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PROLIFERATING CELLS IN THE ADOLESCENT RAT AMYGDALA: CHARACTERIZATION AND RESPONSE TO STRESS

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Abstract—The amygdala is a heterogeneous group of nuclei that plays a role in emotional and social learning. As such, there has been increased interest in its development in adolescent animals, a period in which emotional/social learning increases dramatically. While many mechanisms of amygdala development have been studied, the role of cell proliferation during adolescence has received less attention. Using bromodeoxyuridine (BrdU) injections in adolescent and adult rats, we previously found an almost fivefold increase in BrdU-positive cells in the amygdala of adolescents compared to adults. Approximately one third of BrdU-labeled cells in the amygdala contained the putative neural marker doublecortin (DCX), suggesting a potential for neurogenesis. To further investigate this possibility in adolescents, we examined the proliferative dynamics of DCX/BrdU-labeled cells. Surprisingly, DCX/BrdU-positive cells were found to comprise a stable subpopulation of BrdU-containing cells across survivals up to 56 days, and there was no evidence of neural maturation by 28 days after BrdU injection. Additionally, we found that approximately 50% of BrdU+ cells within the adolescent amygdala contain neural-glial antigen (NG2) and are therefore presumptive oligodendrocyte precursors (OPCs). We next characterized the response to a short-lived stressor (3-day repeated variable stress, RVS). The total BrdU-labeled cell number decreased by ~30% by 13 days following RVS (10 days post-BrdU injection) as assessed by stereologic counting methods, but the DCX/BrdU-labeled subpopulation was relatively resistant to RVS effects. In contrast, NG2/BrdU-labeled cells were strongly influenced by RVS. We conclude that typical neurogenesis is not a feature of the adolescent amygdala. These findings point to several possibilities, including the possibility that DCX/BrdU cells are late-developing neural precursors, or a unique subtype of NG2 cell that is relatively resistant to stress. In contrast, many proliferating OPCs are significantly impacted by a short-lived stressor, suggesting consequences for myelination in the developing amygdala.

Key words: bromodeoxyuridine, doublecortin, NeuN, NG2, TUNEL.

INTRODUCTION

The amygdala is a group of clustered heterogeneous subnuclei important for integrating approach-avoidance behaviors, such as social behaviors and fear responses, and determining the salience of stimuli (Ernst and Fudge, 2009; Schumann et al., 2011). We have been interested in the growth and maturation of the amygdala during development, particularly during adolescence (Fudge, 2004; Fudge et al., 2012; Saul et al., 2013). Recently, cell proliferation and neurogenesis within the brain have emerged as important mechanisms of plasticity or change in postnatal animals (Lledo et al., 2006). In the current study, we wanted to further characterize cell proliferation within the amygdala of an adolescent animal.

Using male Sprague–Dawley rats, we previously reported that the amygdala of both adolescent and adult animals contains a population of proliferating cells, identified by the incorporation of bromodeoxyuridine (BrdU) administered either during adolescence or adulthood, and that there is over four times more of these cells found in the adolescent as compared to the adult animal (Saul et al., 2013). This finding of a greater number of proliferating cells in adolescence, compared to the adult, may reflect greater plasticity that is apparent in adolescents compared to adults (Spear, 2013) and is also similar to previous work reporting age-related changes in cell proliferation (He and Crews, 2007; Rubinow and Juraska, 2009; Amrein et al., 2011).

We also found that a proportion of these BrdU-positive cells contains doublecortin (DCX), a microtubule protein commonly found in immature neurons (Brown et al., 2003). In our previous work (Saul et al., 2013), we demonstrated that 30% of BrdU-positive cells within the amygdala are DCX-positive, when analyzed 10 days post-BrdU administration. We chose this time-point because previous work, focused on the classic neurogenic regions of the sub-ventricular zone (SVZ) and the

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Abbreviations: BrdU, bromodeoxyuridine; DCX, doublecortin; NeuN, neuronal nuclei; NG2, neural-glial antigen; OPCs, oligodendrocyte precursors; PND, post-natal day; RVS, repeated variable stress; SGZ, sub-granular zone; SVZ, sub-ventricular zone; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated UTP nick end labeling.

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sub-granular zone (SGZ) of the hippocampus, has indicated that recent proliferating cells that are destined to be neurons express DCX at 10 days post-BrdU (Brown et al., 2003). This raises the question of whether the BrdU-positive cells we detected in the amygdala are neural precursors (Alvarez-Buylla and Garcia-Verdugo, 2002; Brown et al., 2003), or another unspecified cell type. Postnatal cell proliferation (Antzoulatos et al., 2008; Ehninger et al., 2011), and the presence of immature-appearing neurons has been reported in the amygdala of several species (Bernier et al., 2002; Fowler et al., 2003; Orre et al., 2009; Shapiro et al., 2009; Fudge et al., 2012; Lieberwirth et al., 2012), and immature-appearing neurons have been found in circumscribed regions of the amygdala in adult primates (Bernier et al., 2002; Fudge, 2004; Fudge et al., 2012; Marti-Mengual et al., 2013). Furthermore, cell-proliferation and/or young differentiating neurons have been identified within the amygdala of adults of other species, such as voles (Fowler et al., 2003; Lieberwirth et al., 2012), hamster (Antzoulatos et al., 2008; Mohr and Sisk, 2013), marmoset (Marlatt et al., 2011), and mice (Okuda et al., 2009; Shapiro et al., 2009; Ehninger et al., 2011), and in certain strains (i.e. Wistar) (Goncalves et al., 2008; Orre et al., 2009) or states (post-partum) (Akbari et al., 2007) of the laboratory rat (Jiang et al., 2014).

