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EXPRESSION OF REELIN, ITS RECEPTORS AND ITS INTRACELLULAR SIGNALING PROTEIN, Disabled1 IN THE CANARY BRAIN: RELATIONSHIPS WITH THE SONG CONTROL SYSTEM

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Abstract—Songbirds produce learned vocalizations that are controlled by a specialized network of neural structures, the song control system. Several nuclei in this song control system demonstrate a marked degree of adult seasonal plasticity. Nucleus volume varies seasonally based on changes in cell size or spacing, and in the case of nucleus HVC and area X on the incorporation of new neurons. Reelin, a large glycoprotein defective in reeler mice, is assumed to determine the final location of migrating neurons in the developing brain. In mammals, reelin is also expressed in the adult brain but its functions are less well characterized. We investigated the relationships between the expression of reelin and/or its receptors and the dramatic seasonal plasticity in the canary (*Serinus canaria*) brain. We detected a broad distribution of the reelin protein, its mRNA and the mRNAs encoding for the reelin receptors (VLDLR and ApoER2) as well as for its intracellular signaling protein, Disabled1. These different mRNAs and proteins did not display the same neuroanatomical distribution and were not clearly associated, in an exclusive manner, with telencephalic brain areas that incorporate new neurons in adulthood. Song control nuclei were associated with a particular specialized expression of reelin and its mRNA, with the reelin signal being either denser or lighter in the song nucleus than in the surrounding tissue. The density of reelin-immunoreactive structures did not seem to be affected by 4 weeks of treatment with exogenous testosterone. These observations do not provide conclusive evidence that reelin plays a prominent role in the positioning of new neurons in the adult canary brain but call for additional work on this protein analyzing its expression comparatively during development and in adulthood with a better temporal resolution at critical points in the reproductive cycle when brain plasticity is known to occur.

Key words: reelin, Dab1, songbirds, neuroplasticity, *in situ* hybridization, immunocytochemistry.

The acquisition and production in oscine songbirds of learned, often complex, vocalizations is regulated by a set of neural structures often referred to as the song control system (Nottebohm, 1980a; Brenowitz et al., 1997; Nottebohm, 2005). This neural system includes at least two major circuits: a caudal motor pathway necessary for the acquisition and production of learned song (Nottebohm et al., 1976; Yu and Margoliash, 1996) and a rostral pathway necessary for the imitation of an external model but not necessary for its production (Bottjer et al., 1984; Brainard and Doupe 2000; Doupe et al., 2004). The caudal motor pathway originates in HVC (used as a proper name, Reiner et al., 2004), from where it projects to the nucleus robustus arcopallialis (RA), itself connected directly and indirectly with the neurons of the tracheosyringeal part of the hypoglossal nucleus (nXIIts) that innervate the syrinx (vocal organ of birds); in addition, RA innervates brainstem nuclei that control respiration. The anterior pathway also originates in HVC, from where it projects to area X of the medial striatum, that in turn projects to the medial part of the dorsolateral thalamic nucleus (DLM), and from there to the lateral part of the magnocellular nucleus of the anterior nidopallium (LMAN) that projects back to RA (Nottebohm et al., 1976, 1982; Okuhata and Saito, 1987; Bottjer et al., 1989).

Some of the nuclei in this system exhibit in adulthood considerable anatomical plasticity (Ball, 1999; Tramontin and Brenowitz, 2000). In many songbird species that live in the temperate zones, the occurrence of singing and the stereotypy of singing vary seasonally, often being maximal in the spring, when territories are first acquired, followed by pair formation. The volume of song control nuclei HVC and RA varies in parallel with these changes in singing. For example, HVC and RA are twice as large in early spring as during the fall (Nottebohm, 1981). The changes in RA may result in part from hormone-dependent changes in dendritic length, synapse numbers, cell size and spacing

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Abbreviations: A, arcopallium; ApoER2, apolipoprotein E receptor 2; CO, chiasma opticum; CoA, anterior commissura anterior; CoS, commisuralis septi; Dab1, Disabled1; DLL, nucleus dorsolateralis anterior thalami, pars lateralis; DSV, decussatio supraoptica dorsalis; E, entopallium; FA, tractus fronto-arcopallialis; G, globus pallidus; HA, hyperpallium apicale; HD, hyperpallium densocellulare; ICo, mesencephalic nucleus intercollicularis; ir, immunoreactive; LAD, lamina arcopallialis dorsalis; LaM, lamina mesopallialis; LMAN, lateral part of the magnocellular nucleus of the anterior nidopallium; LPS, lamina pallio-subpallialis; M, mesopallium; MLd, nucleus mesencephalicus lateralis, pars dorsalis; N, nidopallium; NIII, nervus oculomotorius; NMDA, *N*-methyl-D-aspartate; NR2B, NMDA receptor 2B subunit; PAG, periaqueductal gray; PBS, phosphate buffer saline; PBST, phosphate buffer saline containing 0.2% Triton X-100; RA, nucleus robustus arcopallialis; SGC, stratum griseum centrale; SGFS, stratum griseum et fibrosum superficiale; SL, nucleus septalis lateralis; SN, substantia nigra; StM, striatum mediale; T, testosterone; TeO, optic tectum; TrSM, tractus septopallio-mesencephalicus; UVa, nucleus uvaeformis; VLDLR, very low density lipoprotein receptor; VTA, ventral tegmental area.

