Chick Hippocampal Formation Displays Subdivision- and Layer-Selective Expression Patterns of Serotonin Receptor Subfamily Genes

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Hippocampal formation (HF) plays a key role in cognitive and emotional processing in mammals. In HF neural circuits, serotonin receptors (5-HTRs) modulate functions related to cognition and emotion. To understand the phylogenetic continuity of the neural basis for cognition and emotion, it is important to identify the neural circuits that regulate cognitive and emotional processing in animals. In birds, HF has been shown to be related to cognitive functions and emotion-related behaviors. However, details regarding the distribution of 5-HTRs in the avian brain are very sparse, and 5-HTRs, which are potentially involved in cognitive functions and emotion-related behaviors, are poorly understood. Previously, we showed that 5-HTR1B and 5-HTR3A were expressed in chick HF. To identify additional 5-HTRs that are potentially involved in cognitive and emotional functions in avian HF, we selected the chick orthologs of 5-HTR1D, 5-HTR1E, 5-HTR5A, and 5-HTR7 and performed in situ hybridization in the chick telencephalon. We found that 5-HTR1D, 5-HTR1E, 5-HTR5A, and 5-HTR7 were expressed in the chick HF, especially 5-HTR1D and 5-HTR1E, which showed subdivision- and layer-selective expression patterns, suggesting that the characteristic 5-HT regulation is involved in cognitive functions and emotion-related behaviors in these HF regions. These findings can facilitate the understanding of serotonin regulation in avian HF and the correspondence between the HF subdivisions of birds and mammals.

Keywords: chick, hippocampal formation, serotonin receptor, subdivision, layer

INTRODUCTION

The modulation of various neural functions by serotonin (5-hydroxytryptamine, 5-HT) is phylogenetically conserved (Marin et al., 2020). In particular, the association of the 5-HT system with cognition, behavior, and emotion is evolutionarily conserved in the animal kingdom (Kandel et al., 2000; Bacque-Cazenave et al., 2020). In mammals, processing of both cognition and emotion have been shown to involve the hippocampal formation (HF) (Fanselow and Dong, 2010; Anacker and Hen, 2017). The macrohistological features of HF are well-conserved in all mammals. More
specifically, HF consists of similarly convoluted and interlocked 3-layered subdivisions: the dentate gyrus (DG), Ammon’s horns or Cornu ammonis (CA) fields 1 to 3 (CA1 to CA3), and the subiculum (Hevner, 2016; Medina et al., 2017b). Information flow in the mammalian HF was described as a “trisynaptic circuit,” in which the entorhinal cortex (EC) projects to the DG, DG provides the projection to CA3 known as “mossy fiber”, and CA3 relays it to CA1 (Sloviter and Lomo, 2012). To understand the phylogenetic continuity of the neural basis for cognition and emotion, it is essential to reveal the neural mechanisms in HF that process cognitive and emotional behaviors in nonmammalian animals. Birds are well-fitted model animals for understanding the evolutionary continuity of the neural basis of cognition and emotion (Rosa Salva et al., 2015; Papini et al., 2019). For instance, the polymorphism of the 5-HT transporter gene, which affects the levels of gene expression, has been suggested to modulate fear-related behavior in chickens (Krause et al., 2017;Phi Van et al., 2018;Krause et al., 2019). In addition, hippocampal formation in birds (HF, hippocampus proper (Hp), and area parahippocampalis (APH)) has been shown to be related to cognitive functions, such as spatial navigation (Colombo and Broadbent, 2000; Matsushima et al., 2003; Bingman et al., 2005; Mayer et al., 2016; Sherry et al., 2017; Payne et al., 2021), and controlling the stress response (Smulders, 2017; Smulders, 2021) and emotions, such as anxiety-like behavior (Mayer et al., 2018; Morandi-Raikov and Mayer, 2020; Parada et al., 2021). However, the neural circuits that control these behaviors and 5-HT regulation in the avian HF are largely unknown.

