Comparative gene expression analysis among vocal learners (Bengalese finch and budgerigar) and non-learners (quail and ring dove) reveals variable cadherin expressions in the vocal system

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INTRODUCTION

Vocal learning is the ability to acquire a new sound through imitation, and three families of birds (songbirds, parrots, and hummingbirds) have this ability (Jarvis, 2004). Because these birds are taxonomically distantly related, it has been suggested that they acquired this ability independently. In the brain of a vocal learner, a series of nuclei and a neural circuit exist called the “song system,” which is specialized for vocal learning and production (Figure 1; Nottebohm et al., 1976, 1982; Brauth et al., 1994; Striedter, 1994; Durand et al., 1997; Brainard and Doupe, 2000; Gahr, 2000; Jarvis and Mello, 2000; Jarvis et al., 2000; Jarvis, 2004; Bolhuis and Gahr, 2006; Bolhuis et al., 2010). In contrast, non-learners such as chickens and pigeons do not have such a system and only produce innate sounds (Bolhuis and Gahr, 2006). Because of these structure-related behavioral differences, the avian vocal system is a good model for studying brain evolution from a morphological and functional perspective (Matsunaga and Okanoya, 2009b).

We previously performed in situ hybridization screening in a songbird, the Bengalese finch, to explore the molecular basis of vocal system development and identified cadherins, neuropilins, and plexins as molecules whose expression is vocal-area related (Matsunaga et al., 2008). Among them, we particularly focused on cadherins, since cadherins show neural circuit-related expressions (each cadherin is expressed in some restricted population of neurons that are connected with each other; Suzuki et al., 1997; Takeichi, 2007). Actually, in the songbird brain, cadherins show vocal-system-related expressions (Matsunaga and Okanoya, 2008a). Cadherin-6B (cad6B) is broadly expressed in vocal control nuclei, whereas R-cadherin (Rcad) is expressed in the surrounding area. Cadherin-7 (Cad7) is transiently expressed in vocal control nuclei and its expression is downregulated during the phase from sensory to sensorimotor learning stage. Overexpression of cadherins affected vocal learning and production, suggesting the involvement in vocal development (Matsunaga and Okanoya, 2008c). Thus, it appears that cadherins are one of key regulators for vocal development.

Previously, we examined cadherin expressions in other vocal learner, budgerigar (Parrots) and non-learner quail (Galliformes), and published part of results in a short proceeding paper briefly (Matsunaga et al., 2008). Here, we performed more extensive comparative gene expression analysis of cadherins in vocal learner (Bengalese finch and budgerigar) and non-learner (quail and ring dove), and found that cadherin expressions are highly diverse in
the vocal system. The auditory system is well connected to the vocal system and related to vocal learning. The visual system is unrelated to the vocal system and functionally conserved between vocal learners and non-learners. By analyzing gene expressions in these regions between vocal learners and non-learners, we can evaluate whether diverse cadherin expressions are related to vocal learning or not. Therefore, in this study, to examine whether such diverse cadherin expressions are vocal area-specific, we examined cadherin expressions not only in the vocal system, but also in other neural systems.

MATERIALS AND METHODS

ANIMALS

We used three 14-day postnatal (P14) and three P30 male Bengalese finches (Lonchura striata var. domestica), two P14, one P16, and three P30 male budgerigars (Melopsittacus undulatus), and three P14 and three P30 male quails (Coturnix japonica), two P24 and one P45 male ring doves (Streptopelia risoria), all of which were bred at our lab facilities. All birds were deeply anesthetized with an intramuscular injection of sodium pentobarbital (50 mg/kg) and then sacrificed. After decapitation, their brains were embedded in OCT medium and frozen on dry ice for cryosectioning. The brainstem of avian species contains a telencephalic vocal learning pathway and the general vocal production pathway in the brainstem. The telencephalic vocal pathway is found only in vocal learning species. In the Bengalese finch, the telencephalic vocal pathway is composed of HVC-RA-(DM)-nXIIts posterior pathway and the HVC-AreaX-DLM-LMAN-RA anterior pathway. A similar but not the same vocal pathway is observed in the budgerigar: the NLC-AAC-(DM)-nXIIts posterior pathway and the AAC-DVMm-NAO-AAC anterior pathway. In contrast, such neural circuits for vocal learning are not found in the quail brain. A similar neural circuit is found in the brainstems of these species to produce learned or innate vocalization. The red and blue lines indicate anterior and posterior pathways for vocal learning, respectively. The yellow line indicates the general vocalization pathway seen in the brainstems of avian species. AAC: central nucleus of the anterior arcopallium, DLM, dorsal lateral nucleus of the thalamus; DM, dorsal medial nucleus of the midbrain; DMm, magnocellular nucleus of the dorsal thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NAO, oval nucleus of the anterior nidopallium; NLC, central nucleus of the lateral nidopallium; RA, robust nucleus of the arcopallium; nXIIts, tracheosyringeal hypoglossal nucleus.

