descriptions based on Otp- and Sim1-single labeling (García-Calero et al., 2021).

5.2. Otp and Sim1 cells of the medial extended amygdala

As noted above, part of the medial extended amygdala contains numerous cells expressing Otp and Sim1. Otp cells have been previously found in the medial extended amygdala of amniotes and anmaniotes (for example, Bardet et al., 2008; Moreno et al., 2010; Abellán et al., 2013; Herget et al., 2014; Biechl et al., 2017; Porter and Mueller, 2020; López et al., 2022), but more data on Sim1 in different species are needed. Our results show that many of the Otp and Sim1 cells of the medial extended amygdala coexpress Foxg1 and appear to derive from TOH. In the avian BSTM, Otp/Foxg1 and Sim1/Foxg1 cells occupy a ventrolateral division called BSTM3 (Abellán and Medina, 2008; included as part of the hypothalamic BSTM in the zebra finch; Vicario et al., 2017). In contrast, BSTM1/2 are rich in Foxg1 single labeled cells, and seem to correspond to the nuclear subdivisions rich in subpallial cells, such as those expressing Lhx6

(derived from the ventrocaudal pallidal/diagonal domain) and those expressing Shh (derived from the commissural preoptic domain) (Abellán and Medina, 2009; Vicario et al., 2017). While cells of subpallial origin found in BSTM1/2 are GABAergic (Abellán and Medina, 2009), those in BSTM3 are mostly glutamatergic (Abellán and Medina, 2008, 2009). The BSTM also includes subpopulations of cells expressing vasotocin and mesotocin (Aste et al., 1998; Jurkevichs et al., 1999; Vicario et al., 2017), and we previously suggested that these likely originate in SPV (Abellán et al., 2013; Medina et al., 2017), based on the critical role of Otp and Sim1 in differentiation of these cells (Acampora et al., 2000). However, this needs to be reexamined to discern between contributions of TOH and/or SPV core, since the BSTM does not only include Otp/Foxg1 and Sim1/Foxg1 cells (from TOH), but also Otp and Sim1 single labeled cells (likely derived from SPV core).

In contrast to the clear delimitation of sectors in the avian BSTM and in the mouse medial amygdala, the medial amygdala of birds is quite small and cells of different origins are rather intermingled (Abellán and Medina, 2009; Vicario et al., 2017). At least at early stages, the Otp/Foxg1 and Sim1/Foxg1 cells of the avian medial amygdala form a clear continuum with those of BSTM3, following the ventral amygdalofugal tract. More studies will be required to know more about these particular cells of the medial extended amygdala and their specific role in social behaviors.

5.3. Sim1 cells of the central extended amygdala and arcopallium

In contrast to the Otp cells, we found Sim1 cells in the central extended amygdala (mostly at lateral levels) and the adjacent arcopallium of chicken. The finding of Sim1 in the arcopallium resembles that of the mouse pallial amygdala, proposed to derive from SPV core (in particular, central SPV; García-Calero et al., 2021). In agreement with this, most of the Sim1 cells observed in the chicken pallium did not coexpress Foxg1. In mouse, SPV-derived Sim1 cells appear to populate layer 2 of the nucleus of the lateral olfactory tract, after a putative long migration through the posterior pole of the pallium to end in the subpallium (García-Calero et al., 2021). In chicken, Sim1 cells may also contribute to populate the LOT nucleus, suggested to be located near the surface, under the end of the lateral branch of the anterior commissure (Puelles et al., 2019, see Fig. 14 in the 2 nd edition of the Atlas of the Chicken Brain). In agreement with this proposal, this area receives olfactory input (Reiner and Karten, 1985) and contains Sim1 cells (present results). This area is rich in glutamatergic cells and is continuous

laterally with those in the arcopallium and other parts of the avian pallial amygdala (present results, Fig. 11; Abellán et al., 2009). This area also extends a bit rostral to commissural levels, always close to the pial surface, and has been identified in our previous publications as part of the pallial extended amygdala, a glutamatergic cell corridor extending from the SPV to the pallial amygdala (Abellán et al., 2009, 2010; also identified as extended amygdala in the chicken brain atlas by Puelles et al., 2019). Comparison to Foxg1 shows that the subpial part of this area is poor in Foxg1 cells (present results), indicating an external origin of at least some of the cells of this cell corridor. However, this area is quite complex and contains other type of cells with different molecular profiles and apparent origins, such as those with Lhx5 and Tbr1 derived from the prethalamic eminence, and those with Lhx9 of pallial origin (as discussed previously by Abellán et al., 2009; see also Abellán and Medina, 2009; Abellán et al., 2010; Vicario et al., 2017). The mouse LOT also appears to include a variety cell subtypes with different molecular profiles and embryonic origins (discussed by García-López et al., 2008; García-Calero et al., 2021).

One striking finding of our study is the observation of a group of Sim1 cells in the chicken central extended amygdala, some of which coexpress Foxg1 and some do not. It is unclear whether similar cell populations are present in other vertebrates. In chicken, most of these Sim1 are located laterally, in the capsular central amygdala and the intercalated cell area, interposed between the central extended amygdala and the arcopallium. While the Sim1 cells found in the intercalated area at early stages may be in transit to neighboring areas, those in the capsular central extended amygdala resemble a subset of glutamatergic cells observed at later stages in this area (Abellán and Medina, 2009; present results). Based on the presence of a subset of Lhx9 cells in this same location, we previously proposed that they likely originate in the Lhx9-rich amygdalar pallium (Abellán et al., 2009). However, based on the results with Sim1, it is likely that at least part of the glutamatergic cells of the central extended amygdala originates in the TOH and the SPV core, and migrate tangentially to the subpallium during development. Further investigation will be required to know the exact origin of the different glutamatergic cells found in the central extended amygdala, and their functional relation to the predominant GABAergic cells found in this same territory.

