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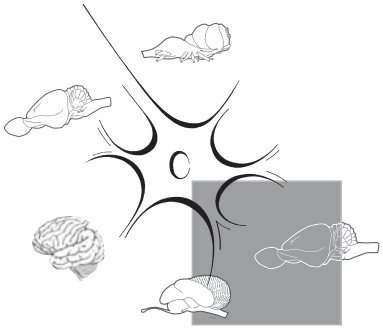

Doublecortin Is a Highly Valuable Endogenous Marker of Adult Neurogenesis in Canaries

Commentary on Vellema M et al. (2014): Evaluating the predictive value of doublecortin as a marker for adult neurogenesis in canaries *(Serinus canaria)*. J Comparative Neurol 522:1299–1315

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Introduction

Doublecortin (DCX) has emerged as an important marker of neurogenesis for comparative neurobiologists. In a recent paper published in the *Journal of Comparative Neurology*, Vellema et al. [2014] questioned the validity of DCX expression as a reliable marker of adult neurogenesis in songbirds (and other vertebrates in general). The authors have some important and useful observations about DCX, but they try to codify these into general rules. This threatens to generate serious doubts in the minds of neurobiologists about the usefulness of DCX as a reliable marker, with their conclusions suggesting to some readers that DCX is not being expressed in relation to neurogenesis in the same way in birds as it is in other vertebrate taxa.

We focus on these conclusions as they might apply to canaries. We contend that the arguments presented are not novel or are based on very limited evidence and generally inconclusive data. We review six observations that Vellema et al. [2014] present to support their contention that DCX is not a reliable marker of adult neurogenesis, and we discuss in a concise manner why, in our opinion, their conclusions are not supported by the facts that they present.

The Neuroanatomical Distribution of DCX Expression Does Not Match the Pattern of Neurogenesis in the Canary Brain

Vellema et al. [2014] report that DCX-expressing cells, based both on immunohistochemistry for the protein and in situ hybridization for the corresponding mRNA, are found throughout the canary brain, with the most pronounced expression in the telencephalon, predominantly in the mesopallium and caudal nidopallium. Based on other methods, this does indeed correspond to brain areas where an active incorporation of new neurons is observed in adult birds. The authors indicate that scattered labeled neurons were also found 'occasionally' in other regions outside the telencephalon (e.g. in the ventral tegmental area, the substantia nigra and the locus ceruleus).

These are not new observations, as this broader distribution was already known from our previous immunohistochemical study in canaries [Boseret et al., 2007]. A similar pattern has also been described in other avian species, and mammalian studies have likewise described DCX in brain regions that are not normally considered to be neurogenic, but might well be, upon closer examination [Kokoeva et al., 2007; Ernst et al., 2014]. These nontelencephalic DCX-expressing cells are rare compared to telencephalic populations, and their staining is usually of a different nature, i.e. weaker and not as crisp (fuzzy) [Boseret et al., 2007].

It has been accepted that DCX in mammals is a marker of young neurons, but that it also labels some cells that are reorganizing their dendritic arbor (another form of plasticity that requires microtubule reorganization and thus DCX expression). It is thus possible that these DCX cells do not represent young newborn neurons, but this conclusion cannot be firmly established at this point in time. Our current understanding of adult neurogenesis in avian and mammalian brains is incomplete, and adult neurogenesis may occur in currently

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unidentified locations [Kokoeva et al., 2007; Ernst et al., 2014]. A broader than expected distribution of neurogenesis in the canary brain is suggested by the fact that Vellema et al. [2014] detected cells labeled by bromodeoxyuridine (BrdU) in subtelencephalic brain regions that are not thought to recruit adult-born neurons [see Vellema et al., 2014: fig. 7B].

Seasonal Changes and Hormonal Effects on DCX Expression Do Not Match Previously Described Changes in Neurogenesis

Vellema et al. [2014] claim that the pattern of DCX distribution in males and females is similar and does not vary across seasons (based on the two examined time points), except in the high vocal center (HVC) and area X. They quantified the area covered by DCX-immunoreactive material in some brain areas, but it is unclear how extensive this quantification was; it appears to have concerned only area X and the surrounding tissue. Furthermore, only the relative expression is reported (with plus and minus signs) and the labeling in the HVC subregions is discussed only qualitatively. Based on these data, they claim that changes in DCX expression in HVC and area X 'did not correlate with known patterns of neuron recruitment'.

Two comments are in order here. First, neurogenesis in the songbird brain is highly variable and is controlled by a multitude of factors (strain, sex, testosterone, photoperiod, singing activity and social environment [Nottebohm, 2008]). The impact of these factors on different aspects of neurogenesis (proliferation at the ventricle and the migration, recruitment, differentiation and survival of neurons) is still largely unknown. The study by Vellema et al. [2014] did not investigate neurogenesis in different groups of birds (e.g. examining different stages in the annual cycle, males vs. females and whether or not they were testosterone-treated) by an independent method, such as BrdU incorporation. This meant it was impossible for them to predict the actual differences in neurogenesis between groups. Claiming that DCX does not correlate with neurogenesis was, therefore, not justified.