ISOLATION AND CLONING OF cDNA

The budgerigar cDNA fragments for Rcad (Genbank accession no. AB329583), cad6B (AB329584), and cad7 (AB329582), ring dove cDNA fragments for Rcad (AB610760), cad6B (AB610761), and cad7 (AB610762) were isolated from the adult brain by the reverse transcription-PCR. The same primer sets used for Bengalese finch were used to isolate budgerigar and ring dove cDNAs, as previously described (Matsunaga and Okanoya, 2008a), except for ring dove cad6B. For ring dove cad6B, the primers were used as follows: 5’-CTTGTGTTGCGCTGATGAGA-3’ and 5’-GTTTATAGCCTGGGCACGAA-3’. Each cDNA fragment was inserted into the pGEM-T Easy Vector (Promega, Madison, WI, USA). We used the same plasmids for probe preparation that were used for Bengalese finch brain, as previously described (Matsunaga and Matsunaga and Okanoya

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FIGURE 1 | Phylogenetic tree of avian species and schematic representation of the vocal system in songbird, parrot, and non-learner.

Phylogenetic relationship among vocal learners and non-learners (based on Hackett et al., 2008) (A). Sagittal view of a songbird (B), parrot (C), and non-learners such as quail and dove (D). The avian vocal system is composed of the telencephalic vocal learning pathway and the general vocal production pathway in the brainstem. The telencephalic vocal pathway is found only in vocal learning species. In the Bengalese finch, the telencephalic vocal pathway is composed of HVC-RA-(DM)-nXIIts posterior pathway and the HVC-AreaX-DLM-LMAN-RA anterior pathway (B). A similar but not the same vocal pathway is observed in the budgerigar: the NLC-AAC-(DM)-nXIIts posterior pathway and the AAC-DVMm-NAO-AAC anterior pathway (C). In contrast, such neural circuits for vocal learning are not found in the quail brain (D). A similar neural circuit is found in the brainstems of these species to produce learned or innate vocalization. The red and blue lines indicate anterior and posterior pathways for vocal learning, respectively. The yellow line indicates the general vocalization pathway seen in the brainstems of avian species. AAC: central nucleus of the anterior arcopallium, DLM, dorsal lateral nucleus of the thalamus; DM, dorsal medial nucleus of the midbrain; DMm, magnocellular nucleus of the dorsal thalamus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; NAO, oval nucleus of the anterior nidopallium; NLC, central nucleus of the lateral nidopallium; RA, robust nucleus of the arcopallium; nXIIts, tracheosyringeal hypoglossal nucleus.

The budgerigar cDNA fragments for Rcad (Genbank accession no. AB329583), cad6B (AB329584), and cad7 (AB329582), ring dove cDNA fragments for Rcad (AB610760), cad6B (AB610761), and cad7 (AB610762) were isolated from the adult brain by the reverse transcription-PCR. The same primer sets used for Bengalese finch were used to isolate budgerigar and ring dove cDNAs, as previously described (Matsunaga and Okanoya, 2008a), except for ring dove cad6B. For ring dove cad6B, the primers were used as follows: 5’-CTTGTGTTGCGCTGATGAGA-3’ and 5’-GTTTATAGCCTGGGCACGAA-3’. Each cDNA fragment was inserted into the pGEM-T Easy Vector (Promega, Madison, WI, USA). We used the same plasmids for probe preparation that were used for Bengalese finch brain, as previously described (Matsunaga and Matsunaga and Okanoya).
Okanoya, 2008a). We used plasmids encoding chicken cadherin cDNAs for quail brain, kindly gifted by Dr. Masatoshi Takeichi (Inuzuka et al., 1991; Nakagawa and Takeichi, 1995). The plasmids were digested with enzymes to release the fragments, and probes were synthesized using SP6, T3, or T7 RNA polymerase (Roche, Indianapolis, IN, USA) with digoxigenin (DIG)-labeling mix (Roche).