5.4. Glutamatergic versus GABAergic cells of the telencephalon

Telencephalic function on regulation of goal-directed behaviors by contextual information, motivation and emotions depends on an exquisite modulation of glutamatergic and GABAergic networks. Unbalance in these excitatory/inhibitory networks is behind many mental and neurodevelopmental disorders (Gao and Penzes, 2015; Sohal and Rubenstein, 2019). Most telencephalic nuclei and areas contain a mixture of both cell subtypes, in different proportions. In the cerebral cortex, the basolateral amygdala and the cortical amygdala, the predominant cells are glutamatergic, while in the septum, basal ganglia and centromedial extended amygdala, the predominant cells are GABAergic (reviewed by Medina and Abellán, 2009, 2012; Moreno et al., 2009). Glutamatergic and GABAergic neurons of the telencephalon are considered to primarily derive from one of the two major embryonic divisions of the telencephalon, the pallium or the subpallium, respectively. Until now, only minor cell subpopulations were thought to originate outside, in the prethalamic eminence and alar hypothalamus, and migrate tangentially to the telencephalon during development. Our study challenges this view, with the identification of a new embryonic domain, the TOH, that expresses Otp, Sim1 and Foxg1, and is located at the ventralmost part of the telencephalon. This domain, observed in sauropsids (present results) and mouse (Morales et al., 2021), produces a large population of glutamatergic cells for the medial extended amygdala, and subsets of glutamatergic cells for the subpallium and pallium. This new division contributes to the heterogeneity of cells found in the amygdala and other telencephalic areas, and open new venues to further study the relation of TOH-derived cells to other cells derived from the pallium or the subpallium.

5.5. Main findings of section 4.2: Ontogenetic expression of Crf peptides and receptors

Our results demonstrate mRNA expression of Crf, Crfr1, Crfr2 and Crfbp in the chicken telencephalon from early embryonic stages, at least from embryonic incubation day 8 (E8). At middle embryonic ages (E14), intense Crf expression is observed in several sensory areas of the telencephalic pallium, such as the visual areas of the hyperpallium and nidopallium (entopallium), and the multisensory and auditory areas of the nidopallium (medial intermediate nidopallium and field L, respectively). At E14, Crfr1 and Crfr2 expressions are intense in associative areas of the pallium, including areas important for sensorimotor integration and emotion processing (such as the apical hyperpallium, mesopallium, caudolateral nidopallium

and arcopallium), showing partially alternating patterns: for example, Crfr1 is strongly expressed in the apical hyperpallium, nidopallium and arcopallium, while Crfr2 signal is intense in the densocellular hyperpallium and mesopallium (including an area related to imprinting). Both receptors also show moderate to intense expression in the hippocampal formation, a structure involved in memory formation and stress control. Expression of Crf receptors in the pallium continues to increase during embryonic and posthatching development, and this is accompanied by an increment in Crfbp expression. In contrast, expression of Crf and its receptors in the subpallium is generally low at middle embryonic stages, with a few exceptions (such as part of the corticopetal basal magnocellular complex), although Crfbp signal is high in parts of the subpallium from middle embryonic stages. Expression of Crf and its receptors in the central extended amygdala (a region playing a critical role in fear and anxiety) increases near hatching, and continues rising after hatching. The results point to an early development of Crf systems in pallial areas regulating sensory processing, sensorimotor integration and cognition, and a late development of Crf systems in subpallial areas regulating the stress response. However, Crfbp buffering system develops late in the pallium and early in the subpallium.

5.6. Expression of Crf mRNA in the HPA and telencephalon in chicken and comparison to previous findings in birds

A few previous studies have described the brain CRF immunoreactive elements in domestic fowl (*Gallus domesticus*) throughout ontogenesis (Józsa et al., 1986), and have quantified variations in expression (Vandenborne et al., 2005) and activity of HPA (Jenkins and Porter, 1984). These studies provided data relevant for knowing when during development the HPA axis might be functional, which seems to be E14, because this was the earliest incubation day when CRF immunoreactive cells were observed in the paraventricular hypothalamic nucleus (PVN), concomitant to the presence of the first immunoreactive fibers in the median eminence (Józsa et al., 1986). Our data agree with the presence of Crf expressing cells in the PVN at E14, but according to our results the first cells are detectable in this nucleus earlier, at E8. However, with our technique we detect mRNA (but not the peptide) and cannot detect expression in axonal projections, so our approach does not permit to know the exact day when the HPA becomes mature enough to be functional. This could be quite early, since the vascular connection between hypothalamus and pituitary is seen from E6, although the portal vascular

plexus is visible at E12 (Jenkins and Porter, 2004). Interestingly, in chicken the adrenal glands are able to release glucocorticoids from embryonic day 8 independently of the pituitary (Jenkins and Porter, 2004). Based on both the first observation of CRF fibers in the median eminence and the increase of corticotrophs in the pituitary, E14 appears to be the age around which the pituitary starts to influence adrenocortical activity (Jenkins and Porter, 2004). Thus, it appears that the HPA is active during the last week of embryonic development in chicken (Jenkins and Porter, 2004). The abundance of immunoreactive fibers and terminals in the median eminence increases from E14 until posthatching day 7 (Józsa et al., 1986). The density of the CRF immunoreactive neuropile in the median eminence of adult domestic fowls is quite high (Józsa et al., 1984; Richard et al., 2004). Based on radioimmunoassay measurement, the content of CRF peptide significantly increases in the hypothalamus (excluding the median eminence) during the last embryonic week and remains elevated after hatching (Vandenborne et al., 2005). In contrast, Crf mRNA decreases in this region just before hatching (Vandenborne et al., 2005). Moreover, CRF peptide content decreases in the median eminence from E16 until E20, possibly due to release into the portal system (Vandenborne et al., 2005). After hatching, there is a robust increase of CRF content in the median eminence (Vandenborne et al., 2005). As discussed by Vandenborne et al. (2005), these fluctuations observed around hatching might be related to the stress hyporesponsive period observed in newly hatch chickens (Freeman and Manning, 1984). A similar period has been described in rodents before and after birth (Grino et al., 1989; Schmidt et al., 2003).