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(DeVoogd and Nottebohm, 1981; Canady et al., 1988; Brenowitz, 2004; Thompson and Brenowitz, 2005). The volume changes in HVC are accompanied by the death and subsequent replacement of some of its cells (Kirn and Nottebohm, 1993; Kirn et al., 1994; Tramontin and Brenowitz, 2000), a phenomenon that does not occur in RA. New neurons continue to be added, too, to the area X of adult songbirds (Lipkind et al., 2002) though seasonal changes in neuronal recruitment were not observed in studies designed to explain the cellular basis of seasonal variation in area X volume in song sparrows (Thompson and Brenowitz 2005).

The birth, migration, recruitment into existing circuits and replacement of neurons in adult brain (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984; Alvarez-Buylla and Nottebohm, 1988; Kirn et al., 1994) and their molecular underpinnings remain poorly understood. It seems reasonable, though, to suppose that molecular agents that control these events developing in mammals might also be expressed in parts of the adult avian brain that engage in neurogenesis and the recruitment and replacement of neurons. We showed recently that the protein doublecortin, which is expressed in post-mitotic neurons and, as part of the microtubule machinery, is required for neuronal migration, is still expressed at high levels in the adult canary (*Serinus canaria*) telencephalon (Boseret et al., 2007), but at much lower levels in the adult mammalian brain (des Portes et al., 1998; Couillard-Despres et al., 2005) where adult neurogenesis is more restricted.

Similarly, the glycoprotein reelin seems broadly expressed in the brain of adult starlings (*Sturnus vulgaris*) (Absil et al., 2003) suggesting that it may play a role in the positioning of migrating neuroblasts (e.g. in HVC where the protein is expressed at relatively high density) but also that it should be implicated in other functions since expression is also detected in areas that are not known to incorporate new neurons in adults. The focus of studies of reelin expression in mammals has been on the critical role it plays during the embryonic period (Schiffmann et al., 1997; Bar et al., 2000). In mammals, reelin is secreted by several different classes of cells, including in particular the Cajal-Retzius cells in the marginal cortical area that produce the protein during development of the cortex, hippocampus and dentate gyrus (Forster et al., 2006). A deficit in reelin secretion produces in humans a major malformation of the cortex called lissencephaly characterized by the absence of folds at the surface of the brain (Hong et al., 2000). Reelin-deficient (reeler) mice similarly show major cytoarchitectonic abnormalities in the CNS, as well as marked neurological dysfunctions including ataxia, tremors and imbalance (D'Arcangelo et al., 1995; Tissir and Goffinet, 2003; Forster et al., 2006). Based on these and other observations, it has been hypothesized that reelin plays a significant role in the control of neuronal migration and determination of the final position of neurons in the developing brain, perhaps even acting as a cellular "stop" signal (Pearlman and Sheppard, 1996). This led us to investigate whether reelin is also expressed in the adult canary brain, in particular in areas that incorporate new neurons, and if so to determine whether reelin expression relates in any way with the localization and numbers of new neurons that are incorporated.

Reelin is secreted in the extracellular space and reelin-signaling is then mediated through its binding to two types of membrane-associated receptors, the very low density lipoprotein receptor (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) expressed at the surface of migrating neurons and of radial glial cells (Forster et al., 2006). When occupied, VLDLR and ApoER2 associate with the intracellular protein Disabled1 (Dab1). This induces the phosphorylation of Dab1 and triggers an intracellular signaling cascade that finally causes the neurons to assume their final shape and location. The importance of this signaling pathway is confirmed by the observation that the inactivation of Dab1 or the double inactivation of VLDLR and ApoER2 results in the same neurological phenotype as reelin deficiency (see (Forster et al., 2006) for review).

As would be expected from its role in neuronal positioning, in the rodent brain reelin expression during ontogeny is generally correlated with the anatomical differentiation of the CNS (Schiffmann et al., 1997). Later in postnatal life, there is evidence that reelin continues to be expressed and takes on other functions (Fatemi, 2005a). For example, it has been shown to stimulate dendrite development (Niu et al., 2004) and to modulate synaptic plasticity by enhancing LTP (Weeber et al., 2002). Reelin also modifies *N*-methyl-D-aspartate (NMDA) and dopamine function (Qiu et al., 2006; Matsuzaki et al., 2007). In addition, reelin has been implicated in a number of psychiatric diseases such as bipolar psychotic disorder and schizophenia (Guidotti et al., 2000).

Reelin expression has been previously detected in the brain of a number of avian and reptilian species (Bernier et al., 1999, 2000; Goffinet et al., 1999; Absil et al., 2003; Li et al., 2007) indicating that this protein has been conserved during evolution. Its structure is also similar across species as attested by the observation that a same antibody detects this protein in the brain of various species belonging to all classes of vertebrates from fishes to mammals (see (Absil et al., 2003) for discussion). Comparative studies of the role of reelin and Dab1 in neural development in a range of species including chicks lead to the conclusion that its developmental function for the vertebrate brain is highly conserved (Bar et al., 2000). Indeed Bar et al. (2000) argue that despite the fact that there are significant taxonomic differences among reptiles, birds and mammals in cortical/pallial organization and the developmental pattern of cellular migration that the fundamental role of reelin and Dab1 for architectonic development is the same in these different lineages (Bar et al., 2000). Given its role in brain development and plasticity, we investigated here the possible relationships between the expression of reelin and/or its receptors and the dramatic seasonal plasticity that has been extensively characterized in the canary brain (Nottebohm, 1981; Brenowitz, 2004). The specific goals of the present experiments were therefore: 1) to verify the presence of the reelin protein and mRNA and its neuroanatomical distribution in relation to the sites such as the