RESULTS
In this study, we analyzed Rcad, cad6B, and cad7 expression in two vocal learners (Bengalese finch and budgerigar) and non-learner (quail) at two different developmental stages (postnatal 2 and 4 weeks). Since gene expression patterns were similar in most regions between these different developmental stages, we only show expression patterns of P30 brains in figures. Furthermore, we analyzed these cadherin expressions in the second non-learner, ring dove. All comparative gene expression results are summarized in Table 1 (we used the terminology of Reiner et al., 2004).

CADHERIN EXPRESSION IN THE VOCAL SYSTEM
We first examined cadherin expression in the vocal pathways among Bengalese finch, budgerigar, and quail.

Cad6B was widely expressed in the vocal system of the Bengalese finch brain including in the HVC, robust nucleus of the arcopallium (RA), lateral magnocellular nucleus of the anterior nidopallium (LMAN), dorsal lateral nucleus of the thalamus (DLM), and nucleus retroambiguus (RAm) and it was weakly expressed in the tracheosyringeal hypoglossal nucleus (nXIIts; Figures 2B,K, 3B,E, and 4K), as previously described (Matsunaga and Okanoya, 2008a). Cadad was not expressed in these vocal control nuclei (Figures 2A, 3A,D, and 4J), except for DM (Figure 2J). At P30, the LMAN had not been clearly formed yet, but cad6B expression and weak Rcad expression were already seen in the corresponding LMAN region (Figures 4A,B). In the juvenile stage, cad7 was also expressed in

Table 1 | Summary of comparative gene expression among vocal learners (Bengalese finch and budgerigar) and non-learners (quail and ring dove).

|                 | Bengalese finch | budgerigar | ring dove | quail | ring dove | LMAN (quail) | MP | 
|-----------------|----------------|------------|-----------|-------|-----------|--------------|----| 
| vocal system    |                |            |           |       |           |              |    | 
| HVC (NCX)      | +             | -          | -         | -     | -         |              |    | 
| MCL or NLu     | -             | +          | -         | -     | -         |              |    | 
| RA (ACL)       | -             | -          | -         | -     | -         |              |    | 
| Ai              | -             | -          | -         | -     | -         |              |    | 
| DM             | -             | -          | -         | -     | -         |              |    | 
| xVNCs          | -             | -          | -         | -     | -         |              |    | 
| xVNCs          | -             | -          | -         | -     | -         |              |    | 
| Acx (DMN)      | -             | -          | -         | -     | -         |              |    | 
| Str             | -             | -          | -         | -     | -         |              |    | 
| DLM, DMN       | -             | -          | -         | -     | -         |              |    | 
| LMAN (DMN)     | -             | -          | -         | -     | -         |              |    | 
| visual system   |                |            |           |       |           |              |    | 
| Wulb            | -             | -          | -         | -     | -         |              |    | 
| Entopeduncular | +             | -          | -         | -     | -         |              |    | 
| optic tectum   | +             | -          | -         | -     | -         |              |    | 
| GLM             | +             | -          | -         | -     | -         |              |    | 
| Auditory system |                |            |           |       |           |              |    | 
| ANH            | -             | -          | -         | -     | -         |              |    | 
| MOC             | -             | -          | -         | -     | -         |              |    | 
| SO              | -             | -          | -         | -     | -         |              |    | 
| LU              | -             | -          | -         | -     | -         |              |    | 
| NA             | -             | -          | -         | -     | -         |              |    | 
| Ou              | -             | -          | -         | -     | -         |              |    | 
| field L, NCM   | +             | -          | -         | -     | -         |              |    | 
| GNM            | -             | -          | -         | -     | -         |              |    | 
| tegmental system |              |            |           |       |           |              |    | 
| STN             | -             | -          | -         | -     | -         |              |    | 
| PV              | -             | -          | -         | -     | -         |              |    | 
| motor system    |                |            |           |       |           |              |    | 
| GP              | -             | -          | -         | -     | -         |              |    | 
| LG              | -             | -          | -         | -     | -         |              |    | 
| vestibular system |            |            |           |       |           |              |    | 
| VII             | -             | -          | -         | -     | -         |              |    | 

Note that gene expression differed more among species in vocal-related areas than other areas. Dark blue filled region: highly similar expression pattern among species; light blue filled region: similar but developmentally different expression pattern; light red filled region: different expression between vocal learners; white region: different expression between non-learners.
some vocal control nuclei such as the HVC and RA (Figures 3C,F), although their expression is downregulated during development (Matsunaga and Okanoya, 2008a).