The avian HF is homologous to that of mammals (Reiner et al., 2004; Herold et al., 2015; Striedter, 2016). The ancient origin of HF in the amniote was supported by both recent large-scale and single-cell transcriptome studies (Belgard et al., 2013; Tosches et al., 2018). However, the avian HF is composed of a layered arrangement of densely packed neurons with poorly defined boundaries, whereas the mammalian HF has a clear laminar organization (Atoji and Wild, 2006; Herold et al., 2015; Striedter, 2016). The existence of many subdivisions in the avian HF has been proposed to arise from multiple aspects, such as developmental origin, connectivity, histochemistry, and immunohistochemistry (Kuenzel and Masson, 1988; Atoji and Wild, 2004; Atoji and Wild, 2006; Suarez et al., 2006; Puelles et al., 2007; Gupta et al., 2012; Herold et al., 2014; Abellan et al., 2014; Atoji et al., 2016; Medina et al., 2017b). However, to date, a one-to-one correspondence of subdivisions between the avian and mammalian HF has not been established, especially owing to the controversy regarding its homology with the mammalian Hf (dentate gyrus and Ammon’s horn) (Atoji and Wild, 2004; Atoji and Wild, 2006; Kempermann, 2012; Herold et al., 2014; Abellan et al., 2014; Striedter, 2016; Hevner, 2016; Atoji et al., 2016; Medina et al., 2017b).

In mammals, various types of the 5-HT receptor (5-HTR) subfamily genes are expressed in the HF and are thought to play important roles in cognitive and emotional functions (Tanaka et al., 2012; Strac et al., 2016; Zmudzka et al., 2018; O’Leary et al., 2020; Viláro et al., 2020). In our previous study, we pointed out that 5-HTRI B and 5-HTR3A were expressed in chick HF in a clear and characteristic manner (Fujita et al., 2020). In the present study, we performed a detailed analysis and determined the subdivision of HF in which 5-HTRI B and 5-HTR3A are expressed. To comprehensively identify more 5-HTRs that are potentially involved in cognitive and emotional functions in avian HF, we investigated the expression of 5-HTR subfamily genes that were not analyzed in our previous study (Fujita et al., 2020). We selected the chick orthologues of 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR5A, and 5-HTR7 and found that 5-HTRI D, 5-HTRI E, 5-HTR5A, and 5-HTR7 were expressed in the chick HF. Among them, 5-HTR1D and 5-HTR1E showed subdivision- and layer-selective expression patterns, suggesting a characteristic 5-HT regulation in these regions. Our findings can be used as a basis for understanding 5-HT regulation in avian HF and the correspondence between the HF subdivisions of birds and mammals.

MATERIALS AND METHODS

Animals

Fertilized eggs of domestic chicks (Gallus domesticus, Cobb strain) were purchased from a local dealer (3-M, Aichi, Japan) and incubated at Teikyo University (Kaga, Itabashi-ku, Tokyo, Japan). Animal experiments were performed as previously described (Yamaguchi et al., 2008a; 2008b). Newly hatched chicks (P0) were transferred to dark plastic enclosures in a dark, warm cage at 30°C for 1 day (P1). We used seven chicks for 5-HTR1D probes, nine for 5-HTR1E, eight for 5-HTR1F, seven for 5-HTR2B, six for 5-HTR5A, six for 5-HTR7, three for 5-HTR3A, five for 5-HTR1B, and six for lymphoid enhancer factor 1 (LEF1) (Supplementary Table S1). As for 5-HTR3A and 5-HTR1B, we have already investigated the expression of 5-HTR3A and 5-HTR1B throughout the entire brain in our previous study. In this study we focused on the expression of 5-HTR3A and 5-HTR1B in the HF. All procedures were reviewed and approved by the Committee on Animal Experiments of Teikyo University and were conducted in accordance with the guidelines of the national regulations for animal welfare in Japan.

Tissue Preparation

P1 chicks were anesthetized by intraperitoneal injection (0.40 ml/individual) of a 1:1 solution of ketamine (10 mg/ml, Ketalar-10, Sankyo Co., Tokyo, Japan) and xylazine (2 mg/ml, Sigma, St. Louis, MO, United States). For brain fixation, the anesthetized chicks were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.5, PFA-PBS). Whole brain specimens were dissected and immediately immersed in PFA-PBS for 1 or 2 days at 4°C. For cryoprotection, fixed brain samples were placed in an 18% sucrose/PFA-PBS solution for 2 days at 4°C. Subsequently, brains with sucrose substitution were embedded in Tissue-Tek OCT compound (Sakura Finetchnical, Tokyo, Japan), frozen immediately on dry ice, and stored at −80°C until sectioning. Frozen brain blocks were cut into 18 µm-thick sections using a cryostat (Leica CM3050S or Leica CM 1850, Leica Biosystems, Nüflossch, Germany). Serial coronal sections were prepared at the level of A14.4–A4.4,
corresponding to those of the atlas by Kuenzel and Masson (1988).

