Published in final edited form as:

J Comp Neurol. 2014 December 15; 522(18): 4100–4120. doi:10.1002/cne.23661.

Endogenous versus exogenous markers of adult neurogenesis in canaries and other birds: Advantages and Disadvantages

Jacques Balthazart¹ and Gregory F. Ball²

¹University of Liège, GIGA Neurosciences, 1 Avenue de l'Hôpital (Bat. B36), Liège, Belgium

²Department of Psychological and Brain Sciences, Johns Hopkins University, Baltimore, Maryland 21218, USA

Abstract

Although the existence of newborn neurons had originally been suggested, but not broadly accepted, based on studies in adult rodent brains, the presence of an active neurogenesis process in adult homoeothermic vertebrates was first firmly established in songbirds. Adult neurogenesis was initially studied with the tritiated thymidine technique, later replaced by the injection and detection of the marker of DNA replication 5-bromo-2'-deoxyuridine (BrdU). More recently, various endogenous markers were used to identify young neurons or cycling neuronal progenitors. We review here the respective advantages and pitfalls of these different approaches in birds, with specific reference to the microtubule-associated protein, doublecortin (DCX), that has been extensively used to identify young newly born neurons in adult brains. All these techniques of course have limitations. Exogenous markers label cells replicating their DNA only during a brief period and it is difficult to select injection doses that would exhaustively label all these cells without inducing DNA damage that will also result in some form of labeling during repair. On the other hand, specificity of endogenous markers is difficult to establish due to problems related to the specificity of antibodies (these problems can be, but are not always, addressed) and more importantly because it is difficult, if not impossible, to prove that a given marker exhaustively and specifically labels a given cell population. Despite these potential limitations, these endogenous markers and DCX staining in particular clearly represent a useful approach to the detailed study of neurogenesis especially when combined with other techniques such as BrdU.

Keywords

Markers of Neurogenesis; Doublecortin; Bromodeoxyuridine; HVC; Thymidine analogues; Neuronal plasticity

Corresponding author: Jacques Balthazart, University of Liège, GIGA Neurosciences, Research Group in Behavioral Neuroendocrinology, Avenue de l'Hôpital, 1 (BAT. B36), B-4000 Liège 1, Belgium, Phone 32-4-366 59 70 -- FAX 32-4-366 59 71 -- jbalthazart@ulg.ac.be.

Conflicts of interest statement

Both authors have no financial or personal relationships with people or organizations that could have influenced their work and the opinions expressed in this review.

Authors contributions

Both authors had full access to all data mentioned in this review and take full responsibility for the integrity of data presented and accuracy of their analysis. JB and GFB wrote the original manuscript and its revision; they both obtained the funding acknowledged in the review.

1. Introduction

Adult neurogenesis is one of the most exciting phenomena to emerge in the field of neuroscience in the last few decades (Gage et al., 2008a). The history and origin of the novel idea that the brain can exhibit plasticity in adulthood via the incorporation of new neurons is somewhat complex, as we will briefly review in the next section of this paper. One important point to note at the outset of this paper is that studies of songbirds played an important role in establishing the phenomenon of adult neurogenesis (Goldman, 2008) and have the potential to play a significant role in the future. Our labs are investigating how environmental and hormonal factors regulate adult neurogenesis in the song system of canaries (Ball et al., 2004; Balthazart et al., 2008; Boseret et al., 2006; Sartor et al., 2005; Yamamura et al., 2011). During the course of our studies we have found that the microtubule-associated protein doublecortin (DCX) serves as a valuable marker of adult neurogenesis in canaries (Balthazart et al., 2008; Boseret et al., 2007; Yamamura et al., 2011). Its use has been adopted by other laboratories with success (Fox et al., 2010; LaDage et al., 2010; LaDage et al., 2011; Melleu et al., 2013; Mezey et al., 2012) although recently a paper by Vellema, Hertel, Urbanus, Van der Linden and Gahr suggested that DCX might not be a reliable and specific marker of adult neurogenesis in birds (Vellema et al., 2014). The Vellema et al. paper however highlights a problem that is more general in the field of neurogenesis and concerns the limitations of tools that are employed to study this phenomenon.

Two types of approaches have been used to analyze neurogenesis. The first one is based on the injection and subsequent detection of exogenous markers of DNA replication such as tritiated thymidine or 5-bromo-2'-deoxyuridine (BrdU). Alternatively, more recently, several endogenous markers of cell cycling or of newborn neurons have been identified and validated to a variable extent so that they could be used to quantify adult neurogenesis without having to inject first an exogenous compound. Both approaches have their advantages but also their problems and limitations that might not be obvious to new investigators as they join the field and attempt to apply in a somewhat naïve manner the diverse histological techniques available for such investigations. Our goal in this paper is therefore to review in a concise manner the different techniques available now to investigate neurogenesis in birds. Special attention will be given to the now broadly used BrdU approach and to a discussion of the usefulness but also limitations of the use of DCX to study neurogenesis in particular in the song system of adult oscine birds. We will argue that limitations associated with the DCX technique are different but not necessarily more important than for BrdU and that no technique in isolation is flawless and provides absolute results. This presentation will therefore highlight the challenges faced by investigators interested in identifying adult neurogenesis and studying its possible regulation of endogenous and exogenous factors. We will start with a brief history to provide a context for our review.

2. History of adult neurogenesis in birds

Studies of the songbird brain galvanized the field of adult neurogenesis in the 1980s. These studies were spearheaded by the efforts of Fernando Nottebohm and resulted directly from his investigations of the neural basis of song behavior (Nottebohm, 2008). Nottebohm was trained as an ethologist and was interested in studying the neural regulation of vocal behavior in songbirds due to the fact that they learn a particular vocalization, their “song”, in a manner akin to how humans learn language (Nottebohm, 1980a). While trying to ascertain whether proprioceptive feedback modulates song development he discovered that the vocal production organ, the syrinx, was neurally controlled in a lateralized fashion in the wasserschlager breed of canaries (*Serinus canaria*). These observations prompted him to investigate how the central nervous system controls the syrinx. This quest in turn resulted in the discovery of a network of brain nuclei now referred to as the song control system, a neural adaptation unique to the songbird suborder that controls the learning and production of song (Nottebohm, 1980a; Nottebohm et al., 1976). The discovery of the song system has engendered a wealth of studies of interest to behavioral neuroscientists of various sorts (Ziegler and Marler, 2008). One dimension relates to the fact that song is a reproductive behavior and is produced in a reproductive context, i.e., when the concentration of steroid hormones in the blood is high. Nottebohm and his student Art Arnold investigated how gonadal steroids such as testosterone regulated the production of song and studied the distribution of sex steroid receptors in the brain of songbirds. They and others discovered that key forebrain nuclei are indeed a target of androgens and in some cases estrogens as well (Arnold et al., 1976; Balthazart et al., 1992; Gahr et al., 1993). Studies of seasonal changes in song behavior engendered studies of whether the song control system change with season. Surprisingly it was discovered that there are marked changes in the volume of key forebrain regions with the song control areas being larger in the spring than in the fall (Nottebohm, 1981). Just prior to the discovery of seasonal changes in the brain, Nottebohm and Arnold reported that these forebrain song nuclei also tended to be larger in volume in males than in females depending on the species investigated (Nottebohm and Arnold, 1976). In adult canaries this sex difference in volume can be reversed to some extent by treating a female with doses of testosterone characteristic of what male’s experience (Nottebohm, 1980b). Thus two contexts were identified when the action of sex steroid hormones could induce marked changes in the size of volumes of song control nuclei, sex and season.

Volume measurements of course only provide gross measures of cellular morphology. They are generally calculated based on the quantification of nuclear volumes based on cytoarchitectonic criteria that are revealed when brain sections are Nissl stained. In the early 1980s, Nottebohm attracted an MD-PhD student to his lab named Steven Goldman who was well versed in developmental neurotechniques. He was fascinated by the marked brain plasticity induced by hormones that Nottebohm had described. Goldman in collaboration with Nottebohm, employed the method of ^3H thymidine a marker that is incorporated into dividing cells to investigate whether increases in brain nucleus volume in adult female canaries that are the result of testosterone treatment are associated with the occurrence of newly born neurons. Surprisingly they found dramatic evidence that new neurons are indeed incorporated into HVC (Goldman and Nottebohm, 1983). However, in this initial study,

HVC volume growth in response to testosterone treatment was not related to a clear increase in the number of new neurons labeled by thymidine but rather to an endothelial and glial proliferation (Goldman and Nottebohm, 1983). Subsequent work has demonstrated though that increases in plasma testosterone concentration are associated with a marked enhancement of neuronal incorporation in HVC (for reviews see (Brenowitz, 2008; Chen et al., 2013; Nottebohm, 2005)).

3. Adult neurogenesis is rediscovered in mammals and multiple markers are established for its study

The songbird brain thus emerged as an influential model for the study of adult neurogenesis. The first claims of widespread adult neurogenesis had been done by Joseph Altman at Massachusetts Institute of Technology (MIT) in the 1960s who found evidence for newly born neurons in the adult hippocampus of rats and guinea pigs (Altman and Das, 1965; 1967). His findings were generally ignored (Gross, 2009). After the initial findings of Goldman and Nottebohm (Goldman and Nottebohm, 1983), Nottebohm and colleagues established that the new neurons are indeed integrated functionally into the brain (Paton and Nottebohm, 1984), that a large fraction of these new cells are projection neurons from HVC to RA (Alvarez-Buylla et al., 1990) and that they develop and migrate using radial glia cells that are, contrary to what is observed in mammal, maintained well into adulthood in songbirds (Alvarez-Buylla and Nottebohm, 1988). These findings provided a strong stimulus for the re-examination of adult neurogenesis in the mammalian brain (Gould and McEwen, 1993; Gould et al., 1999)). This phenomenon was definitively confirmed in rodents by the early 1990s and slightly later in humans (Eriksson et al., 1998) despite much controversy (Gould and Gross, 2002; Gross, 2009; Rakic, 2002a). Though there is some residual skepticism (Gage et al., 2008b), the field is now a vibrant domain of research that has been the object of multiple books and reviews (Gage et al., 2008a; Kemperman, 2011).

4. Exogenous markers of neurogenesis in birds and mammals

4.1. The tritiated thymidine technique

All early studies investigating adult neurogenesis in birds as well as in mammals were based on the quantification of the incorporation of tritiated thymidine into cells replicating their DNA in preparation of the last mitosis of stem cells before they become post-mitotic (Nottebohm, 2008). This proved to be a very useful approach and allowed great advances in the description and understanding of mechanisms underlying the production, incorporation and survival of new neurons in the adult brain. This technical approach was however time consuming and challenging both in terms of producing labeled sections (working with photographic emulsion requires difficult work in a dark room, exposure time of the emulsion to the radioactivity can be quite long) and in terms of quantifying results (counting reduced silver grains over cells and comparing their density to background is very tedious).

4.2. Bromodeoxyuridine

The identification of a thymidine analog, 5-bromo-2'-deoxyuridine (BrdU) that could be injected into an animal, would be incorporated in its replicating DNA and could then be

easily identified by a standard immunohistochemical procedure was therefore a major technological improvement that has allowed neuroscientists over the last few decades to perform more easily a substantial number of studies in a variety of animal species and physiological conditions. Much of what we know today about adult neurogenesis (and in fact neurogenesis in general) was obtained by this method that has consequently become a sort of “gold standard” in the field (Kemperman, 2011; Taupin, 2007).