Similar to the Bengalese finch, cad6B and cad7 were expressed in the vocal control nuclei of budgerigar; however, the expression pattern differed. Cad6B was expressed in the nXIIIs, RA, and magnocellular nucleus of the dorsomedial thalamus (DMm), and central nucleus of the lateral nidopallium (NLC; Figures 2E, 3H, and 4N) as in Bengalese finch, but cad6B expression was not observed in the DM, central nucleus of the anterior arcopallium (AAC) and oval nucleus of the anterior nidopallium (NAO; Figures 2N, 3H, and 4E). As in the Bengalese finch, Cad7 was expressed in the nXIIIs, DMm, and
Although no telencephalic vocal control nuclei occur in quail, they have vocal nuclei in the brainstem to produce innate vocalization. Rcad was expressed in the RAm (data not shown) and DM, but not expressed in the nXIIts of the quail brainstem (Figures 2G, P). As cad6B, cad7 expression was observed in the nXIIts (Figures 2H, I), but no cad7 expression was seen in the DM (Figures 2Q, R). Although no vocal nuclei occur in the thalamus NLC, and not expressed in DM (Figures 2C, F, L, O, 3E, H, and 4L, O). However, cad7 was not expressed in the AAC (Figure 3I). Sparse cad7 expression was seen in the NAO (Figures 4C, F). Additionally, some of the vocal control nuclei did not express Rcad in the budgerigar brain (Figures 3G and 4M), but, in contrast to the Bengalese finch, Rcad was expressed in some vocal control nuclei such as the nXIIts, RAm, DM, NLC, and NAO (Figures 2D, M, 3G, and 4D).

NLC, and not expressed in DM (Figures 2C, F, L, O, 3E, H, and 4L, O). However, cad7 was not expressed in the AAC (Figure 3I). Sparse cad7 expression was seen in the NAO (Figures 4C, F). Additionally, some of the vocal control nuclei did not express Rcad in the budgerigar brain (Figures 3G and 4M), but, in contrast to the Bengalese finch, Rcad was expressed in some vocal control nuclei such as the nXIIts, RAm, DM, NLC, and NAO (Figures 2D, M, 3G, and 4D).

![Image](image-url)

**FIGURE 3** | In situ hybridization for Rcad, cad6B, and cad7 at the telecephalic level. (A–F) Cadherin expression in P30 male Bengalese finch in the RA (A–C) and HVC (D–F). (G–I) Cadherin expression in P30 male budgerigar in the NLC and AAC. (J–O) Cadherin expression in P30 male quail in the intermediate arcopallium (Ai) and caudolateral nidopallium (NCL). Arrowheads indicate expression boundary between the strong Rcad expression domain and the strong cad6B and cad7 expression domain. Scale bars are 1 mm.
region of the arcopallium (Ai).

Cad6B was expressed in the dorsal region of the intermediate Ai, whereas cad7 was expressed in the dorsal and ventral regions of the Ai (Figures 3B,C). Cad6B and cad7 were weakly expressed in the nidopallium around the HVC, although cad6 expression is upregulated and cad7 expression is downregulated during development (Matsunaga and Okanoya, 2008a; Figures 3E,F).

The expression patterns in the vocal control nuclei and their surrounding regions were basically similar in budgerigar brain, as in the Bengalese finch. For example, no clear expression border was found between the NLC vocal control nucleus and the supracentral nucleus of the lateral neostriatum (NLs; data not shown), the surrounding area of NLC (Plummer and Striedter, 2002). However, in contrast to Bengalese finch, the expression pattern in the NAO and AAC was quite different in the budgerigar brain. Cad7 expression

of quail brain, a corresponding area exists. Rcad was expressed, but cad6B was not expressed, in the dorsolateral area of the thalamus (Figures 4P,Q). This expression pattern was different to that in the Bengalese finch and budgerigar (Rcad was not expressed but cad6B was expressed in both species), though cad7 expression was similar (Figures 4L,O,R).

CADHERIN EXPRESSION IN THE REGION SURROUNDING THE VOCAL SYSTEM

Consistent with the idea that avian vocal learning system might be evolved from neighboring motor learning system (Feenders et al., 2008), cadherin expression patterns in the vocal control nuclei were similar to those in surrounding regions of Bengalese finch, although the expression level and density were different (Matsunaga and Okanoya, 2008a; Table 1). The RA was located in the dorsomedial region of the arcopallium (Ai). Cad6B was expressed in the dorsal region of the intermediate Ai, whereas cad7 was expressed in the dorsal and ventral regions of the Ai (Figures 3B,C). Cad6B and cad7 were weakly expressed in the nidopallium around the HVC, although cad6 expression is upregulated and cad7 expression is downregulated during development (Matsunaga and Okanoya, 2008a; Figures 3E,F).