**cDNA Cloning**

Total RNA was extracted from chick brains using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed using the SuperScript III kit (Invitrogen) with an oligo (dT) primer, according to the manufacturer’s protocol. Reverse transcription polymerase chain reaction (RT-PCR) for the amplification of LEF1 was performed using gene-specific primers: forward, 5′-GATCCCCTTCAAGGACGAAG-3′; and reverse, 5′-GCCAAGAGGTGGTGTTATCTG-3′. PCR products were subcloned into the pGEM-T easy vector (Promega, Madison, WI, United States), the sequence of which was validated using Sanger sequencing. For 5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR3A, 5-HTR5A, and 5-HTR7 probes, we used previously generated plasmids (Fujita et al., 2020; Fujita et al., 2022).

**RNA Probe Preparations**

Plasmids containing cDNA fragments for 5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR3A, 5-HTR5A, 5-HTR7, and LEF1 were amplified by PCR using the M13 primer pair. Amplicons containing T7 and SP6 promoter sites were purified using a PCR purification kit (Qiagen, Valencia, CA, United States). Digoxigenin (DIG)-labelled sense and antisense RNA probes were prepared by in vitro transcription using a DIG RNA labelling kit (Roche, Basel, Switzerland) according to the manufacturer’s protocol.

**In situ Hybridization**

ISH experiments were performed as previously described in Fujita et al. (2019), with some modifications. Brain section specimens were fixed in 4% PFA-PBS, pretreated, and hybridized with DIG-labelled RNA probes at 70°C. After stringent washes with a series of saline-sodium citrate (SSC) buffers, hybridized probes were detected via immunohistochemical examination using an alkaline phosphatase-conjugated anti-DIG antibody (1:1,000; Roche). For signal visualization, a chromogenic reaction with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was performed at 25°C for the following durations: 5-HTR1D, 5-HTR1E, 5-HTR1F, and 5-HTR2B, 18–42.5 h; 5-HTR5A, 18.3–39.5 h; 5-HTR7, 19.5–39.8 h; 5-HTR1B, 18.2–39 h; 5-HTR3A, 19.5 h; and LEF1, 18–38.8 h. Sense probes were used as negative controls in every experiment.

**Image Acquisition and Data Processing**

Bright-field images of whole sections on each slide glass were semiautomatically taken using the NanoZoomer 2.0 HT or NanoZoomer XR systems (Hamamatsu Photonics, Shizuoka, Japan). Microscopic fields of interest were cropped using the

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**TABLE 1 | Comparison of terminology regarding avian HF.**

| Chicken | Pigeon |
|---------|--------|
| This study, 2022 | Kuenzel and Masson, (1988) | Suarez et al. (2006); Puelles et al. (2007); Abellan et al. (2014) | Atoji and Wild, 2004; Atoji and Wild, 2006; Herold et al. (2014) |
| V | Hp | DGP |
| DM | APH | APHm, APHi |
| APHre | — | — |
| — | — | — |
| DL | APHi |

APH, area parahippocampalis; APHi, intermediate APH; APHl, medial APH; APHm, ectopic part of rostral APH; DGP, dentate gyrus primordium; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; Hp, hippocampus, Ma, magnocellular region of HF; Pa, parvocellular region of HF; Po, cell-poor region of HF; Tr, triangular region of HF; V, V-shaped complex; VI, V-shaped layer region of HF.
In situ hybridization of 5-HTR1D in P1 chick telencephalons. Digoxigenin-labelled RNA antisense (A–F) and sense (A’–F’) 5-HTR1D probes were used for in situ hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of 5-HTR1D, sections from seven chicks were analyzed, and representative images from four chick brain sections are shown. Diagrams of coronal sections are shown in the rightmost panels (A’–F’). Levels of sections (A 14.0 to A 4.6) were in accordance with those mentioned in Kuenzel and Masson’s chick atlas (Kuenzel and Masson, 1988). A, arcopallium; CDL, area corticodorsolateralis; DL, dorsal lateral region of HF; DM, dorsal medial region of HF; E, entopallium; FL, field L; H, hyperpallium; LSt, lateral striatum; M, mesopallium; N, nidopallium; PoA, posterior pallial amygdala; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm.
FIGURE 3 | In situ hybridization of 5-HTR1E in P1 chick telencephalons. Digoxigenin-labelled RNA antisense (A–F) and sense (A’–F’) 5-HTR1E probes were used for in situ hybridization in coronal sections of the P1 chick telencephalon. To evaluate the expression patterns of 5-HTR1E, sections from nine chicks were analyzed, and representative images from three chick brain sections are shown. Diagrams of coronal sections are shown in the rightmost panels (A’–F’). Levels of sections (A 14.2 to A 6.0) were in accordance with those mentioned in Kuenzel and Masson’s chick atlas (Kuenzel and Masson, 1988). A: arcopallium; B: basorostralis; CDL: the area corticoida dorsolateralis; DL: the dorsal lateral region of HF; DM: the dorsal medial region of HF; E: entopallium; H: hyperpallium; LSt: lateral striatum; M, mesopallium; MSt, medial striatum; N, nidopallium; TnA, nucleus taeniae of the amygdala; V, V-shaped complex; P1: posthatch day 1. Scale bars = 2.5 mm.
Terminology of Avian Hippocampal Formation