BrdU labeling of new neurons has indeed many advantages. The technique is cheap, relatively fast and easy to implement, and it does not require the use of radioactivity thus avoiding the associated regulatory challenges and risks. Many antibodies to BrdU are commercially available that have been raised in a variety of host species (e.g., rabbit, mice, rats, sheep, ...) making it quite feasible to combine BrdU detection with the chemical characterization of labeled cells through the visualization of other proteins in the same cells via double-label immunohistochemistry or of the mRNAs of interest by combining BrdU immunohistochemistry with *in situ* hybridization histochemistry procedures. Results derived from the BrdU method can be obtained quite rapidly (e.g., the staining of sections can be completed in 1-2 days) and their quantification is much easier than with ^3H -thymidine. The label concentrates in the cell nuclei where it will remain for several months (even years) if the cell does not undergo additional divisions. Labeled cells can thus be quantified semi-automatically with computer-assisted image analysis.

This is not to say though that this technical approach is devoid of any problems (Gould and Gross, 2002). Firstly, BrdU has a relatively short half-life in living organisms and remains available for incorporation into cells replicating their DNA for a rather limited period of time. This duration has not been determined in many species and under many physiological conditions. Most studies using BrdU to label mitotic cells have assumed a duration of bioavailability of approximately 2 h after an injection, based primarily on studies detecting its clearance in rodents (Kriss and Revesz, 1962; Packard et al., 1973; Staroscik et al., 1964) or on earlier studies measuring clearance of radioactive thymidine (Nowakowski and Rakic, 1974; Rubini et al., 1960). This duration may however be much shorter (see Mandyam et al., 2007) and may well not be consistent across species and physiological conditions; it was for example demonstrated that pregnant rhesus monkeys clear tritiated thymidine more rapidly (Nowakowski and Rakic, 1974) than 2 hours.

Concerning species differences, we recently discovered that in canaries BrdU injected at a dose of 100 mg/kg is no longer available for incorporation into DNA between 30 and 60 min post-injection (see Figure 1). This delay is shorter than what was anticipated from results in rats but not completely unexpected given the higher body temperature and metabolism of birds as compared to mammals (Barker et al., 2013).

These differences could potentially affect the number of cells that will be labeled following a BrdU injection and thus markedly distort the interpretation of the related results. A host of studies have for example identified differences in the number of new neurons labeled with BrdU as a function of the sex or endocrine conditions of the subjects (for recent reviews see: Charalampopoulos et al., 2008; Galea et al., 2006; Schoenfeld and Gould, 2012)). This could reflect true differences in the rate of neurogenesis, but undetected changes in BrdU

clearance from the serum could have the same effect. Similarly, a situation of increased general metabolism such as hyperthyroidism, could also decrease the apparent rate of neurogenesis because the half life of BrdU in the body has been decreased.

Researchers have partially dealt with this potential problem by administering multiple BrdU injections to experimental subjects (typically 4-5 injections over a 12 hr period) to ensure that most if not all replicated DNA would be labeled over a standard period of time. However, it is obvious that, depending of the half-life of BrdU in the specific conditions that are investigated, this multiple injection procedure only provides an incomplete solution.

Various studies have also tried to determine the minimal doses of BrdU that must be injected to label all cells that are dividing at a given time to generate new neurons. Ascertaining this dose has proven very difficult and discordant results have been published to date. For example, it has been suggested that a dose of 100 mg/kg could lead to such an asymptote in the number of labeled cells but other studies indicated that this number still increases up to 200 or even 300 mg/kg (see: Manyam et al., 2007; Cameron and McKay, 2001). Increasing injected doses is however not necessarily the best solution because several studies indicate that large concentrations of BrdU tend to induce DNA damage and that BrdU will then be incorporated in the cells during DNA repair (Taupin, 2007). These cells could then be mistakenly counted as new neurons. A compromise has therefore to be made between labeling only a fraction of the cells that are going to divide and labeling cells involved in DNA repair. This compromise is most often set at a dose of 50 to 100 mg/kg injected once or a few times.

All this being said, the quantification of BrdU incorporation in cells replicating their DNA has been a very valuable tool to study (adult) neurogenesis even if it has some limitations. It probably does not provide **absolute** measures of neurogenesis but when used with appropriate caution, especially paying attention to BrdU peripheral metabolism and brain uptake, it can easily provide accurate and very sensitive **comparative** measures of neurogenesis in the adult brain.

4.3. Other non radioactive thymidine analogs (EdU, IdU, CldU)

More recently a number of alternative thymidine analogs that can be incorporated when cells replicate their DNA and later visualized in histological sections have become available. In the chemical structure of these compounds, the bromide atom of BrdU is replaced by iodine (IdU, 2'-deoxy-5-iodouridine)(Leuner et al., 2009; Llorens-Martin and Trejo, 2011), chloride (CldU, 2'-deoxy-5-chlorouridine)(Leuner et al., 2009; Llorens-Martin and Trejo, 2011) or a small organic molecule in 2'-deoxy-5-ethynyl-uridine (EdU)(Chehrehasa et al., 2009; Warren et al., 2009). These compounds, especially EdU have gained popularity due to a number of obvious advantages including their easy detection in tissue. They can and have now been used as a substitute for BrdU.

However because there is a high chemical similarity of CldU and IdU with BrdU, the anti-BrdU antibodies usually react with these modified nucleosides, although exceptions have been identified (Liboska et al., 2012). As a consequence, it is very difficult to use them simultaneously with BrdU to identify by double-label immunohistochemistry in the same

tissue two different populations of neurons born and thus labeled at different times by injections of BrdU and one of its alternatives. EdU does not have this problem. EdU incorporated into DNA can be visualized by a simple histochemical reaction in which a small fluorescent tag is added to the incorporated EdU, this so-called “CLICK” reaction does not require a specific antibody (Salic and Mitchison, 2008).

This mode of visualization of the EdU thymidine analog has multiple advantages. First, because the staining is based on the attachment of a small fluorophore to the EdU as opposed to a much larger antibody molecule for BrdU, there is no need of denaturing the DNA (e.g., by HCl) to allow access of the fluorophore. The HCl denaturation is known to degrade some of the antigens in the cells therefore preventing its neurochemical characterization by a double-label immunohistochemistry procedure. This problem is not associated with EdU that can easily be combined with other immunohistochemical reactions. EdU visualization can also be combined with a visualization of BrdU provided that the antibody used to visualize BrdU does not cross react with EdU, which is the case for a few but not all anti-BrdU antibodies (Liboska et al., 2012). It is in this way possible to simultaneously follow the fate of two independent populations of neurons that were labeled by injections of BrdU and EdU at different time points.

The combination of BrdU and EdU was recently used to label two cohorts of neurons in the brain of one songbird species, the white-crowned sparrow (*Zonotrichia leucophrys gambelli*). These two cell cohorts could be successfully followed individually despite the cross reaction of the BrdU antibody with EdU by staining two sets of adjacent sections for BrdU and EdU and subtracting the number of cells resulting from the cross-reaction of the anti-BrdU antibody with EdU from the total number of labeled cells to obtain a reasonably accurate estimate of the number of cells that truly contained BrdU (Thatra et al., 2013).

On the downside, EdU staining ends up being more costly than BrdU and users should be aware of the fact that injection of BrdU and EdU in equimolar concentrations will not necessarily label the same number of neurons (Leuner et al., 2009; Llorens-Martin and Trejo, 2011). Whether this relates to a different half-life, different access to the brain or different detection sensitivity of these compounds is not known to our knowledge. This limitation is not a problem when comparing EdU or BrdU labeling across different experimental groups but is of course a serious limitation when trying to compare labeling of two neuronal populations by the two markers.

4.4. General limitations of exogenous markers

All these thymidine analogs have facilitated the completion of very sophisticated investigations of adult neurogenesis in many animal models. As a group, they have however three major problems. Firstly, as discussed before the thymidine analogue injections probably never label all new neurons at a given time given the short half life of these compounds in the blood and the compromise that must be accepted between selecting a saturating high concentration and avoiding as far as possible the BrdU-induced DNA damage.

Secondly, it must be realized that these exogenous markers label all cells replicating their DNA. This therefore includes new neurons in the adult brain but also a variety of other cell types that are dividing including glia, endothelial cells and undifferentiated cells (Taupin, 2007. Rakic 2002b). The assessment of the true number of new neurons thus requires producing and quantifying sections that are double-labeled for thymidine, BrdU or one of their analogs on the one hand and a specific marker of neurons on the other hand (see next section). This can be technically challenging and at the same time labor-intensive and time consuming.

Thirdly, there are a number of situations where it is very difficult if not impossible to perform (multiple) injections of the thymidine analog at a fixed time before brain collection. This is for example the case in studies of free-living animals (e.g. birds) in which (multiple) captures at pre-programmed times are clearly impossible. Analysis of neurogenesis in very large species (e.g., sheep) by this technique is also difficult or at least limited due to the high cost of the large amount of BrdU needed to inject each subject (e.g. at 100 mg/kg, each sheep [75-100 kg] must be injected with 7.5-10 g BrdU; with a decreased dose of 20 mg/kg this still means several g of BrdU/subject (i.e. several hundreds to thousands of euros/subject; see (Migaud et al., 2011)).

Studies of neurogenesis with exogenous markers in the human brain are also not possible because their injection in human beings is ethically not acceptable. BrdU has been used during a brief period for diagnostic purposes in some forms of cancer and this provided material for a few post mortem studies of human brains in subjects that had received BrdU at a variable time before death (Eriksson et al., 1998). Investigators could in this way confirm the existence of some neurogenesis in the human brain but detailed studies of this process are now impossible by this approach.

Neurogenesis in the adult human brain was recently confirmed via quantification of radioactive isotopes (^{14}C) that had accumulated in neurons of people exposed to radiation produced by above-ground nuclear tests that took place between 1945 and 1963 (Kempermann, 2013; Spalding et al., 2013). But besides the extremely high technical difficulty of his approach, this does not represent a viable technique that could be exploited broadly since it is now the laudable goal of international nuclear regulatory agencies that no such above-ground atomic explosion will happen again.

5. Endogenous markers of neurogenesis

Bearing these limitations in mind, another complementary approach was thus progressively developed based on what we will broadly call endogenous markers of neurogenesis. Work in mammals, largely rats and mice, established that stem cells and neurons can be characterized at different stages of their life cycle by the expression of a number of specific proteins. Various types of such markers have been identified in the study of neurogenesis in the mammalian brain (Kempermann et al., 2004; von Bohlen Und Halbach, 2007)(See Figure 2) and they can broadly be divided in 3 categories:

1. Markers of cell cycling that allow the identification of stem cells or progenitor cells that are cycling and replicating their DNA. Examples of these proteins include:

- Ki67, a nuclear protein associated with ribosomal transcription (Bullwinkel et al., 2006). During the interphase, Ki67 immunoreactivity is selectively located in the cell nucleus but during mitosis it is associated with the surface of the chromosomes. Ki 67 is present during all active phases of the cell cycle (G1, S, G2 and mitosis) but it is absent in cells that are not cycling (Go) (Scholzen and Gerdes, 2000).
- The Proliferating Cell Nuclear Antigen or PCNA. This protein is associated with the catalytic activity of DNA polymerase α and is critical to the extension of the DNA strand during replication. PCNA is thus expressed specifically during the cell cycle but absent from inactive cells in Go. However, it remains detectable for several days after cell cycle exit so that use of this marker could thus lead to an overestimation of the duration of the cell cycle and consequently of the rate of neurogenesis (Mandyam et al., 2007).. Importantly PCNA is also expressed in cells repairing their DNA (Essers et al., 2005; Shivji et al., 1992) and therefore cannot be considered, like BrdU, as an absolutely specific marker of cell cycling even if this relative lack of specificity is usually of limited significance.
- The phospho-Histone H3 or pHH3. Histone H3 is one of the five main histone proteins in the structure of chromatin. The protein is phosphorylated at Serine-10 and Serine-28 during mitosis and antibodies are available that selectively recognize the phosphorylated form of the protein, thus making it a specific marker of mitotic cells from the early prophase to metaphase, anaphase and telophase (Hendzel et al., 1997).

Other proteins are also more or less specific markers of stem cells or progenitor cells in the central nervous system without being specific of any specific stage in the cell cycle. This is namely the case for Nestin, the Glial Fibrillary Acidic Protein (GFAP), vimentin or Sox2, the sex determining region Y (SRY)-box 2 (Ming and Song, 2011) but their discussion is beyond the goal of the present review.