The expression patterns in the vocal control nuclei and their surrounding regions were basically similar in budgerigar brain, as in the Bengalese finch. For example, no clear expression border was found between the NLC vocal control nucleus and the supracentral nucleus of the lateral neostriatum (NLs; data not shown), the surrounding area of NLC (Plummer and Striedter, 2002). However, in contrast to Bengalese finch, the expression pattern in the NAO and AAC was quite different in the budgerigar brain. Cad7 expression
was detected in the medial region of the Ai, but its expression was absent in the AAC (Figure 3J). Cad6B was expressed in the frontal nidopallium, and both cad6B and cad7 were expressed in the Ai. However, the expression domains were mostly separated (Figures 3J-L); Cad6B was expressed in the dorsal region, whereas cad7 was expressed in the ventral region. Whereas Rcad was weakly expressed in the caudolateral nidopallium (NCL) of the quail brain, cad6B and cad7 were expressed in the NCL, although their expression domains were generally separated, with some overlap; Cad6B was expressed in the ventral region, whereas Cad7 was expressed in the dorsal region of the NCL (Figures 3M-O). In addition, no clear cad6B expression was observed in the frontal region of the nidpallium (NF; Figure 4H), though Rcad expression was similar to vocal learners (Figure 4G). Only sparse Cad7 expression was seen in this region (Figure 4I).

**Cadherin expression in the visual system**

In avian species, the visual information is transmitted from the retina to the telencephalon via two distinct pathways. One is the tectofugal pathway via the tectum, nucleus rotundus (Rt) and entopallium (Ent; Benowitz and Karten, 1976), and the other is the thalamofugal pathway via dorsal lateral geniculate nucleus (GLd) and visual wulst (Shimizu and Karten, 1993). Since these pathways are seen in various avian species (Mey and Thanos, 2000; Heyers et al., 2007), it appears that the visual system is evolutionally conserved among all avian species. Hence, it was suspected that gene expression in visual nuclei would be tightly constrained. To examine this possibility, we next analyzed cadherin expression in these visual areas.

**Figure 5** Cadherin expression in the visual system at the level of the tectum and pretectum. (A-I) In situ hybridization for Rcad (A,D,G), cad6B (B,E,H), and cad7 (C,F,I) in the Bengalese finch (A-C), budgerigar (D-F), and quail (G-I) in the caudolateral tectum. Cadherins are strongly expressed in layer c, g and i of the stratum griseum et fibrosum superficiale (sfgs) and the stratum griseum centrale (sgc). (J-R) In situ hybridization for Rcad (J,M,P), Cad6B (K,N,Q), and Cad7 (L,O,R) of Bengalese finch (J-L), budgerigar (M-O), and quail (P-R) in the nucleus pretectalis (PT). Scale bars are 250 μm (I) and 500 μm (L,O,R).
The *Rcad*, *cad6B*, and *cad7* expression patterns were basically similar among the three species. *Rcad* was expressed in the tectum, nucleus pretectalis (PT), GLd, Ent, and visual wulst and was sparsely expressed in the Rt (Figures 5A,D,G,J,M,P and 6A,D,G,J,M,P). *Cad7* was expressed in the tectum, GLd, and visual wulst and sparsely expressed in the Rt, whereas *cad7* was not expressed in the PT or Et (Figures 5C,F,I,L,O,R and 6C,F,I,L,O,R). *Cad6B* expression patterns were also basically similar, although the expression pattern in the Et was exceptionally different (Figures 5B,E,H,K,N,Q and 6B,E,H,K,N,Q).

**CADHERIN EXPRESSIONS IN THE AUDITORY SYSTEM**

In the avian species, the auditory information is transmitted from the inner ear to telencephalic auditory areas Field I via a series of auditory nuclei such as the cochlear nucleus (CN), superior olivary nucleus (SO), the intermediate lateral lemniscal nucleus (LLi), lateral mesencephalic nucleus (MLd), and nucleus ovoidalis (Ov). These ascending auditory pathway is seen both in vocal learners and non-learners (Correia et al., 1982; Mello et al., 1998). However, in contrast to the visual system, the auditory system is related to the vocal control sys-

**Figure 6** | Cadherin expression in the visual system at the level of the thalamus and telencephalon. (A–I) In situ hybridization for *Rcad* (A,D,G), *cad6B* (B,E,H), and *cad7* (C,F,I) in the Bengalese finch (A–C), budgerigar (D–F), and quail (G–I) in the nucleus rotondus. (J–R) In situ hybridization for *Rcad* (J,M,P), *Cad6B* (K,N,Q), and *Cad7* (L,O,R) in the Bengalese finch (J–L), budgerigar (M–O), and quail (P–R) in the Entopallium. Scale bars are 1 mm.
tem, particularly in the telencephalon (Bauer et al., 2008). Therefore, it is possible that cadherin expression in the auditory system would differ to some extent among species. As expected, cadherin expression in the auditory system was different in some parts of the brain.