Second, the limited quantitative estimates for area X did not take into account the morphology of labeled cells: Vellema et al. [2014] only measured the surface covered by immunoreactive material. There are two morphological types of DCX-immunoreactive cells: the fusiform, (mostly) bipolar cells are probably very young neurons still engaged in the radial migration to their final destination, and the round multipolar cells are presumably older neurons that have begun their differentiation. The temporal changes in numbers of these two cell types are substantially different [Balthazart et al., 2008; Yamamura et al., 2011]. Therefore, conclusions based on analyses that do not differentiate between these cell types seem unjustified.

DCX Is Expressed in Neurons of up to One Year in Age

In a potentially important experiment, Vellema et al. [2014] injected a small number of male canaries with BrdU, and collected the brains 38 (n = 4), 60 (n = 4) and 365 (n = 2) days later in order to analyze the expression of DCX in the BrdU-labeled neurons. It is unfortunate that no information on the physiological state of these adult canaries was presented, since neurogenesis could, for example, be quite different in photosensitive or photorefractory birds [Balthazart et al., 2008]. More importantly, the injection schedule between birds varied (i.e. 6 injections at 4-hour intervals on 1 day or 3 injections/day for 2 days) and it is not stated whether or not these two types of injections were equally distributed across the 10 birds and 3 survival times. In other words, comparisons here seem to concern birds coming from three different experimental protocols that may not be directly comparable. The two patterns of injections in different groups of birds potentially living in different endocrine or social conditions likely resulted in a different degree of initial labeling that could interfere with the conclusions drawn from results observed 38, 60 and 365 days later.

Vellema et al. [2014] conclude from this experiment that DCX is still expressed in some BrdU-positive neurons at 38 and 60 days and that 'even one year after BrdU injections we were still able to detect neurons expressing both BrdU and DCX, albeit sporadically'. No numbers are associated with this last statement (only one such neuron is shown in a photomicrograph), and we are not told where these few neurons expressing DCX after one year are located. A few adult neurons are known to express DCX because they undergo a plastic reorganization of their dendritic arbor [Kremer et al., 2013]. The 'sporadic' expression observed by Vellema et al. [2014] may have been related to this dendritic reorganization. If it concerns just a small fraction of neurons, this observation has little significance.

Vellema et al. [2014] also suggest that their observation of DCX expression in BrdU-positive neurons 38 and 60 days after BrdU injections is a major issue particularly because the percentage of double-labeled cells decreased only slightly between these two time points in the HVC, caudomedial nidopallium (NCM) and area X [see Vellema et al., 2014: fig. 7A]. The total numbers on which these percentages are calculated are not indicated, which makes it difficult to appreciate their significance (the decreases are said to be nonsignificant, but no statistical analysis is presented). If we consider the reported numbers as representative of the entire nuclei, they remain consistent with our previous observations. We found that 10 days after the BrdU injections, approximately 70% of the DCX-positive fusiform cells were BrdU-positive and that this decreased to 30% by 30 days after the injections [Balthazart et al., 2008]. At 30 days, the percentage of round DCX-positive cells increased from 5 to 25%. In addition, the percentage of BrdU-positive cells that expressed DCX did not decrease between 10 and 30 days after the BrdU injections, i.e. 73.38 ± 7.57 and 75.37 ± 13.22%, respectively (unpubl. calculations based on results presented in Balthazart et al. [2008]). Most, if not all, DCX-positive cells in the HVC are thus young neurons labeled by BrdU, even if 100% colabeling cannot be observed due to the limitations of the BrdU method [Taupin, 2007; Barker et al., 2013]. The limited decrease between 38 and 60 days in the percentage of BrdU-positive cells that were simultaneously DCX-positive, which was observed by Vellema et al. [2014], indicates that the duration of DCX expression in neurons in different parts of the brain requires further investigation. This observation does not, however, invalidate the use of DCX as a marker, since the initial degree of colabeling in their study is unknown and is likely to have differed between groups.

DCX Expression Does Not Correlate with BrdU Labeling Equally throughout the Brain

In a second analysis of the brains of the same BrdU-injected subjects, Vellema et al. [2014] estimated the ratio of BrdU-positive

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to DCX-positive cells in several brain areas by dividing the absolute numbers of these two cell types [see Vellema et al., 2014: fig. 7B]. This ratio decreased from 38 to 60 days (consistent with DCX labeling young neurons) and varied from one brain region to another (from 12% in the HVC to <2% in the medial septum and thalamic region DMA), which the authors then claim indicates that DCX cannot predict neurogenesis.