2. Markers of neurons such as NeuN, Hu, or Tuj1 that allow one to distinguish these cells from other cell types such as glia that can additionally be identified by other more or less specific markers (GFAP, S-100, ...). Among these neuronal markers, NeuN and Hu are probably those that have been most broadly used.

NeuN which stands for Neuronal Nuclei is a neuronal nuclear antigen that was initially identified based on its reaction with a monoclonal antibody in mice but was later shown to be expressed in most neurons of all vertebrates (Herculano-Houzel and Lent, 2005; Mullen et al., 1992). A few neuronal cell types such as Purkinje cells in the cerebellum of birds (Newman et al., 2010) and olfactory mitral cells or rats are however not labeled by NeuN (Winner et al., 2002). It is broadly accepted that immunoreactivity only becomes apparent in mature neurons after

they stop expressing doublecortin, a marker of young neurons (see below) but this is not always the case as will be discussed in the next sections.

The Hu proteins (HuD, HuC, ...) are family of RNA binding proteins broadly and specifically expressed in neurons of vertebrates (Liu et al., 1995; Szabo et al., 1991). They have been widely used to label neurons in the brain of a variety of species among mammals (Batailler et al., 2014; Bauer et al., 2005; Hourai and Miyata, 2013; Mooney and Miller, 2007) but also in birds (Barami et al., 1995; Marusich et al., 1994; Wakamatsu and Weston, 1997) and fishes (Henion et al., 1996).

Tuj1 is a neuron-specific beta tubulin that is specifically recognized by a monoclonal antibody called Tuj1. Tuj1 is also an early marker of new neurons that can sometimes be detected during the final mitosis of neuronal progenitors (Lee et al., 1990; Memberg and Hall, 1995). Presence of Tuj1 is thus correlated with the presence of neurogenesis in the adult brain.

3. Finally markers of new cells that have become post-mitotic and engaged into the neuronal fate. Hu and Tuj1 are to some extent part of this class but since they can also be expressed by much older neurons, at least in the case of Hu, they cannot really be used to label neurons that have relatively recently become post-mitotic.

Calretinin, a calcium-binding protein involved in calcium signaling is transiently expressed in post-mitotic neurons before they begin expressing another calcium binding protein called calbindin. This protein is however also expressed in other cell types (lung, gastro-intestinal and genital tract, tumor cells) and it is not expressed in all brain areas (Camp and Wijesinghe, 2009; Kempermann et al., 2004). This marker has thus been of limited use. This is also the case for several other markers such as Dlx2 (Ming and Song, 2011) and PSA-NCAM (Zhao et al., 2008) that have been used sporadically in specific contexts. But by far, the most widely used cellular marker for young neurons that have recently become post-mitotic is without any doubt doublecortin, the focus of the present review.

6. Doublecortin as marker of new neurons in mammals

Doublecortin (DCX) is a microtubule-associated protein that binds to microtubules and increases their bundling and stabilization (Horeh et al., 1999). It was identified during studies investigating the molecular bases of a X-linked brain malformation called lissencephaly which involves abnormal cortical lamination due namely to neuronal migration defects (Barth, 1987). As could be expected DCX is very highly expressed during brain development (Couillard-Despres et al., 2005; des Portes et al., 1998; Gleeson et al., 1999) but it was discovered that DCX is also expressed in adulthood in post-mitotic migrating and differentiating neurons (Brown et al., 2003; Francis et al., 1999; Gleeson et al., 1999; Rao and Shetty, 2004). Together with another protein called stathmin, DCX controls the polymerization of the leading process and stabilization of the cytoskeleton during neuronal migration (Bai et al., 2003; Jin et al., 2004; Moores et al., 2004). This mechanism in turn mediates the leading edge extension, nuclear translocation and retraction of the trailing edge in migrating neurons and thus plays a critical role in the positioning of newborn neurons (Curmi et al., 1997; Friocourt et al., 2003).

The duration of DCX expression in young mammalian neurons has not been precisely identified but is generally considered to last approximately one month. It may however vary from one neurogenic zone to another; average expression would be around 20 days in the subventricular zone, rostral migratory stream and olfactory bulb but last up to 30-40 days in the subgranular zone of the hippocampus (Zhao et al., 2008). The duration of DCX expression might also vary from one species to another (Bonfanti and Peretto, 2011). Expression begins in late mitotic neuronal precursors and extends in early postmitotic neurons (Brown et al., 2003). These two populations can however be easily distinguished based on their anatomical localization (ventricle wall vs. brain parenchyma).

Thanks to its widespread expression during a limited period of the life of a young neuron, DCX has during the last 10 years become a widely used proxy for neurogenesis in mammals. A Pubmed search with the term “doublecortin” retrieves 1528 references and when combined with “adult neurogenesis” it still retrieves 555 entries in March 2014, most dated after year 2000. Even if a few of these references discuss the limitations associated with the use of this marker for the study of neurogenesis, their large number nevertheless indicates the high interest associated with its use.

7. Problems associated with the use of endogenous markers of neurogenesis

The endogenous markers described in the last two sections clearly avoid the problems described before for BrdU and other thymidine analogs, but unfortunately they are associated with other ones! The most important one obviously relates to the fact that it is difficult if not impossible to demonstrate that any given marker labels in an exhaustive manner all cells in one category (e.g. all neurons) or at a given stage in their life cycle (mitotic, early postmitotic, fully differentiated). NeuN is for example a very widespread marker of neurons but it has been established that this protein is not detectable in neurons of a few brain areas such as the Purkinje cells in the cerebellum (Mullen et al., 1992; Newman et al., 2010) or neuron of the glomerular layer of the olfactory bulbs (Winner et al., 2002).

When markers of new neurons are concerned, it is obviously difficult to demonstrate that they label ALL neurons that were born during a given time window. This difficulty relates to the fact that exogenous markers of neurogenesis such as BrdU do not themselves label all cells replicating their DNA at a given time due to uncertainties related to the dose that must be injected and duration during which the marker is available for incorporation into cells preparing their next mitosis as discussed before. There is thus no absolute reference to which numbers of new neurons determined by an endogenous marker can be compared. In addition a single injection of BrdU will label cells only during a brief period (one to a few hours) whereas an endogenous marker of young neurons such as DCX will be expressed during several weeks at least. These two types of populations thus cannot be compared.

The specificity of the DCX staining for new neurons can to some extent be evaluated through the analysis of the colocalization of BrdU-positive cells with DCX immunoreactivity. It was for example demonstrated that in the dentate gyrus of rats that have been injected with BrdU for 12 days, 90% of DCX-immunoreactive cells are strongly

positive for BrdU (Rao and Shetty, 2004). DCX is thus a reasonably specific and exhaustive marker of newborn neurons in this region of the adult brain.

This specificity is however not absolute in mammals. DCX expression has been detected in brain areas that are not at present generally accepted as being neurogenic including the striatum (Dayer et al., 2005; Winner et al., 2008), the region of the corpus callosum (Koizumi et al., 2006; Kronenberg et al., 2007) and the piriform cortex (Nacher et al., 2002; Nacher et al., 2001). Some of the cells found in these areas might be relatively young migratory neurons (Nacher et al., 2001) but others are clearly not. The piriform cortex of adult mice, for example, contains an abundant population of DCX-positive cells that display features of immature cells normally seen in newly generated hippocampal granule neurons. These cells are however clearly old and strictly postmitotic (Klempin et al., 2011).

Based on these exceptions, it has been concluded that in mammals DCX is not only a marker of neurogenesis but more broadly relates to the extensive neuronal plasticity present in the adult brain (Brown et al., 2003; Klempin et al., 2011; Kremer et al., 2013; Nacher et al., 2001). This is not unexpected given the role of DCX in microtubule reorganization, a process that is critical not only for cell migration but also to support the reorganization of the dendritic arbor, neurite outgrowth and synaptogenesis in plastic neurons (Francis et al., 1999; Gleeson et al., 1999). This conclusion has however not been construed to imply that DCX cannot be used to quantify neurogenesis in selected brain regions where active neurogenesis is known to take place. Caution must simply be exercised before concluding that a DCX-positive cell is a newborn neuron. Its location, anatomical organization and functional characteristics should also be considered when making such a judgment.

8. The use of endogenous markers of neurogenesis in birds

There is one additional problem related to the use of endogenous markers of neurogenesis when trying to investigate this phenomenon in so-called alternative animal models such as birds. These markers are proteins and as such they can be species-specific. The antibodies that are used to recognize them by immunohistochemistry have usually been raised against the rodent (mice, rats) or human epitopes. Homologous antibodies are rarely available. There is thus no guarantee that antibodies raised against mammalian epitopes will specifically recognize the orthologous proteins in other species and their specificity should thus be clearly demonstrated.

Comparisons of the gene and if possible the protein sequences should be made and related to the sequence used as the antigen for producing the antibody. This can provide an initial assessment of the antibody specificity. The classic immunohistochemical specificity tests should then be performed (e.g., antibody pre-adsorption, Western blot confirming the size of the identified protein,...) and eventually *in situ* hybridization, either alone or in combination with immunohistochemistry, should be used to compare the distribution of the marker protein and of the corresponding mRNA. These recommendations are of course entirely consistent with the policy of the Journal of Comparative Neurology concerning antibody characterization (Saper, 2005; Saper and Sawchenko, 2003).

Even if the immunohistochemical procedure identifies the expected protein, it should still be established that in the new species investigated that this protein is a specific and exhaustive marker of the cell type of interest (neurons vs. glia, cell replicating its DNA, young newborn neuron...). This criteria is actually much more difficult to fulfill because this can only be done usually by referring to another type of independent information that was itself collected with methods that have their own pitfalls.

As a consequence, even though adult neurogenesis was (re)discovered in songbirds, many/most potential endogenous markers of neurogenesis have not been used in birds and when they have, it was often without the exhaustive validation of their specificity of the sort we just discussed. However, even with these limitations in mind, a few antibodies have been identified that seem to specifically recognize their expected target in the avian brain. The NeuN mouse monoclonal antibody raised against purified mouse brain cell nuclei (Chemicon Clone A60, Antibody MAB377) that has been broadly used in mammals was, for example, shown to identify neurons in a variety of avian species including songbirds (Hall and Macdougall-Shackleton, 2012; Newman et al., 2010; Vellema et al., 2010), quail (Bardet et al., 2012; Stamatakis et al., 2004) and chickens (Mezey et al., 2012). As is the case in mammals, some neuronal cell types such as the Purkinje cells do not seem to express NeuN in birds (Newman et al., 2010; Stamatakis et al., 2004), which provides some support to the specificity of this label.

Hu C/D staining has similarly been used to label neurons in the avian brain (Agate et al., 2007; Barami et al., 1995; Vellema et al., 2010). In canaries that had been injected with BrdU, it was found that Hu appeared within hours of the final mitotic division in the neural progeny well before the beginning of the parenchymal migration but was never expressed by glia (Barami et al., 1995). Hu C/D can thus be used as an early marker of neuronal differentiation in the ventricular zone (Barami et al., 1995). Hu C/D is, however, still expressed by mature neurons and can be used to delineate specific nuclei (e.g. song control nuclei in songbirds, see Figure 3) and quantify neurons in these structures (Thompson and Brenowitz, 2005).

Hu immunoreactivity is confined to cells displaying a neuronal (as opposed to a glial) morphology at the light and electron microscopic level in both canaries and zebra finches. In canaries in particular, Hu is colocalized in many cells with two well-established markers of neurons (Barami et al., 1995). Hu immunoreactivity was also shown to colocalize with NeuN immunoreactivity in a variety of mammalian and avian species (see (Vellema et al., 2010)).