All histological terminologies used in this study were based on the atlas of Kuenzel and Masson (1988) and the descriptions of the avian brain nomenclature consortium (Reiner et al., 2004), except for HF and its subdivisions. The subdivision schemes of avian HF were based on various aspects and species, such as tract tracing and Nissl staining in pigeons (Atoji and Wild, 2004; Atoji and Wild, 2006), histochemistry of neurotransmitter radioligands in pigeons (Herold et al., 2014), immunohistochemistry of several neurochemical markers in developing chickens (Suarez et al., 2006), and combinatorial expression patterns of morphogenetic genes in developing chickens (Abellan et al., 2014). No uniform nomenclature has been applied across species (Herold et al., 2015). In this study, the histological position of HF in chicks was based on the nomenclature set by Puelles et al. (2007). Based on previous studies (Kuenzel and Masson, 1988; Atoji and Wild, 2004; Atoji and Wild, 2006; Suarez et al., 2006; Puelles et al., 2007; Abellan, 2004).
et al., 2014; Herold, et al., 2014), we used the following subdivision terms: V-shaped complex (V), dorsal medial region (DM), ectopic part of the rostral APH (APHre), and dorsal lateral region (DL) (Figure 1). The relationships between the terminologies of avian HF used in this study and those in previous studies are summarized in Table 1.

RESULTS

Selection of Chick Orthologues of Mammalian 5-HTR Genes and LEF1 as APHre Marker Gene

We initially selected the chick orthologs of mammalian 5-HTR genes, namely 5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR3A, and 5-HTR7. Among these, the expression patterns of 5-HTR1B and 5-HTR3A in the chick telencephalon have been previously reported (Fujita, et al., 2020). The chicken genome also contains 5-HTR6 (International Chicken Genome Sequencing Consortium, 2004); yet we could not obtain a subclone in our study. LEF1 is an important transcription factor for granule cell production in the dentate gyrus (Galcera, et al., 2000). We found that the chick LEF1 ortholog exhibited sequence similarities with that of humans: 95% (protein) and 85.2% (DNA). In our previous studies, we searched for sequence similarities between chick 5-HTR1B, 5-HTR1D, 5-HTR1E, 5-HTR1F, 5-HTR2B, 5-HTR3A, 5-HTR5A, and 5-HTR7 with those from other animals (Fujita et al., 2020; Fujita et al., 2022). The accession numbers and molecular characteristics of ortholog gene probes are summarized in Supplementary Table S2. We accordingly designed probes to detect multiple transcript variants of orthologs registered in the database. Consecutively, we performed in situ hybridization (ISH) and analyzed the expression patterns of these orthologs in the telencephalon of chicks.

Expression of 5-HTR1D in Chick Telencephalon

To comprehensively examine the expression pattern of 5-HTR1D in the chick telencephalon, we performed ISH using the 5-HTR1D probe on coronal sections at approximately A14.4 to A 4.6 of naive chicks on posthatch day 1 (P1). We detected signals in a large part of the mesopallium (Figure 2A–E, A9–E9), entopallium (Figure 2B, B9), field L (Figures 2D,E, D9–E9), a part of the hyperpallium (Figure 2C, C9), a large part of the arcopallium (Figure 2C,D, C9–D9), and the lateral part of the nidopallium (Figure 2C–F, C9–F9). In addition, we detected signals in a part of the DM (Figure 2C–F, C9–F9), DL in a characteristic layered manner (Figure 2C–F, C9–F9), and the corticoidea dorsolateralis area (CDL) (Figure 2D–F, D9–F9).