The major focus of our study was to map CRF systems in the chicken telencephalon during development, since CRF and its receptors are expressed in several telencephalic areas known to regulate HPA, as well as emotional and cognitive aspects of stress in other vertebrates (Bale and Vale, 2004; Henckens et al., 2016; Herman et al., 2016). Our results on the distribution of Crf mRNA expressing perikarya in different pallial and subpallial areas of the early posthatch chicken telencephalon agree with data on CRF expressing cells in early posthatch and adult chicken and quail, based on immunohistochemistry (Richard et al., 2004). However, we provide new details on cell groups of the pallium and subpallium, and in some of the areas we found more expressing cells than in the description by Richard et al. (2004): for example, in the auditory field L of the caudomedial nidopallium, we found abundant perikarya, while Richard et al. (2004) only described a few. This is likely due to differences in the technique employed, since we used in situ hybridization to detect mRNA (very sensitive to detect neuropeptidergic perikarya; present results), while they employed immunohistochemistry to

detect the peptide (a technique than often requires pretreatment with colchicine to reach detectable levels; Richard et al., 2004). In contrast, immunohistochemistry is better than in situ hybridization for determining the presence of the peptide/protein as well as for analyzing fiber systems (Richard et al., 2004). Thus, both types of approaches provide relevant, complementary information and should be considered for a better understanding of CRF systems.

5.7. Development of CRF in functional systems of the chicken telencephalon

5.7.1. Sensory, high-order association and sensorimotor integration areas

Our results agree with those of Richard et al. (2004) on the presence of CRF expressing cells in several first order sensory areas of the pallium, such as the interstitial nucleus of the apical hyperpallium (IHA), the entopallium (mostly in its periphery) and field L. We also found CRF expressing cells in the multimodal sensory area of the medial intermediate nidopallium (NIM). The major part of IHA receives visual input from the retinorecipient anterior dorsolateral thalamic nucleus (comparable to the lateral geniculate nucleus of mammals) (Miceli et al., 1990; Shimizu et al., 1995), and its frontal pole receives somatosensory input from the lemnothalamic dorsointermediate ventral anterior [DIVA] nucleus (Wild, 1987; Funke, 1989) and somatomotor input from the ventral intermediate [VIA] nucleus (Medina et al., 1997). The entopallium receives visual input from the collothalamic nucleus rotundus, while field L receives auditory input from the collothalamic nucleus ovoidalis (comparable to the medial geniculate nucleus of mammals) (Karten and Hodos, 1970; Wild et al., 1993; Husband and Shimizu, 1999; Krützfeldt and Wild, 2005). Regarding NIM, it receives input of several sensory modalities from multimodal thalamic nuclei (including posterior dorsolateral and posterior dorsomedial nuclei), from the visual parts of the hyperpallium and from the auditory field L (Wild, 1987; Funke, 1989; Shimizu et al., 1995; Kröner and Güntürkün, 1999).

One striking new finding of the present study is that abundant cells expressing Crf mRNA are observed in all of the above-mentioned areas from middle embryonic stages. In mammals, CRF expressing cells of many areas of the pallium are interneurons, which contact pyramidal cells expressing CRF receptors (Swanson et al., 1983; Chen et al., 2004a; Gallopin et al., 2006). However, this is not a general rule, since in mammals the majority of the mitral cells of the olfactory bulb and their projections contain CRF (Imaki et al., 1989; Basset et al., 1992). In chicken, Crf expressing cells in many areas of the pallium (including the mitral cell layer of

the olfactory bulb, but also many other areas like the IHA, the entopallium and field L) seem too abundant to only include interneurons; thus, in addition to interneurons they might include cells involved in pallio-pallial connections, a possibility that would require further investigation. Richard et al. (2004) also proposed that CRF cells of the chicken and quail arcopallium may give rise to commissural projections as well as descending projections, based on the finding of CRF immunoreactive fibers in the anterior commissure and the ventral amygdalofugal tract. Interestingly, our data show high levels of Crf receptors from early embryonic stages in many of the chicken pallial sensory areas that contain Crf cells, plus many association areas of the hyperpallium, mesopallium and nidopallium, which receive inputs from the sensory areas and/or from other integration areas (Kröner and Güntürkün, 1999; Atoji and Wild, 2012). Receptors in thalamorecipient sensory areas may mediate CRF-related transmission in thalamopallial projections, which agrees with the presence from E14 of high numbers of Crf expressing cells in some of the thalamic nuclei that project to the pallium, such as the dorsolateral and dorsointermediate thalamic nuclei (present results, Fig. 1H, detail in H'). In the collothalamus, the shell of nucleus ovoidalis, conveying auditory information to part of field L and to caudal mesopallium (Durand et al., 1992; Kröner and Güntürkün, 1999; Atoji and Wild, 2012), also contains CRF immunoreactive cells (Richard et al., 2004).

As noted above, Crf receptors found in the chicken pallium might also mediate CRF transmission in pallio-pallial connections. This could be the case for the olfactory projections from the olfactory bulb to the prepiriform, piriform and entorhinal cortices (Reiner and Karten, 1985; Atoji and Wild, 2014), since our data in chicken show that mitral cells express Crf and the olfactorecipient areas contain Crfr1 and Crfr2. In addition, based on our data, CRF and its receptors may also be involved in connections between auditory areas within field L, and between field L and the association areas of the caudolateral nidopallium, mesopallium, and arcopallium (Kröner and Güntürkün, 1999; Atoji and Wild, 2012). Furthermore, Crf and its receptors could be involved in part of the projections from the visual entopallium to the mesopallium (including the core nucleus of the ventrolateral mesopallium, MVco, also abbreviated MVL; Krützfeld and Wild, 2004), and from here to the caudolateral nidopallium and arcopallium (Atoji and Wild, 2012). In addition, Crf and their receptors could participate in and/or modulate transmission of visual and somatosensory information from IHA to apical hyperpallium (Shimizu et al., 1995), and might modulate sensorimotor integration and descending outputs from apical hyperpallium (Wild, 1987, 1997; Medina et al., 1997; Kröner and Güntürkün, 1999; Wild and Williams, 2000; Medina and Reiner, 2000).