A limited number of studies have also used endogenous markers to identify cycling progenitors in the avian brain. The primary sequence of the Ki67 protein, the most ubiquitous of these markers that labels cycling cells at every stage of the cell cycle is unfortunately quite variable from one species to another based on our searches in sequences databases. Most antibodies that identify Ki67 in the mammalian brain thus do not seem to work in birds. To the best of our knowledge, one single study used this marker to identify cycling cells in birds and this study concerned the testicular tissue (Reitemeier et al., 2011).

PCNA is in contrast highly conserved among eukaryotes (Bravo et al., 1987; Valero et al., 2005) and has thus been used to compare cellular proliferation during ontogeny in avian species that possess relatively large (e.g., songbirds, parakeets) vs. small (e.g., galliforms) brains in adulthood (Charvet and Striedter, 2008; 2009). The same method was also used in adult quail to demonstrate the presence of a limited age-dependent cellular proliferation in the preoptic area of juvenile Japanese quail (Bardet et al., 2012; Mouriec and Balthazart, 2013).

Similarly pHH3 is expressed during most phases of mitosis and antibodies are available to identify reliably this protein in the avian brain. pHH3 immunohistochemistry was also used to compare between species the process of cell proliferation in the brain during development (Charvet and Striedter, 2009). It has also been employed in adult birds to study cell proliferation in the retina (Ornelas et al., 2013). Recent work in our laboratory has also successfully labeled a pHH3 specific cell population at the level of the lateral ventricles in the canary brain (Barker et al., 2011)(Shevchouk O. and Balthazart, J. unpublished data).

9. Doublecortin as a marker of new neurons in birds

After being broadly adopted as a marker of neurogenesis in mammals, DCX has more recently been identified in the avian brain and then, in a second step, used in studies of adult neurogenesis. In birds, the DCX protein or its mRNA were first described in the developing brain of chickens (Capes-Davis et al., 2005; Hannan et al., 1999) and zebra finches (Kim et al., 2006). The latter study also demonstrated that DCX expression is still present in adults albeit at much lower levels than those observed at post-natal day 9. These findings prompted us to investigate whether DCX expression was related to adult neurogenesis in birds.

9.1. Neuroanatomical distribution of DCX expression in canaries

Our initial immunohistochemical mapping of DCX expression (Boseret et al., 2007) identified very large populations of DCX-immunoreactive (DCX-ir) cells in many areas of the adult canary brain. As reported in mammals, densely stained DCX-ir neurons could be divided into two distinct subgroups based on their morphology. Some displayed a fusiform uni- or bi-polar shape and obviously represent young migrating cells, whereas others had a multipolar shape with more or less round perikarya and are presumably slightly older neurons that are in the early stages of differentiation (Fig. 4). These two different morphologies of DCX-ir cells were also described in a study of the developing chicken brain (Mezey et al., 2012) and of the adult pigeon (*Columba livia*)(Melleu et al., 2013). Subsequent studies found that these two populations are differentially affected by experimental treatments (see next paragraphs)

These densely stained DCX-ir cells were found almost exclusively in parts of the telencephalon that are known to incorporate new neurons in adulthood, in particular parts of the nidopallium where adult neurogenesis has consistently been reported (Alvarez-Buylla et al., 1990; Alvarez-Buylla and Nottebohm, 1988; Nottebohm, 2008). In the dorsal part of this brain region, the boundaries of the song control nucleus HVC could be clearly distinguished from surrounding structures by a higher density of DCX-ir within the nucleus as compared to the surrounding nidopallium (Fig. 4).

Although DCX is a widely recognized marker of newborn neurons in the mammalian brain and the densely stained DCX-ir cells were specifically present in regions of the canary brain that display a high level of neurogenesis in adulthood (Boseret et al., 2007), it was important to confirm its specificity as a marker of new neurons in canaries. To this aim, photosensitive male canaries were injected with BrdU (50 mg/kg) twice daily for 5 consecutive days. Their brain was collected either 10 or 30 days later and sections through HVC were then double-labeled by immunohistochemistry for BrdU and DCX (Balthazart et al., 2008). Large numbers of DCX-ir cells that contained a BrdU-labeled nucleus were detected in the nidopallium, including HVC. At the 10-day time point, more than 70% of the DCX fusiform cells in HVC contained BrdU-positive nuclei (see table 1), demonstrating that the vast majority of these DCX fusiform cells represent new neurons that were born (or, more precisely, entered the DNA synthesis phase) concurrent with the time of the BrdU injections or very soon thereafter. Because after a BrdU injection, the compound remains available for incorporation into the DNA for only a relatively brief period (see before, section 1.3.2.), even with the repeated injection schedule that was used, there were still some of the DCX cells that were born either before or too long after a BrdU injection to be double-labeled. In addition, it should be remembered that BrdU labels all cells replicating their DNA including glia and endothelial or undifferentiated cells in addition to neurons (see section 4.4). Together these factors explain why the percentage of DCX-ir cells containing a BrdU-positive nucleus did not and actually can not reach the level of 100%.

From day 10 to day 30, the percentage of DCX-ir fusiform cells containing BrdU decreased, whereas the number of DCX-ir round cells containing BrdU increased (from 5% to 24%) (Balthazart et al., 2008). These observations are thus consistent with the idea that fusiform DCX-ir cells are young neurons that will later mature into round multipolar DCX cells before they stop expressing this protein.

These published percentages were based on counts of the numbers of DCX-ir and double labeled (DCX-ir+BrdU-ir) cells in HVC. As indicated in the original publication (Balthazart et al., 2008), we had also counted during this study the total number of BrdU-ir cells in the same area although we did not originally use these numbers to compute the percentages of BrdU-ir cells that expressed DCX. We have now made these calculations and demonstrate that the majority of the BrdU-positive cells were indeed expressing DCX at 10 days post injection (73.38%). Most of the cells that had replicated their DNA during the period of BrdU injections were thus labeled by this marker of new neurons. This percentage also did not reach 100 % presumably because some of these new cells have a glial phenotype. Quite interestingly, the total number of BrdU-positive cells decreased between day 10 and 30 (many of the new neurons die during their first month after birth: (Kirn et al., 1999)) but the percentage of BrdU-positive cells that were immunoreactive for DCX had not decreased at 30 days post-injections (77.88%) indicating that this marker of young neurons is expressed for at least 30 days, and probably longer after, cells become post-mitotic. DCX expression has been shown to last approximately one month in the mammalian brain but variation between brain areas was detected (see before, section 1.5.) and this duration has never been investigated in birds. Together, these results are nevertheless consistent with the notion that many, if not all, DCX-ir cells in the canary HVC represent neurons that went through their final division (and DNA synthesis phase) not too long before brain collection.

It is important to note that in addition to the densely stained DCX-ir cells found in the telencephalon that displayed the fusiform or round multipolar morphology described above, our studies in canaries also identified several populations of generally weakly stained cells in various diencephalic and mesencephalic nuclei. These are brain regions not known to be typically neurogenic areas in the avian brain (Balthazart et al., 2008; Boseret et al., 2007). Although one cannot exclude that small numbers of new neurons are born in these regions in the adult brain (see for example (Bardet et al., 2012; Mouriec and Balthazart, 2013)), it is most likely that DCX expression in the regions relates to some other process such as plasticity of the dendritic arbor.

9.2. Hormonal and environmental regulation of DCX expression in birds

In a suite of experiments, we then used the DCX marker to investigate changes in numbers of new neurons in the HVC of canaries that had been exposed to various physiological situations that are known to affect HVC volume and thus presumably the rate of neurogenesis in this nucleus (Balthazart et al., 2008). As illustrated in figure 5, in parallel with changes in HVC volume, the number of DCX-ir cells was increased specifically in the HVC of T-treated males as compared to control castrates (Fig.5 A, B). In castrated males in which plasma testosterone concentrations were clamped to a stable level by a subcutaneous implant of testosterone, the number of round DCX-ir cells in HVC was higher in birds living in a cage with a female (M-F) than in males paired with another male (M-M). Elongated fusiform DCX-ir cells that are presumably migrating neurons were also more numerous in M-F than in M-M subjects (Fig. 5 C,D).

Elongated DCX-ir cells that are presumably migrating neurons were also more numerous in males than in females (panel E) and round multipolar DCX-ir cells that represent differentiating neurons were more numerous in photostimulated but also in photosensitive non-photostimulated birds than in matched photorefractory subjects (panel F) (Balthazart et al., 2008). In all of these cases, the changes in DCX-ir cell numbers paralleled the changes in HVC volume. Thus, in canaries the endocrine state (testosterone-treated vs. control) as well as the social (M-F vs. M-M) or photoperiodic (photosensitive / photostimulated vs. photorefractory) condition, independently of variation in steroid hormones, affect the expression of a protein involved in neuronal migration specifically in brain areas that incorporate new neurons. The DCX gene may thus be a target by which T and social stimuli induce seasonal changes in the volume of song nuclei.

Interestingly, all of these treatments did not generally affect the numbers of fusiform or round DCX-ir cells located in the nidopallium adjacent to HVC (sampled area on the same section just lateral or ventral to the song control nucleus; see Fig. 5G). The anatomical specificity of these experimental effects (seen in but not around HVC) provides important information concerning the mechanisms that mediate the increases in HVC volume. Given that new neurons apparently reach HVC and adjacent tissue by random movement (Vellema et al., 2010), these data suggest that the increase in HVC volume and in the number of DCX-ir cells in the nucleus cannot be the result only of an effect of the steroid on the production of new neurons (otherwise, a similar effect would be seen in adjacent areas), but result from a direct effect of testosterone on the recruitment of new neurons in the nucleus.

In a more recent experiment, we also demonstrated that the testosterone induced changes DCX-ir cells of HVC in canaries are mediated by the synergistic action of the two metabolites, 5 α -dihydrotestosterone and estradiol-17 β (Yamamura et al., 2011). This experiment also confirmed that the effects of testosterone on the numbers of fusiform or round DCX-ir cells are observed specifically in HVC and not in adjacent areas. Doublecortin was also used in several experiments as an indicator of neurogenesis in studies assessing neural plasticity in the avian hippocampus (Fox et al., 2010; LaDage et al., 2010; LaDage et al., 2011).

In summary, all these data indicate that DCX-ir cells in the avian brain represent to a large extent, if not exclusively in some brain regions such as HVC, newly generated neurons that can for the most part be co-labeled with BrdU after injections of this thymidine analogue. Due to the dose-response and timing problems that were described before, it is impossible to label exhaustively ALL neurons that are born in a brain area during an extended period of time. As a consequence, it is impossible to demonstrate that all DCX-ir cells contain this marker of DNA replication but studies performed after BrdU injections demonstrate that this is the case for the majority (over 70%) of the DCX-ir fusiform cells in HVC at a time point (10 days post injections) that is close to the peak of arrival of new neurons in HVC (Kirn et al., 1999). Conversely, at this time point, most BrdU-positive cells co-express DCX (over 70% again) but obviously other non-neuronal cell types also contain BrdU and do not express the neuronal specific marker DCX.

Multiple studies also indicate that changes in HVC volume that are presumably associated with changes in neurogenesis correlate with similar changes in the number of DCX-ir neurons in this nucleus. This protein thus represents a very useful proxy of neurogenesis in the canary HVC that has the definite advantage over thymidine analogues such as BrdU of labeling neurons born over an extended period of time (weeks to months as opposed to hours), which provides an integrated view of changes in neurogenesis as opposed to a brief snap shot. This fact is especially important for investigators interested in assessing the effects of the endogenous and exogenous environment on various aspects of neurogenesis. Many of these environmental factors exert their effects over days or weeks and the brief snapshot provided by a BrdU injection is sometimes inadequate.