In the CN, though the cadherin expression pattern in the magnocellular sub-nucleus (MCC) varied among the three species (Figures 7A–I), Rcad, cad6B, and cad7-expressed cells were distributed in the angular sub-nucleus (AN) of the three species (data not shown). The cadherin expression pattern was similar in the SO and LLi (Figures 7J–R and 8J–R). However, in the MLD and Ov, the cadherin expression patterns differed among species (Figures 2J–R). In the telencephalic auditory area, Field L, the caudomedial mesopallium (CMM), cadherin expression patterns were diverse (Table 1).

CADHERIN EXPRESSION IN THE TRIGEMINAL SYSTEM

Among trigeminal nuclei, we analyzed cadherin expression in the principal sensory nucleus of the pons (PrV), the nucleus et tractus descendens nervi trigemini (nTDV), and the basorostral pallial nucleus (Bas). PrV neurons project to the Bas to regulate tongue and beak movement (Wild et al., 1984, 1997). The Bas receives trigeminal input from the PrV and auditory input from the LLi (Farabaugh and Wild, 1997). Auditory input from the Bas is connected with vocal control nuclei via the NF in the budgerigar (Farabaugh and Wild, 1997), but is not connected in songbirds (Wild and Farabaugh, 1996). Therefore, the trigeminal system seems to differ among the three species, and in the case of the budgerigar, this system is connected to the vocal control system.

As expected, cadherin expression in the PrV and Bas were different in some extent (Figures 9A–R), whereas Rcad, cad6B, and cad7 expression patterns were similar among the three species in the nTDV (data not shown). At P30, although cad6B expression in the Bas was similar in all species, Rcad was expressed in the budgerigar and quail and cad7 was only expressed in the budgerigar (Figures 9J–R).

CADHERIN EXPRESSIONS IN OTHER SYSTEM

In telencephalic motor nuclei such as GP and LSt, cadherin expressions were similar among three species (Figures 10). In vestibular system, nucleus vestibularis medialis (VeM) and lateral nucleus of cerebellum (Cbl), cereberum nucleus, cadherin expressions were also similar (data not shown).
DEVELOPMENTAL DIFFERENCE IN CADHERIN EXPRESSIONS

By comparing gene expressions among three species, it seemed that cadherin expressions were diverse particularly in the vocal and auditory systems. However, it was suspected that these differences did not reflect phylogenetic differences among three species but differences in developmental progress among three species (e.g., budgerigars develop more slowly than Bengalese finches). To examine the possibility, we compared gene expressions between two different developmental stages P14 and P30.

As expected, some cadherin expressions were upregulated or downregulated in some brain areas of the same species between P14 and P30 (Figure 11; summarized in Table 1). By comparing between these different stages, cadherin expressions were particularly variable in the auditory areas during the development (Table 1). Difference in these cadherin expressions among three species may be due to differences in developmental progress rather than phylogenetic differences (summarized in Table 2).

CADHERIN EXPRESSION IN ANOTHER NON-LEARNER RING DOVE

By comparing among two vocal learners (Bengalese finch and budgerigar) and an non-learner quail, we found diverse cadherin expressions in the vocal system. However, if cadherin expressions differ between two different non-learners, the differences might have been caused by evolutional changes unrelated to vocal learning. To exclude the possibility, we also examined cadherin expressions in another non-learner, ring dove.

Some cadherin expressions were different between quail and ring dove [Rcad in nXIIIs (Figures 2G and 12A), DM (Figures 2P and 12D), and DLM (Figures 4P and 12M); cad6B in NF (Figures 4H and 12Q), DLM (Figures 4Q and 12N), GLd (Figures 6H and 13E), and PrV (Figures 9H and 13N); cad7 in the second auditory areas]. However, most cadherin expression patterns were similar between two species (Figures 2H,I,Q,R, 3J–O, 4G,I,R, 5P–R, 6G,I, 7G–I, 8G–I, 9G,I, 10G–I, 12B,C,E–L,O,P,R and 13A–D,F–M,O–R).