Based on the differential expression of Crfr1 and Crfr2 in the pallium (present results), it appears that different receptors are preferentially involved in distinct functional subsystems, but this changes with age. During embryonic development, projections to the mesopallium preferentially involve receptor 2 (except those involving MVco, which shows high levels of both receptors), while projections to the nidopallium and arcopallium preferentially target receptor 1 (the most abundant in nidopallium, except for nucleus basorostralis, which shows high expression of both receptors). It used to be thought that Crfr1 activation promotes anxiogenesis and Crfr2 has the opposite effect (reviewed by Bale and Vale, 2004). However, it now appears that the functions of these receptors depend on the specific cell types where they are expressed, and the circuits in which they are engaged (Henckens et al., 2016). Moreover, to understand the function it is important to consider the type of fast neurotransmitter (such as GABA or glutamate) coexpressed with CRF at the level of presynaptic terminals, since CRF will potentiate their effect (Partridge et al., 2016). In chicken, there is high coexpression of glutamate and aspartate in the principal neurons of the arcopallium and caudal nidopallium, and the ratio between both might differ between cell subpopulations and/or their projections (Adam and Csillag, 2006). Thus, this is another factor to consider for understanding CRF function in cases of coexpression.

5.7.2. Areas related to learning, emotion and memory

Our results demonstrate the presence of Crf expressing cells, as well as Crfr1 and/or Crfr2 in high-order associative pallial areas involved in different types of learning and memory, such as the caudolateral nidopallium (involved in associative learning, value coding, and working memory; Güntürkün, 2005; Dykes et al., 2018), the medial intermediate mesopallium (involved in filial imprinting and passive avoidance learning; Horn et al., 1985; Rose and Csillag, 1985; Horn, 1998; Bourne et al., 1991; McCabe and Horn, 1994; Dermon et al., 2002) and the hippocampal formation (involved in spatial navigation, memory, and stress control; Gagliardo et al., 2001; Hough and Bingman, 2004;Bingman et al., 2006; Sherry et al., 2017; Smulders, 2021). The medial intermediate mesopallium projects to the arcopallium (Csillag et al., 1994, 1997), while the caudolateral nidopallium and the hippocampal formation are reciprocally connected with the arcopallium (Kröner and Güntürkün, 1999; Atoji and Wild, 2012). The arcopallium, together with the caudal nidopallium, is considered part of the avian pallial amygdala based on embryonic origin, topological location, and transcription factors expression during development, as well as other features (reviewed by Martínez-García et al., 2007; Medina et al., 2022, 2023). Like the amygdala of mammals (Swanson et al., 1983; Chalmers

et al., 1995; Van Pett et al., 2000; Weera et al., 2022), the arcopallium of chicken and other birds includes subsets of CRF expressing cells (present results; Richard et al., 2004) and expresses high levels of Crfr1 (present results). Moreover, it is involved in regulation of fear behavior (Saint-Dizier et al., 2009), passive avoidance learning (Lowndes and Davies, 1994; Csillag, 1999), taste detection (in particular, the amygdalohippocampal area, identified as nucleus taeniae in the study by Protti-Sanchez et al., 2022), and regulation of ingestive behavior (da Silva et al., 2009). In addition, it projects to the medial striatum, extended amygdala and the hypothalamus, with descending axons travelling in the ventral amygdalofugal tract (Davies et al., 1997; Csillag, 1999; Kröner and Güntürkün, 1999; Atoji et al., 2006; Hanics et al., 2017). In the rodent pallial amygdala (in particular, the basolateral complex), CRFR1 is expressed mostly in glutamatergic neurons, which receive input from local CRF neurons, and these receptors are critical for CRF-mediated stress responses (Weera et al., 2022). This may be similar in chicken and other birds. However, some differences are observed between rodents and birds, because in rodents CRF cells of the pallial amygdala appear to be interneurons (Swanson et al., 1983), while in birds they appear to include projection neurons. As noted above, in chicken and other birds, it appears that CRF cells of the arcopallium are involved in contralateral projections through the anterior commissure and in descending projections (Richard et al., 2004). Interestingly, the situation in birds is similar to that described in primates (Basset and Foote, 1992). The consequences of this for understanding differences between species in neural regulation of stress require further investigation.

5.7.3. Corticopetal cells of the ventral telencephalon and relation to the ascending reticular activating system

One striking finding of this study is the expression of Crf mRNA in a subset of large perikarya of the so-called basal forebrain corticopetal system (located in the subpallial telencephalon). These cells were previously observed in chicken and quail using immunohistochemistry, but were proposed to belong to the extended amygdala (Richard et al., 2004). Like in mammals (Alheid and Heimer, 1988), corticopetal subpallial cells in chicken partially overlap the extended amygdala, the pallidal parts of the basal ganglia, and the lateral and medial forebrain bundles, but they belong to a different functional system characterized by projections to the pallium (Leutgeb et al., 1996; discussed by Abellán and Medina, 2009; Kuenzel et al., 2011). Corticopetal cells include cholinergic, GABAergic and glutamatergic neuronal subpopulations (Medina and Reiner, 1994; Abellán and Medina, 2009), and it would be interesting to investigate coexpression of these neurotransmitters with CRF. In mammals, minor

subpopulations of CRF neurons in the central extended amygdala (in the sublenticular part and posterior subdivision of lateral BST) and adjacent ventral pallidum were described to coexpress glutamatergic markers (Fudge et al., 2022), but it is possible that these neuronal subsets -or part of them- may belong to the corticopetal system.