9.3. Recent critiques of DCX use as endogenous marker of neurogenesis in birds

As previously discussed in section 7 devoted to DCX in mammals, a number of publications have discussed the limitations of DCX as a marker of new neurons (Brown et al., 2003; Francis et al., 1999; Gleeson et al., 1999; Klempin et al., 2011; Kremer et al., 2013; Nacher et al., 2001). In a recent paper published in the *Journal of Comparative Neurology*, Vellema, Hertel, Urbanus, Van der Linden and Gahr (Vellema et al., 2014) questioned the validity of DCX expression as a reliable marker of neurogenesis in songbirds (as well as other vertebrates in general) based on studies they performed in canaries. There are relatively few studies of this sort in songbirds so even a single case can be widely interpreted as an indication that this technique does not apply to avian species. In the specific case of DCX use in canaries, the demonstration that it marks exclusively new neurons could theoretically be obtained by labeling all new neurons with BrdU and then confirming that DCX is

exclusively expressed in BrdU-positive cells. This goal is however more difficult to reach than it appears at first sight because as explained earlier in this review, it is nearly impossible to label all new neurons for an extended period of time with BrdU. Additionally, BrdU also labels other cell types (glial and endothelial cells) and to some extent cells repairing their DNA. The label in the glial and endothelial cell lines that are not necessarily post-mitotic will be progressively diluted as cells undergo successive mitoses but it is usually accepted that the label remains visible by standard immunohistochemical procedures during at least 4 cell cycles (Hayes and Nowakowski, 2002). A substantial number of BrdU-positive cells in the brain are thus not neurons and the study of neurogenesis *sensu stricto* requires that BrdU-positive cells be double-labeled with a neuronal marker that has its own specificity problems (see sections 4.4 and 9.1 before). In contrast, DCX is a generally reliable marker of the neuronal cell lineage (see however (Verwer et al., 2007) for one notable exception in diseased human brain).

The paper recently published by Vellema et al (2014) goes however beyond these words of caution that have been previously presented and lists five separate types of arguments which according to them support the notion that DCX is not a valid marker of neurogenesis namely: a) The neuroanatomical distribution of doublecortin expression does not match the pattern of neurogenesis in the canary brain, b) The seasonal and hormonal changes in doublecortin expression do not match the previously described changes in neurogenesis, c) Doublecortin is expressed in neurons of up to one year of age, d) Doublecortin expression does not predict neuron recruitment equally throughout the brain, and finally e) Doublecortin is expressed in adult active neurons labeled by NeuN that are expressing the immediate early gene *egr-1* and project to RA

A detailed discussion of these propositions is not appropriate in this general review. We do contend, however, that the arguments presented in this paper are either not novel (see section 7), or are based on limited evidence or inconclusive data. We concede that some of these arguments are probably generally correct for some brain areas and for a small proportion of DCX-ir cells in other brain regions, these arguments certainly do not apply to the HVC of canaries or, if they do, they concern rare exceptions. We present below in a concise fashion the reasons that lead us to this conclusion.

- a. It is certainly true that, as already reported in Boseret et al. (Boseret et al., 2007), DCX-ir cells are found outside well characterized, putative neurogenic areas of canaries and other birds but cells in these areas are either rare or have a low staining density. DCX expression in these areas either reflects other forms of plasticity (dendritic arbor reorganization) or neurogenesis in areas where it was not expected to take place as has been observed before (see namely (Chen et al., 2006; Chen and Cheng, 2007; Cheng, 2013) for birds and (Kokoeva et al., 2005; 2007; Migaud et al., 2010; Perez-Martin et al., 2010) in mammals.
- b. The correlation between changes in neurogenic activity and DCX-ir cell density should certainly be studied in more brain areas and species but the canary studies described above (section 1.8.1) support in our opinion this relationship for the canary HVC at least. The suggested lack of correlation claimed in the Vellema et al. paper is based on subjects whose endocrine and environmental conditions were

poorly defined and in which neurogenesis as such was not determined so that no real conclusion can be extracted.

- c. To our knowledge, the duration of DCX expression in young neurons has never been studied in detail in birds. We found that 30 days after BrdU injections over 70% of DCX-ir cells contain BrdU (Balthazart et al., 2008) and conversely that at the same time more than 70% of the BrdU-positive cells still express DCX (Table 1 in this paper). The observation that DCX expression would persist up to 60 days in NCM and Area X is interesting and certainly is worth additional follow-up research but this observation alone does not contradict previous data indicating that at 30 days post injection DCX expression was still largely present. The presence of DCX in cells labeled by BrdU one year previously is more puzzling but it is described in the paper by Vellema et al. (2014) as being “sporadic” in the two birds that were studied. Only one neuron is illustrated and there is no quantitative analysis, also the brain region where this observation was made is not mentioned. For these reasons we think that this criticism should be interpreted with caution these sporadic cells may well represent exceptions and could simply be related to the adult reorganization of the dendritic arbor in a few neurons.
- d. The observation that the ratio of BrdU-positive and DCX-ir cell numbers is variable across brain regions certainly cannot be used to decide whether DCX is or is not a reliable marker of neurogenesis. The duration of DCX expression is likely to differ between tissues (see (Zhao et al., 2008) for such studies in mammals) and survival of new neurons (labeled by BrdU) is clearly different from one brain region to another or as a function of endocrine conditions (Kirn et al., 1994; Kirn and Schwabl, 1997; Rasika et al., 1994). BrdU labels cells (neurons, glia, endothelial cells) that are replicating their DNA during a few hours after the BrdU injection (Barker et al., 2013) and this label will be permanent in cells that undergo their last division and will afterwards become post-mitotic which is the case for new neurons in the adult brain. DCX labels new neurons for extended period of times that can be variable. While it makes sense to quantify cells double-labeled for BrdU and DCX, the physiological meaning of counting these two populations in single-labeled material and calculating ratios is questionable. This is especially a concern given that Vellema et al (2014) conducted the cell counts at 38 and 60 days post BrdU injections when a large fraction of the new cells would have died. This analysis does not really address the question of whether DCX is a reliable marker of newly born neurons. It is especially important to keep in mind that the labeling and survival of BrdU cells and expression of DCX are two different phenomena with their own time dynamics.
- e. Finally Vellema et al. (2014) put emphasis on their observations that DCX expression was present in “mature” cells, i.e., cells that were expressing the neuronal marker NeuN or the immediate early gene *egr-1*, or had made connections to song nuclei robustus arcopallialis (RA) as demonstrated by retrograde tracing from this nucleus. These observations are in our opinion not surprising and not inconsistent with the fact that DCX labels new neurons. So, for example, it is not known exactly when NeuN begins to be expressed in avian neurons but, in the

mammalian hippocampus, it is clearly accepted that new neurons begin expressing NeuN before they stop expressing DCX (Kempermann et al., 2004). Egr-1 is known to be expressed in the canary nidopallium caudale following exposure to conspecific song (Mello and Ribeiro, 1998) but there is no indication in the present paper that such stimulation was provided to the experimental birds and no indication of where these Egr-1 positive cells were located. The reason why Egr-1 was present in the cells under study is therefore completely unknown. Young developing DCX-positive neurons synthesize a large number of proteins during their differentiation and it is quite possible that transcription of these proteins is under the control of egr-1. Some of these proteins may even be necessary for neuronal maturation since it was shown in mice that inactivation of the egr-1 gene results in alterations of the neurogenesis process (Veyrac et al., 2013). Finally, given that DCX is expressed in new HVC neurons for 30 days or more (Balthazart et al., 2008; Vellema et al., 2014), it is not surprising that some of them were retrogradely labeled from RA since, based on the available data (Kirn et al., 1999), initial connections take place around two weeks after the neurons become post-mitotic and these connections probably reach their maximum within a month. NeuN and egr-1 expression or retrograde labeling for RA are thus not incompatible with the idea that DCX-ir neurons are young neurons.

In summary, we think that this paper by Vellema et al., (2014) that provides a strong critique of the use of DCX as a marker of new neurons certainly presents a number of interesting observations that deserve further study but this critique also needs to be considered with great caution. A careful evaluation of the critique reveals that it systematically highlights some potentially rare exceptions to generalizations about the occurrence and significance of DCX labeled cells or in some case the critique actually involves the misinterpretation of some of its observations.

It is, at the same time, obvious that the specificity of DCX as a marker of newborn neurons is not absolute. This protein is part of the microtubule machinery that is used by migrating neurons but also by neurons reorganizing their dendritic arbor. A few cells, especially those with a multipolar ("round") phenotype that express DCX are thus not necessarily young newborn neurons and this has been previously recognized in studies of rodents. DCX-positive cells have for example been identified in parts of the mammalian telencephalon that probably do not incorporate new neurons (see section 7). However, even with this limitation, to this date DCX is probably the best available endogenous marker of new neurons in the mammalian brain and the only one validated the canary HVC and possibly more broadly for the avian brain.

10. Conclusions

The present review briefly summarizes some of the advantages and pitfalls of the methods that have been commonly used to study adult neurogenesis in birds. The injection of thymidine analogues (BrdU and more recently EdU, IdU and CldU) is without a doubt considered the method of choice at this point but sometimes these analogues cannot be applied easily (e.g., in free-living species) and this approach only provides a brief snap-shot

of the rate of DNA replication at a specific point in time; multiple groups of subjects must therefore be studied to obtain information on the fate of these new cells. In parallel, there has recently been a clear increase in the use of endogenous markers in studies of avian neurogenesis. We clearly agree with the idea that such markers must always be considered with due caution. This is true for markers of cell lineages (e.g. neurons vs. glia) as well as for marker of cell cycling (Ki67, PCNA, pHH3..) and for DCX as a marker young new neurons is no exception to this caveat. However, even if we take into account these limitations, this reasonably specific endogenous marker of new neurons, alone or in combination with a standard method such as BrdU, remains extremely useful.

Studying all steps of neurogenesis (proliferation, migration, recruitment, differentiation and survival) by injections of exogenous markers such as BrdU is extremely difficult and tedious. This approach involves the repeated injection to a very large number of subjects of saturating concentrations of BrdU or a similar thymidine analog to label progenitor cells dividing during a given period of time (e.g. 12-24 hours) and then the collection of brains in subgroups of subjects at various times post-injection to capture the fate of the labeled cells. If the goal is to investigate the effect of an experimental treatment (e.g., a treatment with testosterone) on the different steps of the neurogenesis process, this approach becomes almost impossible because the treatment should now technically be applied separately at each different step in the neurogenesis process (before division, during migration, recruitment...etc) as captured by the BrdU injections and brains should then be collected in multiple sub-groups of subjects at various latencies after BrdU injections. This implies a two-dimensional experimental design with a huge number of subjects to study the effect of a single factor (a tri-dimensional design becomes necessary if several levels of the experimental factor are considered) and a thorough experiment of this type has to our knowledge never been carried out.

There are therefore still many unknown facts concerning neurogenesis in canaries as well as in more commonly studies species such as mice and rats; active research is still ongoing to uncover the underlying mechanisms. The use of endogenous markers can be an invaluable help to make progress in this research especially when used in combination with the injection of exogenous markers of DNA replication. The value of DCX in addition to BrdU relates to the fact that at a given time point, when brains are collected, DCX potentially labels ALL new neurons instead of just a small sub-sample of cells that duplicated their DNA within the few hours that followed the BrdU injection(s). Furthermore, by quantifying separately the bipolar fusiform cells that are presumably very young migrating neurons and round multipolar cells that have presumably reached their destination and initiated their final differentiation one can obtain additional information on two largely independent populations of cells that became post-mitotic in different time windows. The usefulness and validity of this approach has been extensively demonstrated in studies of laboratory rodents and to some extent in canaries. It is especially useful for researchers studying birds in particular in field studies where (multiple) injections of thymidine analogs would be difficult if not impossible at very specific time before brain collection.

It is clearly the case that the specificity of DCX as a marker of new neurons is not absolute. However, the relative number of DCX cells that are not newborn neurons is in all

probability quite minimal especially in the canary HVC and in most circumstances will not interfere with the conclusions of studies investigating neurogenesis in the avian telencephalon. Ignoring the potential of this tool would be damaging to the field of adult neurogenesis. DCX is, and remains, a very useful tool to study neurogenesis in canaries and other birds especially in combination with other markers such as BrdU. It is important that we don't throw the proverbial baby out with the bathwater!