DISCUSSION

HIGHLY DIVERSE CADHERIN EXPRESSIONS IN THE VOCAL CONTROL SYSTEM

We performed a comparative gene expression analysis among two vocal learners, the Bengalese finch (oscine songbirds) and budgerigar (parrots), and non-vocal learners, the quail (Galiformes) and ring dove (Columbiformes). Both vocal learners had a similar but not the same series of telencephalic nuclei and neural circuits specialized for vocal learning and production, whereas non-learners had no such structure in the telencephalon (Bolhuis and Gahr, 2006). Consistent with diverse vocal system among avian species, cadherin expressions were highly variable, not only in the vocal telencephalic nuclei, but also in the vocal nuclei at the level of the brainstem. In contrast, the visual system may be more tightly conserved and less variable than the vocal system during evolution. Actually, cadherin expression in the visual system was almost similar among all species.

Diverse cadherin expressions in the vocal system were also seen between two non-learners quail and dove. The ratio of different cadherin expressions in brainstem vocal areas between these species were almost similar to the ratio of different cadherin expressions between two vocal learners (see Table 2), suggesting that diverse cadherin expressions are not vocal learner-specific and change of cadherin expressions may not have been related to the acquisition of vocal learning ability itself, rather, related to diversity in vocal behaviors among avian species.
Some cadherin expression patterns change during development. For example, cadherin expression was not the same between chick embryos (Redies et al., 2001) and postnatal quail in the present study. Cad7 downregulation and Cad6B upregulation are observed in the RA nucleus during the transition from the sensory to sensorimotor learning stage (Matsunaga and Okanoya, 2008a). In this study, to verify the possibility that some gene expression differences were caused by developmental differences among species, we used juvenile birds at two developmental stages to perform the comparative gene expression analysis. We found cadherin expressions were changed in some areas between two different developmental stages of the same species (light blue region, Table 1). However, in the vocal system, cadherin expressions differed in many areas among different species, even their expressions were similar between two different developmental stages of the same species (dark blue region, Table 1), suggesting that these gene expression differences were caused by species difference rather than developmental differences. Thus,
Since the vocal system is closely related to auditory system (Margoliash et al., 1994; Fortune and Margoliash, 1995; Mello et al., 1998; Bauer et al., 2008) and vocal learning is auditory-dependent (Konishi, 1965), changes of gene expression in auditory system might have been related to species differences in vocal learning and behavior. Actually, our results showed that cadherin expressions were diverse in auditory nuclei such as MLd, Ov, telencephalic auditory area. In avian species, there are two auditory systems: primary auditory pathway via thalamus and secondary auditory pathway without thalamic relay via Bas. Songbirds only use the former, while parrots use both auditory pathways (Wild and Farabaugh, 1996; Farabaugh and Wild, 1997). Thus, not only the vocal system but also the auditory system are different between songbirds and parrots. In accordance with this, some cadherin expressions were different in the Bas between Bengalese finch and budgerigar. However, comparing between different stages, cadherin expressions were diverse between the early and late developmental stages in Bengalese finch and budgerigar, and it seems that these differences were mainly due to ontogenic differences rather than phylogenetic differences (see Table 2). In contrast, comparing between two non-learners quail and dove, expression patterns were similar between two different developmental stages, though their expressions differed between these species. Some phylogenetic variations in cadherin expressions may also exist in the non-learner’s auditory system.

In contrast to vocal and auditory system, cadherin expressions were less variable in the visual, trigeminal, motor, and vestibular system (see Table 2). Compared to vocal and auditory system,
Songbirds, parrots, and hummingbirds have neural circuits for vocal learning. Because these birds are taxonomically distantly related, it has been suggested that these families acquired vocal learning ability independently (Jarvis, 2000, 2004). Anatomical and molecular analyses have suggested the possibility that each vocal system evolved from general primordial structures (Margoliash et al., 1994; Matsunaga and Okanoya, 2011).

**FIGURE 12 | Cadherin expressions in vocal areas of the brainstem and corresponding areas of ring dove to telencephalic vocal areas.** In situ hybridization for Rcad (A,D,G,J,M,P), Cad6B (B,E,H,K,N,O), and cad7 (C,F,I,L,D,R). Cadherin expressions in nXIIts (A–C), DM (D–F), NCL (G–I), Ai (J–L), DLM (M–O), and NF (P–R). Scale bars are 1 mm.