The presence of CRF cells in the corticopetal system is interesting because they can modulate the function of several pallial areas, and could play a role in arousal, attention and memory, functions in which mammalian corticopetal cells of the basal forebrain are involved (Zaborszky et al., 1999; Saper and Fuller, 2017). In mammals, these cells are targeted by different cell groups of the ascending reticular activating system, such as the pedunculopontine nucleus, the parabrachial nucleus, and locus coeruleus (Zaborszky et al., 1999; Saper and Fuller, 2017), and this appears to be similar in birds (Kitt and Brauth, 1986). All of these brainstem centers also contain subpopulations of CRF cells in mammals and birds (Swanson et al., 1983; Richard et al., 2004), so that CRF ascending systems may participate in modulating arousal, attention and memory formation in response to a stressing stimulus.

5.7.4. Fear and anxiety related areas (central extended amygdala)

In mammals, two important CRF expressing neuronal subpopulations have been described in the central amygdala and related lateral part of the BST (both included in the central extended amygdala), which play important roles in fear and anxiety responses (Davis et al., 2010; Phelps and LeDoux, 2005; Ulrich-Lai and Herman, 2009; Zhang et al., 2021). Thanks to the use of targeted transgenic disruption and optogenetics for silencing specific neurons in mouse, it has been possible to study the function of the CRF cell populations found in the central amygdala versus those of lateral BST (Gafford and Ressler, 2015; Asok et al., 2018). CRF cells of the central amygdala receive glutamatergic inputs from the basolateral pallial amygdala, express glutamatergic receptors, and are involved in fear learning and memory (Gafford and Ressler, 2015). However, it seems that CRF cells of the central amygdala are particularly involved in the long-lasting components of long-term contextual fear memory, but not in short-term memory (Asok et al., 2018). CRF cells of the central amygdala have GABAergic projections to the lateral BST, where they target CRF and non-CRF neurons (Partridge et al., 2016). The projections from CRF cells of the central amygdala to the lateral BST appear to be critical to modulate long-term contextual fear memory (Asok et al., 2018). However, GABA receptor-expressing CRF cells of lateral BST play a role in anxiety, but not in fear acquisition, although they may be involved in fear extinction (Gafford and Ressler, 2015). It appears that CRF cells

of the central amygdala preferentially target non-CRF cells of the lateral BST in normal conditions, but chronic unpredictable stressful stimuli potentiate the connections between CRF cells of the central amygdala and CRF cells of lateral BST (Partridge et al., 2016), thus producing sustained fear responses akin to anxiety.

In chicken and quail, an important subpopulation of CRF cells is found in lateral BST (Richard et al., 2004), and our results show that this population expresses CRF mRNA at least from E14. Previous studies also found immunoreactive cells in this same location from E14 (Józsa et al., 1986), indicating that these BSTL cells are producing the neuropeptide already at this embryonic age. This is an important observation because, like in mammals, the lateral BST of adult birds is involved in anxiety (Nagarajan et al., 2014), and shows descending projections to hypothalamic and brainstem centers compatible with a regulation of HPA responses and autonomic system (Atoji et al., 2006). Our results agree with the finding of CRF cells in the territory of the extended amygdala laterally to BST (Richard et al., 2004). However, based on mRNA expression, these cells appear to mature later than those of BSTL. We found mRNA expressing cells in several subdivisions of this territory, including the peri/post-intrapeduncular island field and in the medial part of the capsular central amygdala, which belong to the central extended amygdala (as defined by Vicario et al., 2014; see also Pross et al., 2022). These CRF cells may be homologous to those of the central amygdala of mammals, although more studies are needed to investigate their exact embryonic origin, connections, and function. Previous connectivity studies showed that the territory encompassing the central extended amygdala (as understood now) receives inputs from the arcopallium and caudal nidopallium (Veenman et al., 1995; Kröner and Güntürkün, 1999; Atoji et al., 2006), and also point to the existence of local connections between lateral and medial parts of this region (Atoji et al., 2006; Hanic et al., 2017).

In rodents, the central amygdala only contains low levels of CRF receptors, while the lateral BST contains light to moderate levels of CRF receptors 2 (Chalmers et al., 1995) and 1 (Van Pett et al., 2000). Our results show that the chicken central extended amygdala is quite poor in expression of CRF receptors during most embryonic development, and only starts to show light Crfr1 and Crfr2 expressions around hatching (in the capsular central amygdala, oval central amygdala, peri/post-intrapeduncular island field, and lateral BST). By P7, expression continues to be light to moderate. In contrast, expression of Crfbp is moderate to intense in the peri/post-intrapeduncular island field from E14. Altogether, these results suggest that in chicken CRF transmission and its modulation within the central extended amygdala develops relatively late.

In contrast, some of the known targets of the descending projections of lateral BST to the preoptic area, hypothalamus and brainstem (Atoji et al., 2006) show expression of CRF receptors from E14, as discussed next.

5.8. Possible roles of embryonic expression of CRF and its receptors

As discussed above, in mature mammals and birds, CRF is a major regulator of the neuroendocrine and autonomic stress response (Vandenborne et al., 2005; more references above). In addition, in mammals CRF and its receptors are expressed in many brain regions, playing a modulatory role in relation to the stress response. Based on expression patterns, this may be similar in birds, although there are some species-specific variations noted above that require further investigation. One remarkable finding of our study is that mRNAs of CRF and its receptors are found in many telencephalic areas from early embryonic development, and by E14 expression is quite high in some areas of the pallium, and light to moderate in parts of the subpallium. What is the function(s) of CRF during embryonic development? We will explore some possibilities below.