However, the concept of postnatal neurogenesis outside of the classic neurogenic zones of the SVZ and the SGZ is controversial (Gould et al., 1999, 2001; Rakic, 2002; Breunig et al., 2007; Ehninger et al., 2011). There are other proliferating cell populations within the CNS, in addition to neuroblasts, including glia, neural-glial antigen (NG2) cells, epithelial (RECA) cells and microglia (Banasr et al., 2007). NG2 has been called the '4th type' of glia (Peters, 2004) and is the most proliferative cell type in the brain (Dawson et al., 2003). NG2 cells are unique cells; one primary role is as oligodendrocyte precursors (OPCs), yet NG2 cells in their immature undifferentiated state also receive neural inputs, some may be excitable and form action potentials, and their proliferation and motility are influenced by glutamate (Yuan et al., 1998; Gudz et al., 2006; Ehninger et al., 2011). The fate of determination of NG2 cells is also not completely understood; some remain undifferentiated, many develop into oligodendrocytes and some may differentiate into neurons, although the latter finding is controversial (Belachew et al., 2003; Tamura et al., 2007; Rivers et al., 2008; Richardson et al., 2011; Dimou and Gallo, 2015). Sensory deprivation in newborn mice also increases NG2 proliferation, disrupting the normal distribution pattern in barrel cortex by post-natal day (PND) 6 (Mangin et al., 2012). Since early somatosensory deprivation also strongly affects the development of experience-dependent plasticity (Rema et al., 2003) and eventual myelin distribution in the cortex (Barrera et al., 2013), this suggests that early experience influences on NG2 proliferation and migration may have long-term effects on myelination and neural function.

Since proliferating cells are abundant in adolescent amygdala, in the current series of experiments we wanted to begin to further characterize this population, based on both the phenotype of the cells and whether these cells are affected by changes in the animals' environment. Previous work has indicated that cell proliferation, including neurogenesis, can be inhibited by stress (Gould et al., 1998; Wennstrom et al., 2006; Banasr and Duman, 2007; Banasr et al., 2007). Therefore, after initial characterization of the BrdU-positive cells in the adolescent rodent amygdala, we examined the impact of a repeated variable stress (RVS), a paradigm that we have used previously (Saul et al., 2012).

EXPERIMENTAL PROCEDURES

Overall design

The current set of studies aims to examine the large proliferating cell population we found in the adolescent brain (Saul et al., 2013). Adolescence is a developmental time period that encompasses the changes that occur between infancy and adulthood to create a physically and socially mature organism (Poveda, 1972; Giedd et al., 1999; Spear, 2000; Rubinow and Juraska, 2009). In the rat, adolescence is defined by changes in behavior, including increased exploratory, novelty-seeking and risk-taking behaviors (Siviy et al., 2006; Tsoory and Richter-Levin, 2006). Based on the expression of these behaviors, PND 28 until PND 42 is a generally accepted age range for adolescence in rats of both sexes (Spear and Brake, 1983; Watt et al., 2009), though individual differences exist. We chose to examine changes to proliferating cells during the period coinciding with the onset of these behaviors.

Adolescent male Sprague–Dawley rats (Charles River Laboratories, Wilmington MA, USA) were used in all studies, and were shipped to the University of Rochester Vivarium facility (Table 1). Cohort 1 animals used in the *Characterization studies* arrived at post natal day (PND) 25; Cohort 2 animals used for *Response to Stress studies* arrived at PND 22. All animals in both cohorts were pair-housed with animals of the same treatment group, had *ad libitum* access to food and water, lights on 0730–1930 h, and were allowed to acclimate for 5 days prior to handling. All animals in both Cohorts were handled and weighed three times per week from the point of arrival through completion of the studies. All studies were previously approved by the University of Rochester Committee on Animal Resources.

Characterization of proliferating cells. Since 30% of BrdU-positive cells in the normal adolescent amygdala co-localize with DCX ten-days post BrdU administration (Saul et al., 2013), we first examined the idea that some of these cells were neuroblasts. In neurogenic zones such as the dentate gyrus and olfactory bulb, pulse-chase studies show that BrdU-positive cells express DCX by 10-days post BrdU administration; DCX levels then decline and are replaced by the mature neuronal marker, neuronal nuclei (NeuN) (Brown et al., 2003). We used a similar approach to determine the phenotype, development, and differentiation of BrdU-positive cells in the adolescent amygdala.