**DIVERSE CADHERIN EXPRESSION AND EVOLUTION IN THE VOCAL LEARNING SYSTEM**

Songbirds, parrots, and hummingbirds have neural circuits for vocal learning. Because these birds are taxonomically distantly related, it has been suggested that these families acquired vocal learning ability independently (Jarvis, 2000, 2004). Anatomical and molecular analyses have suggested the possibility that each vocal system evolved from general primordial structures (Margoliash et al., 1994; Matsunaga and Okanoya, 2011).
Cadherins, originally isolated as cell-adhesion molecules, are involved not only in synapse formation, but also in synapse function. For example, cad8 mutant mice show reductions in miniature excitatory postsynaptic currents in temperature-sensitive neurons (Suzuki et al., 2007). Cad11 mutant mice show enhanced long-term potentiation in CA1 neurons (Manabe et al., 2000). Cadherin overexpression by lentiviral vectors affects vocal learning and production (Matsunaga and Okanoya, 2008c).

In vitro analysis using rat hippocampal Mello et al., 1998; Farries, 2001; Feenders et al., 2008; Matsunaga et al., 2008; Matsunaga and Okanoya, 2009b). Actually, some expressed genes are similar in the vocal systems of songbirds and parrots. The androgen receptor, a transcription factor, is widely expressed in vocal control nuclei of the budgerigar as well as in songbirds (Matsunaga and Okanoya, 2008b). The axon guidance molecules neuropilin-1 and plexin-A4 show similar expression patterns between the Bengalese finch and budgerigar (Matsunaga and Okanoya, 2009a). In contrast, cadherin expression in the vocal system is highly diverse between songbirds and parrots. Cadherins, originally isolated as cell-adhesion molecules, are involved not only in synapse formation, but also in synapse function. For example, cad8 mutant mice show reductions in miniature excitatory postsynaptic currents in temperature-sensitive neurons (Suzuki et al., 2007). Cad11 mutant mice show enhanced long-term potentiation in CA1 neurons (Manabe et al., 2000). Cadherin overexpression by lentiviral vectors affects vocal learning and production (Matsunaga and Okanoya, 2008c). In vitro analysis using rat hippocampal

FIGURE 13 | Cadherin expressions in non-vocal areas of ring dove. In situ hybridization for Rcad (A–D,G,J,M,P), cad6B (E,H,K,N,O), and cad7 (C,F,L,O,R).

Cadherin expressions in PT (A–C), Rt (D–F), MCC (G–I), Ov (J–L), PrV (M–O), and GP (P–R). Scale bars are 1 mm (F,I,L,O,R), and 500 μm (C).
Table 2 | Summary of species difference in cadherin expressing areas.

| Species difference in cadherin expressing areas |
|-----------------------------------------------|
| Vocal learners | Non-learners |
|----------------|--------------|
| Telencephalic vocal areas | 5/12 (42%) | – |
| Surrounding areas | 0/12 (0%) | – |
| Vocal areas in the brainstem | 2/12 (17%) | 1/12 (8%) |
| Visual areas | 1/18 (6%) | 0/18 (0%) |
| Auditory areas | 0/27 (0%) | 5/27 (19%) |
| Other areas | 1/21 (5%) | 1/21 (5%) |
|                | 2/21 (10%) | 0/21 (0%) |

The value shown in bold indicates the percentage of species difference in cadherin expressions that may be due to phylogenetic differences, and the value shown in light letters indicates the percentage of species difference that may be due to developmental stage differences among species. Note that diverse cadherin expressions are prominent in vocal and auditory areas.

culture neurons revealed that cad6 increased but cad7 decreased the number of spines and frequency of miniature excitatory post synaptic current (mEPSC; our unpublished data). Therefore, the diversity of cadherin expression may modulate neuronal activity and plasticity to generate diversification in the processes of vocal learning and production. Glutamate receptors also showed diverse expression in the vocal system among three vocal learners (Wada et al., 2004). It appears that transcription factors induce the expression of various genes involved in synapse formation of vocal control nuclei, and subsequently, that the differential expression of synaptic genes such as neurotransmitter receptors and cadherins modulate neuronal activity and plasticity in vocal control nuclei to create species-specific vocal behaviors and evolution.

Many vocal control-area-related genes have been recently identified in the zebra finch and Bengalese finch using comprehensive cDNA chip gene expression analysis (Wade et al., 2004; Lombardino et al., 2005; Wada et al., 2006; Li et al., 2007; Lovell et al., 2008; Replogle et al., 2008; Kato and Okanoya, 2010), and the genomic sequence of the zebra finch has been recently published (Warrant et al., 2010). Using this new technology and knowledge, similarities and differences among vocal learners and non-learners will become clear, and the molecular mechanisms of avian vocal system evolution will be elucidated.

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