5.8.1. HPA and autonomic regulation

As discussed above, in chicken the HPA may be functionally active from E14, if not earlier. Cells expressing mRNA of Crf (present results), as well as Crfr1 (see Fig. 6C) are found in the medial paraventricular hypothalamic region from E8. One of the centers that regulates HPA as well as autonomic nervous system activation is the lateral BST, which is involved in fear and anxiety responses in mammals and birds (Ulrich-Lai and Herman, 2009; Nagarajan et al., 2014). The influence of lateral BST on HPA can be direct or indirect, through neurons of the preoptic area, hypothalamus or brainstem (such as cells of the solitary nucleus), that in turn project to the paraventricular hypothalamic nucleus (Ulrich-Lai and Herman, 2009). Like in mammals, the lateral BST of birds is rich in GABAergic neurons, although it also contains minor subpopulations of glutamatergic cells (Abellán and Medina, 2009; Vicario et al., 2015; Metwalli et al., 2022). Depending on the neurons involved, it may lead to inhibition or disinhibition of HPA (Ulrich-Lai and Herman, 2009). In mammals, the lateral BST includes a subpopulation of GABAergic cells that coexpress CRF (Partridge et al., 2016), and these are particularly involved in sustained fear responses (akin to anxiety) (Gafford and Ressler, 2005; Davis et al., 2010). CRF expressing cells are also found in the avian lateral BST (Richard et al., 2004; present results). Our results show that these CRF cells are visible from E14,

suggesting that they may already be regulating HPA and possibly the autonomic system to adapt to environmental changes within the egg.

In mammals, the lateral BST is itself regulated by the central amygdala, the pallial amygdala and the hippocampal formation (among other centers; Ulrich-Lai and Herman, 2009). Based on the known connections of lateral BST in pigeons (Atoji et al., 2006), this appears to be similar in birds. In mammals and birds, the central amygdala, pallial amygdala and hippocampal formation contain subpopulations of CRF cells.

Regarding CRF cells of the central amygdala, in mammals they are involved in fear learning (Partridge et al., 2016). These cells are mostly GABAergic, and preferentially have inhibitory contacts with non-CRF cells of lateral BST, and less frequently with CRF cells of this nucleus (Partridge et al., 2016). In this study we identified similar CRF cells in the lateral parts of the central extended amygdala, but they are not visible until peri-hatching stages. Moreover, CRF receptors are not visible in lateral BST until E18 (present results). Thus, regulation of lateral BST (and indirectly of HPA) by CRF cells of the central amygdala seems to start relatively late. However, such regulation might occur by way of other cell types of the central extended amygdala, such as those expressing enkephalin, which are seen from early embryonic stages in chicken and zebra finch (Vicario et al., 2014, 2015, 2017; Pross et al., 2022).

Regarding the hippocampus, in mammals this exerts a negative influence on HPA by way of lateral BST (Ulrich-Lai and Herman, 2009), and this appears to be similar in birds (reviewed by Smulders, 2017). Like in mammals, the avian hippocampus expresses glucocorticoid and mineralocorticoid receptors, and becomes active during stress by responding to high levels corticosterone (reviewed by Smulders, 2017). The avian hippocampus also projects to the lateral BST and can thus indirectly influence HPA activity (Atoji et al., 2006). Since most neurons of lateral BST are GABAergic (Abellán and Medina, 2009), this pathway would lead to inhibition of HPA, similarly to mammals (Ulrich and Herman, 2009; Smulders, 2017). In mammals, a subpopulation of stress-responsive CRF interneurons is also found in the hippocampus (Chen et al., 2004a). These CRF expressing interneurons modulate hippocampal activation of principal cells through receptors (Chen et al., 2004a), and this may be similar in birds based on the presence of scattered Crf cells and abundant Crfr1 and Crfr2 in the hippocampal formation (present results). These CRF cells and both receptors are visible from E8, suggesting that CRF is modulating hippocampal function from early embryonic stages. Curiously, we found more cells during embryonic development than at hatching and at P7. One

possibility is that part of the expression is transient, like in mammals (Chen et al., 2004b). In rats and mice, there is a transient population of Cajal-Retzius-like cells that express CRF, and during development this stress-activated neuropeptide appears to modulate dendritic differentiation of pyramidal hippocampal neurons, acting through CRFR1 (Chen et al., 2004b). In this context, CRF leads to decrease dendritic growth. This finding helps to explain the negative effects of CRF, if released in excess, on dendritic arbors and synaptic plasticity of hippocampus, and on memory formation (McEwen, 1999; McGaugh and Roozendaal, 2002; discussed by Chen et al., 2004b). This can also contribute to understand long-term effects of early stress in mammals and birds (Nordgreen et al., 2006; De Haas et al., 2021), as discussed in a separate section.

In birds, another telencephalic center that regulates HPA is the nucleus of the hippocampal commissure (Nagarajan et al., 2017; Kadhim et al., 2019). The nucleus of the hippocampal commissure contains stress-responsive CRF expressing cells (Nagarajan et al., 2014, 2017; Kadhim et al., 2019; present results) as well as receptors of CRF and glucocorticoids (Kadhim et al., 2019; present results). Compared to the control situation, this nucleus increases its activity during acute stress, but decreases its activation in chronic stress (Nagarajan et al., 2014). Our previous studies showed that this nucleus and the adjacent septofimbrial nucleus (both including CRF neurons from E14; present results) contain glutamatergic neurons that derive from the pallial septum, and share some similarities with the hippocampus like expression of the transcription factors Emx1 and Lhx9 during development (Abellán et al., 2009; 2010). Like the hippocampal formation, it is reciprocally connected with lateral and medial septal nuclei (Atoji and Wild, 2004). The presence of glutamatergic neurons in the nucleus of the hippocampal commissure helps to explain why its hyperactivation during acute stress leads to increased activation of HPA and increase of blood corticosterone, while its hypoactivation in chronic stress produces a decrease in both HPA activation and blood corticosterone (Nagarajan et al., 2014).