However, the physiological meaning of this ratio is questionable as it was based on single-labeled material and estimated from tissue collected 38 or 60 days after the BrdU injections. The BrdU injections label cells for only a few hours after the actual injection [Barker et al., 2013] and only during DNA replication, whereas DCX labels young neurons continuously. It is therefore quite logical that only a small fraction of DCX-positive cells contain BrdU after 38 or 60 days; most of them were born after the injections (or shortly before). In our previous study, we showed that, of the young (fusiform) neurons arriving in the HVC 10 days after injection, 72% contained BrdU, but that 20 days later, this percentage was already down to 30% [Balthazart et al., 2008]. The low percentages observed here are thus very consistent with our understanding of what DCX and BrdU are measuring in cells.

The differences between brain regions that Vellema et al. [2014] found surprising are to be expected; the migration, recruitment and survival of new neurons in different brain regions are obviously different and take place over different periods of time. In the HVC, for example, half of the new neurons die within a month [Kirn et al., 1999], and their survival depends on the endocrine conditions [Kirn et al., 1994; Rasika et al., 1994; Kirn and Schwabl, 1997] and possibly the social environment, neither of which were described in detail in their paper. The numerator of the BrdU/DCX ratio (number of BrdU-positive cells) is thus expected to differ from one brain area to another. The observations presented [see Vellema et al., 2014: fig. 7B] are therefore consistent with the idea that DCX is a reliable marker of neurogenesis.

DCX Is Expressed in Adult Neurons Expressing the Immediate Early Gene egr-1

Vellema et al. [2014] contend that DCX is expressed in differentiated neurons of the nidopallium because the DCX-positive cells express the neuronal marker NeuN. One such neuron is illustrated [see Vellema et al., 2014: fig. 6]. How frequent is this type of colabeling? They give the figure of 41.3 ± 4.9% for the nidopallium, but what is the basis of this calculation? The percentage of all cells? Was cell type considered, i.e. fusiform or round or both together? How many birds and how many sections were investigated? Is this observation specific to the nidopallium or can a similar observation be made in other brain areas? Contrary to what Vellema et al. [2014] state in their paper, NeuN is not exclusively an adult neuron marker, at least not in mammals. In the mammalian brain, new neurons begin expressing NeuN before they stop expressing DCX [Kempermann et al., 2004; Ernst et al., 2014]. If the same is true in canaries, it would not be surprising to find the degree of colocalization observed by Vellema et al. [2014].

Vellema et al. [2014] report that DCX is expressed in physiologically active neurons, as indicated by the expression of the immediate early gene egr-1. Once again, this observation raises several questions. Was this colocalization specific to the auditory regions (NCM)? What percentage of cells exhibited this pattern of double-labeling? What stimuli induced this expression? Young developing DCX-positive neurons must synthesize a large number of proteins during their differentiation, and it is quite possible that transcription of the corresponding genes is controlled by the immediate early gene egr-1. Some of these proteins may be necessary for neuronal maturation, since in mice, inactivation of the egr-1 gene results in alterations of the neurogenesis process [Veyrac et al., 2013]. In the absence of experimental data demonstrating that egr-1 expression in DCX-positive cells in NCM correlates with neuronal activation by species-specific auditory stimuli, it is difficult to assign a specific meaning to the observation that some DCX-positive cells express egr-1.

DCX Is Expressed in Differentiated Projection Neurons

Finally, Vellema et al. [2014] report that DCX is expressed in neurons that have established functional connectivity and that these have, therefore, presumably been mature for a significant period of time. In three birds, the authors labeled some DCX-expressing neurons in the HVC by injecting a retrograde tracer into the target nucleus robustus arcopallialis (RA) but not into area X. These data are consistent with DCX being a marker of new neurons, since RA-projecting neurons in the HVC are replaced in adulthood whereas area X-projecting neurons are not.

Furthermore, based on available H^3-thymidine or BrdU experiments, it seems that the HVC to RA connections form at around 2 weeks after the neurons become postmitotic, and full connectivity is achieved within a month [Kirn et al., 1999]. It is therefore no surprise that some DCX-positive neurons in the HVC could be labeled afterwards from the RA because DCX is still expressed 1–2 months after the neurons become postmitotic.

Conclusions

Based on these arguments, we see no reason to abandon DCX as a proxy for neurogenesis in the HVC of canaries and possibly in other neurogenic zones of the songbird brain. The report by Vellema et al. [2014] contains interesting results and draws attention to the fact that a marker for a given cell type must always be considered with due caution. We certainly agree that reminding investigators in a particular field of the pitfalls of a particular technique is valuable. However, such critiques should be put in the broader context of the methods available to address a particular question. The fact that there are limitations to a particular method is true for markers of cell lineages (e.g. neurons vs. glia) as well as for markers of the cell cycling stage (e.g. Ki67). DCX as a marker of young new neurons is no exception. It does have major advantages, however; it provides an integrated view of the neuronal proliferation during the last few weeks before brain collection, the separate analysis of fusiform and round cells provides information about cellular events during two different time periods and it requires no injections of exogenous proliferation markers, e.g. BrdU (impossible in some species and some situations). We urge scientists to consider the article by Vellema et al. [2014] with caution and to not abandon a very useful research tool.

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