Expression of 5-HTR1E in Chick Telencephalon

We examined the expression pattern of 5-HTR1E in sections A14.2 to A6.0 of P1 chick telencephalons (Figure 3). We detected strong signals in the nucleus taeniae of the amygdala (TnA) (Figure 3E, F), and a part of the DM, in a cluster manner (Figure 3C–F, C9–F9). We also detected signals in a part of the mesopallium (Figure 3A–F, A9–F9), hyperpallium (Figure 3D, D9), DL in a layered manner (Figure 3D–F, D9–F9), and CDL (Figure 3F, F9).
Comparison of 5-HTR1E and LEF1 Expression Patterns in Chick APH
To compare the expression patterns between 5-HTR1E and LEF1, which is the APHre region marker (Abellan et al., 2014), we performed ISH using 5-HTR1E and LEF1 probes in neighboring sections of A8.8 and A6.6, respectively (Figure 4). Interestingly, we found that the regions of expression of 5-HTR1E and LEF1 in the APHre matched well at both sections (Figure 4D,E, J-K, D’-E’, J’-K’).

Expression of 5-HTR1F in Chick Telencephalon
We then examined the expression patterns of 5-HTR1F in sections A14.4 to A5.0 of P1 chick telencephalons (Figure 5). Our analysis revealed the presence of signals in the interstitial part of the hyperpallium (Figure 5A,B, A’-B’).

Expression of 5-HTR5A in Chick Telencephalon
We also examined the expression patterns of 5-HTR5A in sections A13.8 to A4.4 of P1 chick telencephalons (Figure 6). We accordingly detected the expression of 5-HTR5A in the dorsal arcopallium, lateral nidopallium, DL, and CDL (Figure 6A,B, A’-B’).

Expression of 5-HTR7 in Chick Telencephalon
We further examined the expression patterns of 5-HTR7 in sections A13.6 to A4.4 of P1 chick telencephalons (Figure 7) and found that 5-HTR7 was mainly expressed in a large part of the arcopallium, lateral nidopallium, DM, DL (Figure 7A-C, A’-C’), and CDL (Figure 7B,C, B’-C’).
Expression of 5-HTR2B in Chick Telencephalon

Finally, we examined the expression patterns of 5-HTR2B in sections A13.2 to A5.0, but did not detect any signal (data not shown), suggesting that the levels of expression of 5-HTR2B were either very low or cells expressing 5-HTR2B were very rare.

Comparison of 5-HTR1B, 5-HTR1D, 5-HTR1E, and 5-HTR3A Expression Patterns in Chick Hippocampal Formation

We found that 5-HTR1D and 5-HTR1E were clearly expressed in chick HF. In our previous study, we revealed the characteristic expression patterns of 5-HTR1B and 5-HTR3A (Fujita, et al., 2020). Subsequently, to understand the relationship between the regions of expression of these 5-HTRs in chick HF, we performed ISH using 5-HTR1B, 5-HTR1D, 5-HTR1E, and 5-HTR3A probes on neighboring sections of A8.8 to A8.6 (Figures 8, 9A6.6 to A6.4 (Figures 10–13). We found that 5-HTR1B was expressed in the whole DL (Figure 8A, A’, Figure 9, Figure 10A, A’, Figure 13), whereas sparsely in the DM (Figure 8A, A’, Figure 9, Figure 10A, A’, Figure 12). We also detected the expression of 5-HTR1D in a part of DM (Figure 8B, B’, Figure 9, Figure 10B, B’, Figure 12), and in DL in a layered manner (Figure 8B, B’, Figure 9, Figure 10B, B’, Figure 13). We found that 5-HTR1E was expressed in APHRe (Figure 8C, C’, Figure 9, Figure 10C, C’, Figure 12), and in DL in a layered manner (Figure 8C, C’, Figure 9, Figure 10C, C’, Figure 13). We detected rare signals of expression of 5-HTR3A in both the DM and DL (Figure 8D, D’, Figure 9, Figure 10D, D’, Figure 12, Figure 13). Finally, we observed that 5-HTR1B and 5-HTR3A were sparsely expressed in V, whereas we detected a faint
expression of 5-HTR1E and no expression of 5-HTR1D in this region (Figure 11).

DISCUSSION
Expression Patterns of 5-HTR Subfamily Genes in Chick HF
In the present study, we revealed that 4 5-HTRs, 5-HTR1B, 5-HTR1D, 5-HTR1E, and 5-HTR3A, were expressed in a subdivision- and layer-selective manner in chick HF (Figure 14). We also found that 5-HTR5A and 5-HTR7, which were faintly expressed, did not clearly show subdivision- or layer-selective expression patterns in the chick HF.