In mammals, corticosterone regulates the brain through glucocorticoid and mineralocorticoid receptors, such as those found in the hippocampus leading to inhibition of HPA (references above). In late potshatching and adult birds (vocal and non-vocal learners), both receptors are found not only in the hippocampus (reviewed by Smulders, 2021), but also in areas of the mesopallium, nidopallium, arcopallium, and subpallium, including the extended amygdala (Matsunaga et al., 2011; Senft et al., 2016). In vocal learners, expression of mineralocorticoid receptors is particularly high in telencephalic areas associated to vocal learning and control, as

well as in visual and auditory nuclei of the thalamus (Suzuki et al., 2011; Matsunaga et al., 2011). In contrast, expression in generally light in the telencephalon and thalamus of non-vocal learners, although some signal is seen in visual and auditory related centers (Matsunaga et al., 2011). However, to our knowledge no information exists on embryonic expression in the avian brain. This information is particularly relevant in precocial species, such as quail and chicken, and is crucial to better understand regulation of the stress response as well as the effects of prehatching corticosterone (De Haas et al., 2021).

5.8.2. Auditory stimulation and synchronization of hatching

As noted above, high expression of Crf and its receptors are found in auditory telencephalic areas of chicken. What is the role of CRF in this functional system during embryonic development? In mammals, CRF is involved in acoustic startle (Liang et al., 1992a), and the neural circuit mediating this effect appears to involve the central amygdala, but this is not the primary center that initiates the response (Liang et al., 1992b). It is possible that this neural circuit also involves areas of the pallium, including the pallial amygdala, where sensorimotor integration and gating of the startle reflex in front of a sudden, intense sound takes place (Wan and Swerdlow, 1997). It is possible that CRF and CRF receptors in auditory-related telencephalic areas of chicken embryos contribute to modulate acoustic startle. This functional system may also mediate the long-term effects of noise-associated early life stress (De Haas et al., 2021). In addition, this CRF functional system may participate in modulating sound communication before hatching, and in auditory stimulation for hatching synchronization. Chicken embryos can detect auditory stimuli from E12 (Rogers, 1995), and appear to communicate with their mother before hatching (Edgar et al., 2016). Moreover, auditory stimuli from other chicks, including the clicking sound produced by egg teeth tapping during hatching, are essential for hatching synchronization (White, 1984).

5.8.3. Light stimulation and lateralization

Correct light stimulation during the incubation period is known to be critical for lateralization, and conditions the side preferent pecking after hatching (Rogers et al., 2007; Rogers, 2008). It appears that lateralization improves cognition and the ability of performing the double task of both feed searching and predator watching, which is advantageous in natural conditions (Rogers et al., 2004; reviewed by De Haas et al., 2021). Photopigments are present in the retina from E14, and retinal photoreceptors respond to light/dark cycles from E16. However, light-sensitive cells are found from earlier stages in other brains areas, such the pineal gland and

hypothalamus (De Haas et al., 2021). According to our results, several visual areas of the chicken telencephalic pallium show high levels of Crf expression from E12 and E14, including those in the hyperpallium and entopallium. In addition, Crf expressing cells are found from E14 in the retinorecipient anterior dorsolateral thalamic nucleus, comparable to the lateral geniculate of mammals (present results). It is possible that part of the visual pathways to the pallium and the related centers are active from middle embryonic stages, and that CRF in these areas and pathways modulates their maturation and function. Moreover, CRF and its receptors in these areas may mediate the effects of light-associated early life stress (De Haas et al., 2021, discussed below).

5.9. Variations in stress response after hatching and relationship to imprinting

Mammals and birds pass through a hyporesponsive period to stress around birth/hatching, which coincides with an increase of corticosterone in blood; this period is followed by a rapid normalization during early posthatching (Schapiro et al., 1962; Freeman and Manning, 1984). In birds, the duration of this hyporesponsive period is about 48 hours (Freeman and Manning, 1984). Interestingly, during this period posthatchlings go through a period of filial learning or imprinting, through which they develop a social preference for parents or other individuals, and even for stimuli resembling conspecifics (reviewed by Bolhuis, 1999; Rosa-Salva et al., 2015; Di Giorgio et al., 2017). The brain areas and circuits involved in filial imprinting have been studied, and include one crucial area of the medial intermediate mesopallium (Horn, 1998; Bolhuis, 1999), as well as parts of the septum and arcopallium, including a subdivision identified as nucleus taeniae (Mayer et al., 2017), called amygdalohippocampal area here (as in Puelles et al., 2019). Interestingly, these areas express CRF receptors.

Imprinting involves high-order association and regulation of both reward and fear responses. In this regard, the medial intermediate mesopallium is a highly associative area not only involved in filial imprinting, but also in passive avoidance learning (Rose and Csillag, 1985; Bourne et al., 1991; Dermon et al., 2002). This area is reciprocally connected with a multisensory area of the medial intermediate nidopallium, and projects to a rostromedial accumbens-like subdivision of the striatum that is involved in motivation and reward (Atoji and Wild, 2012; Bálink and Csillag, 2007; Bálink et al., 2011). The medial intermediate mesopallium also projects to the arcopallium (Csillag et al., 1994; Atoji and Wild, 2012), a part of the avian pallial amygdala that is involved in fear responses, by way of projections to the

extended amygdala and hypothalamus (Martínez-García et al., 2007; Saint-Dizier et al., 2009; Medina et al., 2017). Since the arcopallium projects to the medial striatum, the medial intermediate mesopallium projection to the arcopallium also appears to be part of a loop that indirectly modulates the reward system (Csillag et al., 1997). All these interconnected areas contain CRF cells and/or express CRF receptors. However, while in the rostromedial striatum and arcopallium those are present from middle embryonic stages, expression of receptors in the medial intermediate nidopallium and the mesopallium starts from E18, increasing after hatching. The increment in receptor expression (in particular, Crfr1) after hatching is higher in the intermediate/caudal mesopallium. Moreover, the CRF buffering protein starts to increase in the pallium at hatching, and continues to increase moderately during the first week after hatching. It is possible that CRF transmission during the perihatching and early posthatching period contributes to shape synaptic contacts and plasticity involved in filial learning, and the higher expression levels seen in some of the pallial areas at P7 contribute to gate and to a more sophisticated modulation of the fear responses in front of strangers or other novelties.