Acknowledgments

This work was supported by an NIH/NINDS RO1 NS35467 and an Interuniversity Attraction Pole (IAP) grant number SSTC PAI P7/17 from the Belgian Science Policy Office (BELSPO) to JB and GFB.

References

- Agate RJ, Hertel M, Nottebohm F. FnTm2, a novel brain-specific transcript, is dynamically expressed in the song learning circuit of the zebra finch. *The Journal of comparative neurology*. 2007; 504(2): 127–148. [PubMed: 17626267]
- Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *The Journal of comparative neurology*. 1965; 124(3):319–335. [PubMed: 5861717]
- Altman J, Das GD. Postnatal neurogenesis in the guinea-pig. *Nature*. 1967; 214(5093):1098–1101. [PubMed: 6053066]
- Alvarez-Buylla A, Kirn JR, Nottebohm F. Birth of projection neurons in adult avian brain may be related to perceptual or motor learning. *Science*. 1990; 249:1444–1446. [PubMed: 1698312]
- Alvarez-Buylla A, Nottebohm F. Migration of young neurons in adult avian brain. *Nature*. 1988; 335:353–354. [PubMed: 3419503]
- Arnold AP, Nottebohm F, Pfaff DW. Hormone concentrating cells in vocal control areas of the brain of the zebra finch (*Poephila guttata*). *JCompNeurol*. 1976; 165:487–512.
- Bai J, Ramos RL, Ackman JB, Thomas AM, Lee RV, LoTurco JJ. RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat Neurosci*. 2003; 6(12):1277–1283. [PubMed: 14625554]
- Ball GF, Auger CJ, Bernard DJ, Charlier TD, Sartor JJ, Ritters LV, Balthazart J. Seasonal plasticity in the song control system: multiple brain sites of steroid hormone action and the importance of variation in song behavior. *Annals of the New York Academy of Sciences*. 2004; 1016:586–610. [PubMed: 15313796]
- Balthazart J, Boseret G, Konkle AT, Hurley LL, Ball GF. Doublecortin as a marker of adult neuroplasticity in the canary song control nucleus HVC. *The European journal of neuroscience*. 2008; 27(4):801–817. [PubMed: 18333960]
- Balthazart J, Foidart A, Wilson EM, Ball GF. Immunocytochemical localization of androgen receptors in the male songbird and quail brain. *JCompNeurol*. 1992; 317:407–420.
- Barami K, Iversen K, Furneaux H, Goldman SA. Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. *J Neurobiol*. 1995; 28(1):82–101. [PubMed: 8586967]
- Bardet SM, Mouriec K, Balthazart J. Birth of neural progenitors during the embryonic period of sexual differentiation in the Japanese quail brain. *The Journal of comparative neurology*. 2012; 520(18): 4226–4253. [PubMed: 22628012]
- Barker JM, Ball GF, Balthazart J. Sex differences in rate of division and maturation of new neurons in adult canaries; Abst Society for Neuroscience meeting; Washington DC. 2011; Abstract 945.910
- Barker JM, Charlier TD, Ball GF, Balthazart J. A new method for in vitro detection of bromodeoxyuridine in serum: a proof of concept in a songbird species, the canary. *PLoS one*. 2013; 8(5):e63692. [PubMed: 23691086]
- Barth PG. Disorders of neuronal migration. *Can J Neurol Sci*. 1987; 14(1):1–16. [PubMed: 3545413]

- Batailler M, Droguerre M, Baroncini M, Fontaine C, Prevot V, Migaud M. DCX-expressing cells in the vicinity of the hypothalamic neurogenic niche: A comparative study between mouse, sheep, and human tissues. *Journal of Comparative Neurology*. 2014; 522(8):1966–1985. [PubMed: 24288185]
- Bauer S, Hay M, Amilhon B, Jean A, Moyse E. In vivo neurogenesis in the dorsal vagal complex of the adult rat brainstem. *Neuroscience*. 2005; 130(1):75–90. [PubMed: 15561426]
- Bonfanti L, Peretto P. Adult neurogenesis in mammals--a theme with many variations. *The European journal of neuroscience*. 2011; 34(6):930–950. [PubMed: 21929626]
- Boseret G, Ball GF, Balthazart J. The microtubule-associated protein doublecortin is broadly expressed in the telencephalon of adult canaries. *J Chem Neuroanat*. 2007; 33(3):140–154. [PubMed: 17367992]
- Boseret G, Carere C, Ball GF, Balthazart J. Social context affects testosterone-induced singing and the volume of song control nuclei in male canaries (*Serinus canaria*). *J Neurobiol*. 2006; 66(10):1044–1060. [PubMed: 16838373]
- Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature*. 1987; 326(6112):515–517. [PubMed: 2882423]
- Brenowitz, EA. Plasticity of the song control system in adult birds. In: Zeigler, HP.; Marler, P., editors. *Neuroscience of birdsong*. Cambridge University Press; Cambridge: 2008. p. 332-349.
- Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG. Transient expression of doublecortin during adult neurogenesis. *The Journal of comparative neurology*. 2003; 467(1):1–10. [PubMed: 14574675]
- Bullwinkel J, Baron-Luhr B, Ludemann A, Wohlenberg C, Gerdes J, Scholzen T. Ki-67 protein is associated with ribosomal RNA transcription in quiescent and proliferating cells. *J Cell Physiol*. 2006; 206(3):624–635. [PubMed: 16206250]
- Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *The Journal of comparative neurology*. 2001; 435(4):406–417. [PubMed: 11406822]
- Camp AJ, Wijesinghe R. Calretinin: modulator of neuronal excitability. *Int J Biochem Cell Biol*. 2009; 41(11):2118–2121. [PubMed: 19450707]
- Capes-Davis A, Tolhurst O, Dunn JM, Jeffrey PL. Expression of doublecortin (DCX) and doublecortin-like kinase (DCLK) within the developing chick brain. *Dev Dyn*. 2005; 232(2):457–467. [PubMed: 15614772]
- Charalampopoulos I, Remboutsika E, Margioris AN, Gravanis A. Neurosteroids as modulators of neurogenesis and neuronal survival. *Trends Endocrinol Metab*. 2008; 19(8):300–307. [PubMed: 18771935]
- Charvet CJ, Striedter GF. Developmental species differences in brain cell cycle rates between northern bobwhite quail (*Colinus virginianus*) and parakeets (*Melopsittacus undulatus*): implications for mosaic brain evolution. *Brain Behav Evol*. 2008; 72(4):295–306. [PubMed: 19088470]
- Charvet CJ, Striedter GF. Developmental origins of mosaic brain evolution: Morphometric analysis of the developing zebra finch brain. *The Journal of comparative neurology*. 2009; 514(2):203–213. [PubMed: 19266567]
- Chehrehasa F, Meedeniya AC, Dwyer P, Abrahamsen G, Mackay-Sim A. EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. *J Neurosci Methods*. 2009; 177(1):122–130. [PubMed: 18996411]
- Chen G, Bonder EM, Cheng MF. Lesion-induced neurogenesis in the hypothalamus is involved in behavioral recovery in adult ring doves. *J Neurobiol*. 2006; 66(6):537–551. [PubMed: 16555235]
- Chen G, Cheng MF. Inhibition of lesion-induced neurogenesis impaired behavioral recovery in adult ring doves. *Behav Brain Res*. 2007; 177(2):358–363. [PubMed: 17194488]
- Chen Z, Ye R, Goldman SA. Testosterone modulation of angiogenesis and neurogenesis in the adult songbird brain. *Neuroscience*. 2013; 239:139–148. [PubMed: 23291451]
- Cheng MF. Hypothalamic neurogenesis in the adult brain. *Front Neuroendocrinol*. 2013; 34(3):167–178. [PubMed: 23684668]

- Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn HG, Aigner L. Doublecortin expression levels in adult brain reflect neurogenesis. *The European journal of neuroscience*. 2005; 21(1):1–14. [PubMed: 15654838]
- Curmi PA, Andersen SS, Lachkar S, Gavet O, Karsenti E, Knossow M, Sobel A. The stathmin/tubulin interaction in vitro. *J Biol Chem*. 1997; 272(40):25029–25036. [PubMed: 9312110]
- Dayer AG, Cleaver KM, Abouantoun T, Cameron HA. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. *J Cell Biol*. 2005; 168(3):415–427. [PubMed: 15684031]
- des Portes V, Francis F, Pinard JM, Desguerre I, Moutard ML, Snoeck I, Meiners LC, Capron F, Cusmai R, Ricci S, Motte J, Echenne B, Ponsot G, Dulac O, Chelly J, Beldjord C. doublecortin is the major gene causing X-linked subcortical laminar heterotopia (SCLH). *Hum Mol Genet*. 1998; 7(7):1063–1070. [PubMed: 9618162]
- Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. Neurogenesis in the adult human hippocampus. *Nat Med*. 1998; 4(11):1313–1317. [PubMed: 9809557]
- Essers J, Theil AF, Baldeyron C, van Cappellen WA, Houtsmuller AB, Kanaar R, Vermeulen W. Nuclear dynamics of PCNA in DNA replication and repair. *Mol Cell Biol*. 2005; 25(21):9350–9359. [PubMed: 16227586]
- Fox RA, Roth TC 2nd, LaDage LD, Pravosudov VV. No effect of social group composition or size on hippocampal formation morphology and neurogenesis in mountain chickadees (*Poecile gambeli*). *Dev Neurobiol*. 2010; 70(7):538–547. [PubMed: 20336697]
- Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron*. 1999; 23(2):247–256. [PubMed: 10399932]
- Friocourt G, Koulakoff A, Chafey P, Boucher D, Fauchereau F, Chelly J, Francis F. Doublecortin functions at the extremities of growing neuronal processes. *Cereb Cortex*. 2003; 13(6):620–626. [PubMed: 12764037]
- Gage, FH.; Kemperman, G.; Song, HJ. Adult neurogenesis. Cold Spring Harbor Laboratory Press; Cold spring Harbor NY: 2008a.
- Gage, FH.; Song, H.; Kemperman, G. Adult neurogenesis: A prologue. In: Gage, FH.; Kemperman, G.; Song, H., editors. Adult neurogenesis. Cold Spring Harbor Laboratory Press; Cold spring Harbor NY: 2008b. p. 1-5.
- Gahr M, Güttinger H-R, Kroodsma DE. Estrogen receptors in the avian brain: Survey reveals general distribution and forebrain areas unique to songbirds. *JCompNeurol*. 1993; 327:112–122.
- Galea LA, Spritzer MD, Barker JM, Pawluski JL. Gonadal hormone modulation of hippocampal neurogenesis in the adult. *Hippocampus*. 2006; 16(3):225–232. [PubMed: 16411182]
- Gleeson JG, Lin PT, Flanagan LA, Walsh CA. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron*. 1999; 23(2):257–271. [PubMed: 10399933]
- Goldman, SA. Neurogenesis in the adult songbird: A model for inducible striatal neuronal addition. In: Gage, FH.; Kemperman, G.; Song, HJ., editors. Adult neurogenesis. Cold Spring Harbor Laboratory Press; Cold spring Harbor NY: 2008. p. 593-618.
- Goldman SA, Nottebohm F. Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. *Proceedings of the National Academy of Sciences of the United States of America*. 1983; 80:2390–2394. [PubMed: 6572982]
- Gould E, Gross CG. Neurogenesis in adult mammals: some progress and problems. *J Neurosci*. 2002; 22(3):619–623. [PubMed: 11826089]
- Gould E, McEwen BS. Neuronal birth and death. *Curr Opin Neurobiol*. 1993; 3(5):676–682. [PubMed: 8260816]
- Gould E, Reeves AJ, Graziano MS, Gross CG. Neurogenesis in the neocortex of adult primates. *Science*. 1999; 286(5439):548–552. [PubMed: 10521353]
- Gross CG. Three before their time: neuroscientists whose ideas were ignored by their contemporaries. *Exp Brain Res*. 2009; 192(3):321–334. [PubMed: 18641979]