We found that 5-HTR1B was highly expressed in the DL, whereas sparsely in V, DM, APHre, while 5-HTR3A was expressed in all the V, DM, DL, APHre, sparsely. In comparison, in mammals, 5-Htr1b is expressed in Cornu Ammonis 1 (CA1) and CA3 pyramidal cells, GABAergic interneurons of the hilus, granule cells of dentate gyrus (DG), the subgranular zone, the layer II pyramidal cells in the entorhinal cortex (EC) (Tanaka, et al., 2012), while 5-Htr3A is expressed in GABAergic interneurons of the hilus, in the subgranular zone, in scattered EC cells, CA1, CA3, and DG (Tecott, et al., 1993; Fonseca et al., 2001; Morales and Wang 2002; Tanaka, et al., 2012; Koyama, et al., 2017). Considering the expression patterns of 5-HTR1B and 5-HTR3A, our results support the correspondence of V and DM in the avian HF to the mammalian hippocampal proper (DG and CA subfields) (Montagnese et al., 1996; Szekely, 1999; Atoji and Wild, 2004; Atoji and Wild, 2006; Suarez, et al., 2006; Puelles et al., 2007; Herold et al., 2014; Abellan et al., 2014; Atoji et al., 2016; Medina et al., 2017b). To date, a one-to-one correspondence of subdivisions between the avian and mammalian HF has not been established, while various studies have postulated on possible areas in avian HF corresponding to mammalian DG. One suggestion is that the mammalian DG corresponds to V in avian HF (Atoji and Wild, 2004; Suarez, et al., 2006; Puelles et al., 2007; Gupta, et al., 2012; Herold et al., 2014; Abellan et al., 2014; Atoji et al., 2016). Another theory is that the mammalian DG corresponds to DM in avian HF (Montagnese et al., 1996; Szekely, 1999). However, another possibility is that DG might be a novel acquisition in mammals, which would imply that there is no homolog in birds. The avian HF has undergone divergence over hundreds of millions of years of evolution, thus making it difficult to compare the subdivisions of HF between birds and mammals.

FIGURE 8 | Comparison of 5-HTRs expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 8.8 level. In situ hybridization using DIG-labelled RNA antisense and sense 5-HTR1B (A and A’), 5-HTR1D (B and B’), 5-HTR1E (C and C’), and 5-HTR3A (D and D’) probes in coronal sections of P1 chick telencephalons (A–D) Diagrams of coronal sections are shown in the rightmost panels. DL, dorsal lateral region of HF; DM, dorsal medial region of HF; H, hyperpallium; M, mesopallium; V, V-shaped complex; P1: posthatch day 1. Scale bars = 1 mm.
(Hevner, 2016; Striedter, 2016). In fact, although no macrostructure corresponding to mossy fibers in the mammalian DG has been observed in avian HF (Faber et al., 1989; Montagnese et al., 1993, 1996; Tombol et al., 2000; Herold et al., 2014), this does not exclude the existence of a neuron population in the avian HF corresponding to granule cells in the mammalian DG. This candidate neuronal cell population corresponding to granule cells might be unevenly distributed in the avian HF. We assumed that using chick orthologs of another marker gene to distinguish pyramidal from granule cells in mammalian HF could provide new clues for the correspondence between avian and mammalian HF.

In chicks, 5-HTR1E was highly expressed in APHre, and weakly in the V and DL in a layer-selective manner, suggesting that the APHre cell population that preferentially expresses 5-HTR1E has novel characteristics.

In contrast, in mammals, 5-Htr1e was shown to be expressed in the CA fields and DG (Bruinvels et al., 1994a; Mengod et al., 2006; Vilaro et al., 2020). However, because the relationship between APHre in avian HF and other HF subdivisions and the function of APHre are completely unknown (Abellan et al., 2014), it was difficult to determine the correspondence of APHre to the mammalian subdivision of HF. In the future, a better understanding of the features of APHre in chicks with respect to multiple aspects, such as connectivity, electrophysiological properties, and behavioral function, is expected to help determine the correspondence of APHre subdivisions.