5.10. Early development of pallial CRF systems as a mechanism mediating long-term effects of early life stress

Several studies in mammals and birds have shown long-term negative effects of early life stress, including prenatal and early postnatal stress (Braastad, 1998; Hedlund et al., 2019; De Haas et al., 2021). In domestic chicken, prehatching stress is known to increase fearfulness of the offspring (Janczak et al., 2006), and can lead to increased injurious pecking of these animals later in life (De Haas et al., 2021). Moreover, injecting chicken eggs with corticosterone results in increased mortality, reduced growth, impaired laterality (Ericksen et al., 2003), and leads to reduced ability for filial imprinting after hatching (Nordgreen et al., 2006). In these cases, it appears that there is an alteration of 'embryonic programing', leading to changes at physiological, metabolic and epigenetic levels with long-term implications (reviewed by De Haas et al., 2021).

Regarding effects on brain development, to really understand the mechanisms behind short- and long-term effects of early life stress, it is mandatory to study embryonic and early posthatching expression of: 1) Glucocorticoid and mineralocorticoid receptors in brain centers known to be involved in regulation of HPA, but also in centers modulating sensorimotor integration, attention, learning and memory, motivation and emotions. 2) CRF and its receptors

in the brain, since these are known to be stress-responsive and be involved in dendritic and synaptic maturation and plasticity, as well as in modulation of emotional and cognitive aspects of stress (discussion above). Considering that HPA appears to become active early in development, we can speculate that glucocorticoid/mineralocorticoid receptor expression may also develop early, at least in upper and middle centers of HPA (paraventricular hypothalamic nucleus and anterior pituitary), so that part of the negative loop regulating corticosterone production is in place from the very first moment when HPA becomes active (at about E14). Regarding development of CRF systems, our results show that there are many telencephalic centers that contain stress-responsive CRF cells and receptors from E14. In posthatching and adult animals, these centers express glucocorticoid and mineralocorticoid receptors, and it is possible that they start to express these receptors and respond to corticosterone during embryonic and/or early posthatching development.

Overall, the results help to understand the mechanisms underlying the negative effects of noise and light stress during prehatching in chicken (Riedstra and Groothuis, 2004; De Haas et al., 2021), and suggest that mechanisms for regulating stress become more sophisticated with age. We provide baseline data on the presence of CRF, receptors and binding proteins in multiple areas of the telencephalon, which open new venues for investigating their function.

6. Conclusions

1. We identified an embryonic domain near the frontier between telencephalon and hypothalamus of chicken, with overlapping expressions of the transcription factors Foxg1, Otp and Sim1. This division is identical to the telencephalon-opto-hypothalamic domain (TOH) recently found in mouse. Double-labeling showed that chicken TOH is rich in cells coexpressing Otp and Foxg1, and Sim1 and Foxg1. This domain is located between the telencephalic subpallium and the core of the supraopto-paraventricular hypothalamic domain (SPV), which only expresses Otp and Sim1, but not Foxg1.
2. Based on its Foxg1 expression, we consider that the TOH is part of the telencephalon. Since this domain is found in chicken, mouse, the long-tailed lacertid lizard and zebrafish, we propose that it is likely present in the telencephalon of most (if not all) jawed vertebrates.
3. During development, the peduncular prosomeric part of the TOH domain generates a cell corridor that contributes Otp/Foxg1 and Sim1/Foxg1 doubled labeled cells to a ventral part of the medial bed nucleus of the stria terminalis (BSTM3) and part of the medial amygdala (Me).
4. In the BSTM and medial amygdala we also observed single-labelled cells expressing either Otp, Sim1 or Foxg1. While Foxg1 single labeled cells likely derive from the subpallium, the Otp and Sim1 single labeled cells likely originate from the SPV core.
5. In contrast to the Otp cells, Sim1 expressing cells also reach the nucleus of the lateral olfactory tract, the lateral (capsular and intercalated) part of the central extended amygdala, as well as the medial part of the central extended amygdala, which includes the lateral bed nucleus of the stria terminalis (BSTL).
6. In the capsular central extended amygdala, two groups of cells are obserwed. One group consists of Sim1/Foxg1 double labeled cells likely originating from the TOH domain, and another group consists of Sim1 single labeled cells that appear to derive from the SPV core. Based on comparison with VGLUT2, these cells are likely glutamatergic.
7. Regarding the terminal or pre-peduncular prosomeric division of TOH, during development it produces subpopulations of Otp/Foxg1 and Sim1/Foxg1 double labelled cells for the subpreoptic region, and some appear to invade tangentially the preoptic region (in the subpallium). In addition, we identified a a subpial cell corridor that gives rise to an ectopic supraoptic subnucleus in the ventral telencephalon, which appears to migrate from SPV core.
8. With respect to the corticotropin releasing factor (CRF) related system, our results demonstrate an early mRNA expression of CRF and its receptors in pallial areas

regulating sensory processing, sensorimotor integration and cognition, and a late expression in subpallial areas regulating the stress response (such as the central extended amygdala). However, CRF buffering system develops earlier in the subpallium than in the pallium. These results help to understand the mechanisms underlying the negative effects of noise and light during prehatching stages in chicken, and suggest that stress regulation becomes more sophisticated with age.

7. References

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