- Hall ZJ, Macdougall-Shackleton SA. Influence of testosterone metabolites on song-control system neuroplasticity during photostimulation in adult European starlings (*Sturnus vulgaris*). *PloS one*. 2012; 7(7):e40060. [PubMed: 22792214]
- Hannan AJ, Henke RC, Seeto GS, Capes-Davis A, Dunn J, Jeffrey PL. Expression of doublecortin correlates with neuronal migration and pattern formation in diverse regions of the developing chick brain. *J Neurosci Res*. 1999; 55(5):650–657. [PubMed: 10082087]
- Hayes NL, Nowakowski RS. Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. *Brain Res Dev Brain Res*. 2002; 134(1-2):77–85.
- Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP, Allis CD. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma*. 1997; 106(6):348–360. [PubMed: 9362543]
- Henion PD, Raible DW, Beattie CE, Stoesser KL, Weston JA, Eisen JS. Screen for mutations affecting development of Zebrafish neural crest. *Dev Genet*. 1996; 18(1):11–17. [PubMed: 8742830]
- Herculano-Houzel S, Lent R. Isotropic fractionator: a simple, rapid method for the quantification of total cell and neuron numbers in the brain. *J Neurosci*. 2005; 25(10):2518–2521. [PubMed: 15758160]
- Horesh D, Sapir T, Francis F, Wolf SG, Caspi M, Elbaum M, Chelly J, Reiner O. Doublecortin, a stabilizer of microtubules. *Hum Mol Genet*. 1999; 8(9):1599–1610. [PubMed: 10441322]
- Hourai A, Miyata S. Neurogenesis in the circumventricular organs of adult mouse brains. *Journal of Neuroscience Research*. 2013; 91(6):757–770. [PubMed: 23526379]
- Jin K, Mao XO, Cottrell B, Schilling B, Xie L, Row RH, Sun Y, Peel A, Childs J, Gendeh G, Gibson BW, Greenberg DA. Proteomic and immunochemical characterization of a role for stathmin in adult neurogenesis. *Faseb J*. 2004; 18(2):287–299. [PubMed: 14769823]
- Kemperman G. Stem cells and neuronal development in the adult brain. Oxford University Press; New York: 2011. Adult neurogenesis; p. 587
- Kempermann G. Neuroscience. What the bomb said about the brain. *Science*. 2013; 340(6137):1180–1181. [PubMed: 23744936]
- Kempermann G, Jessberger S, Steiner B, Kronenberg G. Milestones of neuronal development in the adult hippocampus. *Trends Neurosci*. 2004; 27(8):447–452. [PubMed: 15271491]
- Kim YH, Peregrine J, Arnold AP. The distribution of expression of doublecortin (DCX) mRNA and protein in the zebra finch brain. *Brain Res*. 2006; 1106(1):189–196. [PubMed: 16814268]
- Kirn J, O'Loughlin B, Kasparian S, Nottebohm F. Cell death and neuronal recruitment in the high vocal center of adult male canaries are temporally related to changes in song. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91(17):7844–7848. [PubMed: 8058721]
- Kirn JR, Fishman Y, Sasportas K, Alvarez-Buylla A, Nottebohm F. Fate of new neurons in adult canary high vocal center during the first 30 days after their formation. *The Journal of comparative neurology*. 1999; 411(3):487–494. [PubMed: 10413781]
- Kirn JR, Schwabl H. Photoperiod regulation of neuron death in the adult canary. *J Neurobiol*. 1997; 33(3):223–231. [PubMed: 9298761]
- Klempin F, Kronenberg G, Cheung G, Kettenmann H, Kempermann G. Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared to the neurogenic dentate gyrus of adult mice. *PloS one*. 2011; 6(10):e25760. [PubMed: 22022443]
- Koizumi H, Tanaka T, Gleeson JG. Doublecortin-like kinase functions with doublecortin to mediate fiber tract decussation and neuronal migration. *Neuron*. 2006; 49(1):55–66. [PubMed: 16387639]
- Kokoeva MV, Yin H, Flier JS. Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science*. 2005; 310(5748):679–683. [PubMed: 16254185]
- Kokoeva MV, Yin H, Flier JS. Evidence for constitutive neural cell proliferation in the adult murine hypothalamus. *The Journal of comparative neurology*. 2007; 505(2):209–220. [PubMed: 17853440]
- Kremer T, Jagasia R, Herrmann A, Matile H, Borroni E, Francis F, Kuhn HG, Czech C. Analysis of adult neurogenesis: evidence for a prominent “non-neurogenic” DCX-protein pool in rodent brain. *PloS one*. 2013; 8(5):e59269. [PubMed: 23690918]

- Kriss JP, Revesz L. The distribution and fate of bromodeoxyuridine and bromodeoxycytidine in the mouse and rat. *Cancer Res.* 1962; 22:254–265. [PubMed: 14459709]
- Kronenberg G, Wang LP, Geraerts M, Babu H, Synowitz M, Vicens P, Lutsch G, Glass R, Yamaguchi M, Baekelandt V, Debyser Z, Kettenmann H, Kempermann G. Local origin and activity-dependent generation of nestin-expressing protoplasmic astrocytes in CA1. *Brain Struct Funct.* 2007; 212(1): 19–35. [PubMed: 17717696]
- LaDage LD, Roth TC 2nd, Fox RA, Pravosudov VV. Ecologically relevant spatial memory use modulates hippocampal neurogenesis. *Proc Biol Sci.* 2010; 277(1684):1071–1079. [PubMed: 19939840]
- LaDage LD, Roth TC 2nd, Pravosudov VV. Hippocampal neurogenesis is associated with migratory behaviour in adult but not juvenile sparrows (*Zonotrichia leucophrys* ssp.). *Proc Biol Sci.* 2011; 278(1702):138–143. [PubMed: 20659933]
- Lee MK, Tuttle JB, Rebhun LI, Cleveland DW, Frankfurter A. The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. *Cell Motil Cytoskeleton.* 1990; 17(2):118–132. [PubMed: 2257630]
- Leuner B, Glasper ER, Gould E. Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. *The Journal of comparative neurology.* 2009; 517(2):123–133. [PubMed: 19731267]
- Liboska R, Ligasova A, Strunin D, Rosenberg I, Koberna K. Most anti-BrdU antibodies react with 2'-deoxy-5-ethynyluridine -- the method for the effective suppression of this cross-reactivity. *PLoS one.* 2012; 7(12):e51679. [PubMed: 23272138]
- Liu J, Dalmau J, Szabo A, Rosenfeld M, Huber J, Furneaux H. Paraneoplastic encephalomyelitis antigens bind to the AU-rich elements of mRNA. *Neurology.* 1995; 45(3 Pt 1):544–550. [PubMed: 7898713]
- Llorens-Martin M, Trejo JL. Multiple birthdating analyses in adult neurogenesis: a line-up of the usual suspects. *Front Neurosci.* 2011; 5:76. [PubMed: 21660291]
- Mandyam CD, Harburg GC, Eisch AJ. Determination of key aspects of precursor cell proliferation, cell cycle length and kinetics in the adult mouse subgranular zone. *Neuroscience.* 2007; 146:108–122. [PubMed: 17307295]
- Marusich MF, Furneaux HM, Henion PD, Weston JA. Hu neuronal proteins are expressed in proliferating neurogenic cells. *J Neurobiol.* 1994; 25(2):143–155. [PubMed: 7517436]
- Melleu FF, Santos TS, Lino-de-Oliveira C, Marino-Neto J. Distribution and characterization of doublecortin-expressing cells and fibers in the brain of the adult pigeon (*Columba livia*). *J Chem Neuroanat.* 2013; 47:57–70. [PubMed: 23137815]
- Mello CV, Ribeiro S. ZENK protein regulation by song in the brain of songbirds. *The Journal of comparative neurology.* 1998; 393(4):426–438. [PubMed: 9550149]
- Memberg SP, Hall AK. Dividing neuron precursors express neuron-specific tubulin. *J Neurobiol.* 1995; 27(1):26–43. [PubMed: 7643073]
- Mezey S, Krivokuca D, Balint E, Adorjan A, Zachar G, Csillag A. Postnatal changes in the distribution and density of neuronal nuclei and doublecortin antigens in domestic chicks (*Gallus domesticus*). *The Journal of comparative neurology.* 2012; 520(1):100–116. [PubMed: 21674497]
- Migaud M, Batailler M, Pilon D, Franceschini I, Malpoux B. Seasonal changes in cell proliferation in the adult sheep brain and pars tuberalis. *J Biol Rhythms.* 2011; 26(6):486–496. [PubMed: 22215607]
- Migaud M, Batailler M, Segura S, Duittoz A, Franceschini I, Pilon D. Emerging new sites for adult neurogenesis in the mammalian brain: a comparative study between the hypothalamus and the classical neurogenic zones. *The European journal of neuroscience.* 2010; 32(12):2042–2052. [PubMed: 21143659]
- Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron.* 2011; 70(4):687–702. [PubMed: 21609825]
- Mooney SM, Miller MW. Postnatal generation of neurons in the ventrobasal nucleus of the rat thalamus. *Journal of Neuroscience.* 2007; 27(19):5023–5032. [PubMed: 17494688]
- Moore CA, Perderiset M, Francis F, Chelly J, Houdusse A, Milligan RA. Mechanism of microtubule stabilization by doublecortin. *Mol Cell.* 2004; 14(6):833–839. [PubMed: 15200960]