Patch RNA-sequencing analysis in mammals showed that 5-Htr1d was expressed in the GABAergic interneurons of CA1 (Luo et al., 2019). We here found that 5-HTR1D was expressed in DM, APHre, and DL in a layer-selective manner. Based on this,
FIGURE 10 | Comparison of 5-HTRs expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 6.6. In situ hybridization using DIG-labelled RNA antisense and sense 5-HTR1B (A and A’), 5-HTR1D (B and B’), 5-HTR1E (C and C’), and 5-HTR3A (D and D’) probes in coronal sections of P1 chick telencephalons (A–D). Diagrams of coronal sections are shown in the rightmost panels. CDL: area corticoidea dorsolateralis; DL: dorsal lateral region of HF; DM: dorsal medial region of HF; M: mesopallium; N: nidopallium; V: V-shaped complex; P1: posthatch day 1. Scale bars = 1 mm.

FIGURE 11 | Comparison of 5-HTRs expression patterns in the HF in the P1 chick telencephalons using neighboring sections around A 6.6 focused on V. Magnified views of HF surrounding regions shown in Figure 10. V: V-shaped complex; P1: posthatch day 1. Scale bars = 250 µm.
we assumed that 5-HTR1D might also be expressed in GABAergic interneurons in chick HF.

In the past, the cytoarchitecture of avian HF was considered quite different from that of the mammalian 3-layered HF, as it appeared to have completely lost its layered structure. Three layers are clearly present in lizards but less visible in crocodiles (closest to birds) as for HF homologue. (Hevner, 2016; Striedter, 2016). However, detailed immunohistochemical analysis and combinatorial expression analysis using developmental regulatory genes revealed the existence of a layered cytoarchitecture orthogonal to radial glial fibers in the chick HF during its developmental stages (Redies et al., 2001; Abellan et al., 2014). Such a layered structure has also been confirmed in the HF of adult pigeons (Suarez et al., 2006; Herold et al., 2019). In our study, we showed that the expression patterns of 5-HTR1D and 5-HTR1E in DL were layered, suggesting a selective regulation of the DL layer by these receptors. Our data indicated that the neuronal population in this layer might have functional roles in cognition and emotion.

We also examined the expression of 5-HTR2B and failed to detect it in chick telencephalons, suggesting either a low level of expression of 5-HTR2B or the rarity of expressing cells. Interestingly, the expression level of 5-Htr2b in the telencephalon of mammals was found to be low (Bonaventure et al., 2002; Vilaro, et al., 2020). This finding was consistent with our obtained results for the expression of 5-HTR2B.

**Possible Functions of Chick 5-HTRs in Chick Telencephalon Other Than HF**

Regarding its the expression pattern, we noticed that 5-HTR1D was expressed in a large part of the mesopallium, arcopallium, and a part of the hyperpallium, nidopallium, HF, CDL, and major part of the intercalated nidopallium (entopallium and field L (Jarvis et al., 2013)). Of note, the intercalated nidopallium receives sensory projections from the thalamus (Reiner et al., 2004; Jarvis et al., 2013). We previously showed that 5-HTR2C was preferentially expressed in intercalated nidopallium in chicks (Fujita et al., 2020). Considering the expression combination of 5-HTR1D and 5-HTR2C, it is possible that these 2 5-HTRs might work together in sensory input information processing in the intercalated nidopallium in birds. In mammals, 5-HTR1D was demonstrated to be distributed in the frontoparietal cortex, primary olfactory cortex, accumbens nucleus, caudate-
putamen, and lateral mammillary nucleus (Bruinvels et al., 1994a, 1994b; Vilaro et al., 2020). However, the regional expression of 5-HT1D in the chick hyperpallium appeared to be limited (Figure 2C, C'), suggesting a serotonergic modulation in the neuronal population of the hyperpallium via 5-HT1D. In the case of the expression pattern of 5-HT1E, we found that the major regions of expression were the APHre, TnA, and a part of the hyperpallium. We previously showed that 5-HT2C and 5-HT4 were preferentially expressed in the TnA in chicks (Fujita et al., 2020), whereas in mammals, 5-HT2C and 5-HT4 are expressed in the amygdala (Huang and Kandel, 2007; Bombardi 2014; Bocchio et al., 2016). TnA is considered to be the counterpart of the mammalian medial amygdala (Reiner et al., 2004; Yamamoto et al., 2005; Yamamoto and Reiner, 2005; Hanics et al., 2017), which is functionally associated with social behaviors, including sexual behavior and social interactions (Ikebuchi et al., 2009; Mayer et al., 2017; Mayer et al., 2019). These findings indicated that 5-HT might play a key role in shaping social responses in birds and mammals. In this study, we found another 5-HT, 5-HT1E, which was preferentially expressed in TnA. Whereas, in mammals, 5-Htr1e is expressed in the amygdala (Lowther et al., 1992; Bruinvels et al., 1994a; Mengod et al., 2006). Our findings regarding 5-HT1E also support the potentially conserved roles of 5-HTRs in the mammalian medial amygdala and avian TnA. Furthermore, the expression patterns of 5-HT1E and 5-HT1D in the hyperpallium, appeared to be similar (Figure 8A, C, A', C'), suggesting a serotonergic modulation in the neuronal population of the hyperpallium via these receptors.

**FIGURE 13** Comparison of 5-HTRs expression patterns in HF in P1 chick telencephalons using neighboring sections at approximately A 6.6 focused on DL. Magnified views of HF surrounding regions shown in Figure 10. DL: dorsal lateral region of HF; P1: posthatch day 1. Scale bars = 250 µm.
Regarding the expression pattern of 5-HTR1F, we observed that it was selectively expressed in the interstitial part of the hyperpallium (IHA). According to developmental studies, the hyperpallium of birds is a region homologous to the mammalian neocortex (Fernandez, et al., 1998; Puelles et al., 2000), and is composed of four pseudolayers: the apical part of the hyperpallium, IHA, the intercalated part of the hyperpallium, and the densocellular part of the hyperpallium (Medina and Reiner 2000; Reiner et al., 2004). Among them, the IHA has projection terminals of sensory information from the thalamus, which is considered equivalent to layer IV of the mammalian neocortex (Bangnoli and Burkhalter, 1983; Miceli and Reperant, 1985; Miceli et al., 1990; Atoji et al., 2018; Atoji and Wild, 2019). Despite some species differences, 5-Htr1F is expressed in the intermediate cortical layers (layers IV and V) in mammals (Waeber and Moskowitz 1995; Mengod et al., 1996; Pascual et al., 1996; Lucaites et al., 2005). Taken together, both 5-HTR1F-expressing neurons in chick IHA and 5-Htr1F-expressing neurons in layer IV of the mammalian neocortex might have conserved functions in processing sensory input under serotonergic modulation.

We also detected that 5-HTR5A was expressed in the dorsal arcopallium, lateral nidopallium, HF, and CDL. In mammalian brains, 5-HTR5A is distributed in the piriform cortex, habenula, and HF, suggesting its involvement in the regulation of cognition, anxiety, and sensory perception (Plassat et al., 1992; Erlander et al., 1993; Matthes et al., 1993; Kinsey et al., 2001; Mengod et al., 2006; Vilaro et al., 2020). The dorsal arcopallium is the proposed region homologous to the basolateral amygdala in terms of embryonic origin and expression combinations of conserved morphogenetic genes (Martinez-Garcia et al., 2009; Medina et al., 2017a; Martinez-Garcia and Lanuza, 2018). The similarity in the regional expression of 5-HTR5A between mammalian and chick telencephalons suggested its conserved function in the serotonergic modulation in telencephalons.

Finally, we observed that 5-HTR7 was expressed in the arcopallium, lateral nidopallium, HF, and CDL. In the mammalian telencephalon, 5-Htr7 is distributed in some brain regions, including the HF and amygdala (Gustafson et al., 1996; Mengod et al., 1996; Martin-Cora and Pazos, 2004; Mengod et al., 2006; Tanaka et al., 2012). This finding suggested the conserved 5-HTR7-mediated serotonergic modulation in the amygdala and HF between birds and mammals.

CONCLUSION

We comprehensively revealed the expression patterns of 5-HTR subfamily genes in the chick telencephalon and specifically found that 5-HTR1D, 5-HTR1E, 5-HTR5A, and 5-HTR7 were expressed in chick HF. These receptors might be involved in the regulation of HF neural circuits that control cognitive and emotion-related functions in birds. In addition, we found that 5-HTR1B, 5-HTR1D, 5-HTR1E, and 5-HTR3A were expressed in HF in a subdivision- and layer-selective manner. Our findings can facilitate the improved understanding of the correspondence between the avian and mammalian HF.
DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Teikyo University.

AUTHOR CONTRIBUTIONS

TF and SY designed the study and performed the experiments; TF, NA, CM, EF, KH and SY analyzed the data; TF, NA, CM, TM, KH and SY wrote the paper. All authors have reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.882633/full#supplementary-material

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