- Mouriec K, Balthazart J. Peripubertal proliferation of progenitor cells in the preoptic area of Japanese quail (*Coturnix japonica*). *Brain Res.* 2013; 1516:20–32. [PubMed: 23623814]
- Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. *Development.* 1992; 116(1):201–211. [PubMed: 1483388]
- Nacher J, Alonso-Llosa G, Rosell D, McEwen B. PSA-NCAM expression in the piriform cortex of the adult rat. Modulation by NMDA receptor antagonist administration. *Brain Res.* 2002; 927(2):111–121. [PubMed: 11821005]
- Nacher J, Crespo C, McEwen BS. Doublecortin expression in the adult rat telencephalon. *The European journal of neuroscience.* 2001; 14(4):629–644. [PubMed: 11556888]
- Newman AE, MacDougall-Shackleton SA, An YS, Kriengwatana B, Soma KK. Corticosterone and dehydroepiandrosterone have opposing effects on adult neuroplasticity in the avian song control system. *The Journal of comparative neurology.* 2010; 518(18):3662–3678. [PubMed: 20653028]
- Nottebohm F. Brain pathways for vocal learning in birds: A review of the first 10 years. In: Sprague, JM.; Epstein, AN., editors. *Progress in Psychobiology and Physiological Psychology.* 0 ed.. Vol. 9. Academic Press; New York: 1980a. p. 85-214.
- Nottebohm F. Testosterone triggers growth of brain vocal control nuclei in adult female canaries. *Brain Res.* 1980b; 189:429–436. [PubMed: 7370785]
- Nottebohm F. A brain for all seasons: Cyclical anatomical changes in song-control nuclei of the canary brain. *Science.* 1981; 214:1368–1370. [PubMed: 7313697]
- Nottebohm F. The neural basis of birdsong. *PLoS Biol.* 2005; 3(5):e164. [PubMed: 15884976]
- Nottebohm, F. The discovery of replaceable neurons. In: Zeigler, HP.; Marler, P., editors. *Neuroscience of birdsong.* Cambridge University Press; Cambridge: 2008. p. 425-448.
- Nottebohm F, Arnold AP. Sexual dimorphism in vocal control areas of the songbird brain. *Science.* 1976; 194(4261):211–213. [PubMed: 959852]
- Nottebohm F, Stokes TM, Leonard CM. Central control of song in the canary, *Serinus canarius*. *JCompNeurol.* 1976; 165:457–486.
- Nowakowski RS, Rakic P. Clearance rate of exogenous 3H-thymidine from the plasma of pregnant rhesus monkeys. *Cell Tissue Kinet.* 1974; 7(2):189–194. [PubMed: 4205927]
- Ornelas IM, Silva TM, Fragel-Madeira L, Ventura AL. Inhibition of PI3K/Akt pathway impairs G2/M transition of cell cycle in late developing progenitors of the avian embryo retina. *PloS one.* 2013; 8(1):e53517. [PubMed: 23301080]
- Packard DS Jr. Menzies RA, Skalko RG. Incorporation of thymidine and its analogue, bromodeoxyuridine, into embryos and maternal tissues of the mouse. *Differentiation.* 1973; 1(6):397–404. [PubMed: 4802502]
- Paton JA, Nottebohm FN. Neurons generated in the adult brain are recruited into functional circuits. *Science.* 1984; 225(4666):1046–1048. [PubMed: 6474166]
- Perez-Martin M, Cifuentes M, Grondona JM, Lopez-Avalos MD, Gomez-Pinedo U, Garcia-Verdugo JM, Fernandez-Llebrez P. IGF-I stimulates neurogenesis in the hypothalamus of adult rats. *The European journal of neuroscience.* 2010; 31(9):1533–1548. [PubMed: 20525067]
- Rakic P. Neurogenesis in adult primate neocortex: an evaluation of the evidence. *Nat Rev Neurosci.* 2002a; 3(1):65–71. [PubMed: 11823806]
- Rakic P. Adult neurogenesis in mammals: an identity crisis. *J Neurosci.* 2002b; 22(3):614–618. [PubMed: 11826088]
- Rao MS, Shetty AK. Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *The European journal of neuroscience.* 2004; 19(2):234–246. [PubMed: 14725617]
- Rasika S, Nottebohm F, Alvarez-Buylla A. Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries. *Proceedings of the National Academy of Sciences of the United States of America.* 1994; 91(17):7854–7858. [PubMed: 8058723]
- Reitemeier S, Hanse M, Hahn A, Schmidt V, Steinbach-Sobiraj K, Krautwald-Junghanns ME, Einspanier A. Evaluating the reproductive status of the male budgerigar (*Melopsittacus undulatus*). *Gen Comp Endocrinol.* 2011; 171(3):350–358. [PubMed: 21377466]

- Rubini JR, Cronkite EP, Bond VP, Fliedner TM. The metabolism and fate of tritiated thymidine in man. *J Clin Invest.* 1960; 39:909–918. [PubMed: 14439902]
- Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. *Proceedings of the National Academy of Sciences of the United States of America.* 2008; 105(7): 2415–2420. [PubMed: 18272492]
- Saper CB. An open letter to our readers on the use of antibodies. *The Journal of comparative neurology.* 2005; 493(4):477–478. [PubMed: 16304632]
- Saper CB, Sawchenko PE. Magic peptides, magic antibodies: guidelines for appropriate controls for immunohistochemistry. *The Journal of comparative neurology.* 2003; 465(2):161–163. [PubMed: 12949777]
- Sartor JJ, Balthazart J, Ball GF. Coordinated and dissociated effects of testosterone on singing behavior and song control nuclei in canaries (*Serinus canaria*). *Hormones and behavior.* 2005; 47(4):467–476. [PubMed: 15777813]
- Schoenfeld TJ, Gould E. Stress, stress hormones, and adult neurogenesis. *Exp Neurol.* 2012; 233(1): 12–21. [PubMed: 21281629]
- Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol.* 2000; 182(3):311–322. [PubMed: 10653597]
- Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell.* 1992; 69(2):367–374. [PubMed: 1348971]
- Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, Huttner HB, Bostrom E, Westerlund I, Vial C, Buchholz BA, Possnert G, Mash DC, Druid H, Frisen J. Dynamics of hippocampal neurogenesis in adult humans. *Cell.* 2013; 153(6):1219–1227. [PubMed: 23746839]
- Stamatakis A, Barbas H, Dermon CR. Late granule cell genesis in quail cerebellum. *The Journal of comparative neurology.* 2004; 474(2):173–189. [PubMed: 15164421]
- Starosck RN, Jenkins WH, Mendelsohn ML. Availability of Tritiated Thymidine after Intravenous Administration. *Nature.* 1964; 202:456–458. [PubMed: 14167821]
- Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, Henson J, Posner JB, Furneaux HM, HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. *Cell.* 1991; 67(2):325–333. [PubMed: 1655278]
- Taupin P. BrdU immunohistochemistry for studying adult neurogenesis: paradigms, pitfalls, limitations, and validation. *Brain Res Rev.* 2007; 53(1):198–214. [PubMed: 17020783]
- Thatra, NM.; Larson, TA.; Benowitz, EA. *Abst Soc Neurosciences.* San Diego CA: 2013. Turnover of adult born neurons in the avian song control system. *Abst* 869.802
- Thompson CK, Brenowitz EA. Seasonal change in neuron size and spacing but not neuronal recruitment in a basal ganglia nucleus in the avian song control system. *The Journal of comparative neurology.* 2005; 481(3):276–283. [PubMed: 15593375]
- Valero J, Weruaga E, Murias AR, Recio JS, Alonso JR. Proliferation markers in the adult rodent brain: bromodeoxyuridine and proliferating cell nuclear antigen. *Brain Res Brain Res Protoc.* 2005; 15(3):127–134. [PubMed: 16024267]
- Vellema M, Hertel M, Urbanus SL, Van der Linden A, Gahr M. Evaluating the predictive value of doublecortin as a marker for adult neurogenesis in canaries (*Serinus canaria*). *The Journal of comparative neurology.* 2014; 522:1299–1315. [PubMed: 24115109]
- Vellema M, van der Linden A, Gahr M. Area-specific migration and recruitment of new neurons in the adult songbird brain. *The Journal of comparative neurology.* 2010; 518(9):1442–1459. [PubMed: 20187140]
- Verwer RW, Sluiter AA, Balesar RA, Baayen JC, Noske DP, Dirven CM, Wouda J, van Dam AM, Lucassen PJ, Swaab DF. Mature astrocytes in the adult human neocortex express the early neuronal marker doublecortin. *Brain: a journal of neurology.* 2007; 130(Pt 12):3321–3335. [PubMed: 18055496]
- Veyrac A, Gros A, Bruel-Jungerman E, Rochefort C, Kleine Borgmann FB, Jessberger S, Laroche S. *Zif268/egr1* gene controls the selection, maturation and functional integration of adult hippocampal newborn neurons by learning. *Proceedings of the National Academy of Sciences of the United States of America.* 2013; 110(17):7062–7067. [PubMed: 23569253]

- von Bohlen Und Halbach O. Immunohistological markers for staging neurogenesis in adult hippocampus. *Cell Tissue Res.* 2007; 329(3):409–420. [PubMed: 17541643]
- Wakamatsu Y, Weston JA. Sequential expression and role of Hu RNA-binding proteins during neurogenesis. *Development.* 1997; 124(17):3449–3460. [PubMed: 9310339]
- Warren M, Puskarczyk K, Chapman SC. Chick embryo proliferation studies using EdU labeling. *Dev Dyn.* 2009; 238(4):944–949. [PubMed: 19253396]
- Winner B, Cooper-Kuhn CM, Aigner R, Winkler J, Kuhn HG. Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. *The European journal of neuroscience.* 2002; 16(9):1681–1689. [PubMed: 12431220]
- Winner B, Couillard-Despres S, Geyer M, Aigner R, Bogdahn U, Aigner L, Kuhn HG, Winkler J. Dopaminergic lesion enhances growth factor-induced striatal neuroblast migration. *J Neuropathol Exp Neurol.* 2008; 67(2):105–116. [PubMed: 18219258]
- Yamamura T, Barker JM, Balthazart J, Ball GF. Androgens and estrogens synergistically regulate the expression of doublecortin and enhance neuronal recruitment in the song system of adult female canaries. *J Neurosci.* 2011; 31(26):9649–9657. [PubMed: 21715630]
- Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. *Cell.* 2008; 132(4):645–660. [PubMed: 18295581]
- Ziegler, HP.; Marler, P. *Neuroscience of Birdsong.* Cambridge University Press; Cambridge, UK: 2008. p. 550

Figure 1.
Serum concentrations of BrdU in canaries at various times (in minutes) after a single injection of BrdU at 100 mg/kg. All values are means $\pm$ SD. Redrawn from data in (Barker et al., 2013).

Figure 2.

Schematic representation of the different stages in the life of a neuron born in the adult mammalian brain and examples of the characteristic proteins expressed at these stages. The figure also indicates when cells become postmitotic. Redrawn from data in (Kemperman, 2011; Kempermann et al., 2004; Ming and Song, 2011).

Figure 3.

The canary HVC can be clearly distinguished from the surrounding nidopallium in section stained by antibodies directed against the neuronal proteins HuC/D. The dotted line indicates the ventral border of the nucleus. Magnification bar= 200 μ m.

Figure 4.

Photomicrographs illustrating the doublecortin-immunoreactive (DCX-ir) cells in the song control nucleus HVC of a male canary. **A.** HVC is identified by a higher density of DCX-ir cells as compared to the surrounding nidopallium. **B.** At higher magnification, two types of DCX-ir cells can clearly be distinguished: uni- or bipolar fusiform cells that are presumably migrating neurons (Arrows) and round multipolar cells that have presumably reached their destination and initiated their final differentiation (Arrow heads). Magnification bar= 200 μm in A and 50 μm in B.

Figure 5.

Effects of endocrine (A-B), social (C-D) and environmental (E-F) stimuli on the number of fusiform (A, C, E) or round (D, D, F) DCX-ir cells in HVC. In these studies, numbers of DCX-ir cells were compared in castrated males canaries (CX) that had been treated or not with testosterone (CX vs. CX+T, panels A-B), in CX+T males that were housed with another male (M-M) or a female (M-F; panels C-D) and in gonadally intact birds that had been exposed to various photoperiodic regimes that make them photorefractory (Refr), photosensitive (Sens) or photostimulated (Stim; panels E-). No change in the number of DCX-ir cells was usually detected in two equivalent areas located laterally and ventrally to HVC. The brain drawing in panel G schematically illustrates the distribution of DCX-ir cells in the brain and the location of areas in which systematic quantification was performed. NCM: nidopallium caudomediale; N: nidopallium; RA: nucleus robustus arcopallialis; V: lateral ventricle. * $P < 0.05$ as compared with the other experimental group. Redrawn from data in (Balthazart et al., 2008).

Table 1

Numbers of fusiform and round doublecortin-immunoreactive (DCX-ir) cells and of BrdU-positive cells in the canary HVC following multiple injections of 50 mg/kg of BrdU (two injections/day for 5 days). The table also indicates the absolute numbers of double-labeled cells and their relative numbers (in %) compared to the total numbers of DCX-ir or BrdU positive cells. All data are means $\pm$ SD. Data from (Balthazart et al., 2008) with additional unpublished calculations (% of BrdU-positive cells that express DCX) on the same data.

	DCX only (nbr)		DCX+BrdU (nbr)		BrdU only (nbr)	DCX+BrdU/ DCX (%)		DCX+BrdU/ BrdU (%)
	Fusiform	Round	Fusiform	Round		Fusiform	Round	
10 days	9.50 $\pm$	30.00 $\pm$	27.25 $\pm$	1.50 $\pm$	11.75 $\pm$	72.14$\pm$	5.03$\pm$	73.38$\pm$
	1.29	8.87	10.81	0.58	9.07	10.25	2.54	7.57
30 days	12.50 $\pm$	34.00 $\pm$	5.75 $\pm$	10.50 $\pm$	5.75 $\pm$	30.95$\pm$	24.17$\pm$	75.37$\pm$
	1.71	12.73	3.10	4.20	4.35	13.65	8.44	13.22