Postnatal Development of the Amygdala: A Stereological Study in Rats

Loïc J. Chareyron,[1] Pamela Banta Lavenex,[1,2] and Pierre Lavenex[1,2]*

[1]Laboratory of Brain and Cognitive Development, Department of Medicine, University of Fribourg, Fribourg, CH-1700 Switzerland
[2]Fribourg Center for Cognition, University of Fribourg, Fribourg, CH-1700 Switzerland

ABSTRACT

The amygdala is the central component of a functional brain system regulating fear and emotional behaviors. Studies of the ontogeny of fear behaviors reveal the emergence of distinct fear responses at different postnatal ages. Here, we performed a stereological analysis of the rat amygdala to characterize the cellular changes underlying its normal structural development. Distinct amygdala nuclei exhibited different patterns of postnatal development, which were largely similar to those we have previously shown in monkeys. The combined volume of the lateral, basal, and accessory basal nuclei increased by 113% from 1 to 3 weeks of age and by an additional 33% by 7 months of age. The volume of the central nucleus increased only 37% from 1 to 2 weeks of age and 38% from 2 weeks to 7 months. At 1 week of age, the medial nucleus was 77% of the 7-month-old's volume and exhibited a constant, marginal increase until 7 months. Neuron number did not differ in the amygdala from 1 week to 7 months of age. In contrast, astrocyte number decreased from 3 weeks to 2 months of age in the whole amygdala. Oligodendrocyte number increased in all amygdala nuclei from 3 weeks to 7 months of age. Our findings revealed that distinct amygdala nuclei exhibit different developmental profiles and that the rat amygdala is not fully mature for an extended period postnatally. We identified different periods of postnatal development of distinct amygdala nuclei and cellular components, which are concomitant with the ontogeny of different fear and emotional behaviors.

INDEXING TERMS: amygdaloid complex; fear; ontogeny; neurons; astrocytes; oligodendrocytes; neuropil

The amygdala is the central component of a functional brain system regulating fear and emotional behaviors (Amaral et al., 2003; LeDoux, 2000a; Rosen, 2004). The emergence of fear responses is of particular interest, because what appears to be a unique learning process in adults separates across different effector systems during development (Prather et al., 2001; Stanton, 2000). Individual components of the fear response are thought to incorporate sequentially as animals mature, eventually giving rise to what are identified as mature fear behaviors (Wiedenmayer, 2009). Indeed, several studies have revealed that distinct fear behaviors emerge at different times during early postnatal life and continue to mature during late postnatal development (Blozovski and Cudennec, 1980; Bronstein and Hirsch, 1976; Chen et al., 2006; Collier et al., 1979; Foster and Burman, 2010; Hefner and Holmes, 2007; Hubbard et al., 2004; Ito et al., 2009; Kim and Richardson, 2007; Moriceau et al., 2004; Raineki et al., 2010; Rudy, 1993; Takahashi, 1992; Wiedenmayer and Barr, 1998, 2001a,b).

The gradual emergence and maturation of fear responses are likely due to the development of the brain structures underlying these behaviors in mature individuals. To date, however, there has been no systematic investigation of the morphological characteristics, such as cell numbers and volumes of the main amygdala nuclei, during the first months of life in the rat. It is thus important to define and characterize the postnatal development of the amygdala in order to understand the emergence of fear behaviors. We have previously used stereological techniques to describe the postnatal development of the monkey amygdala (Chareyron et al., 2012). In

contrast, only scant information is available regarding the postnatal development of the rat amygdala. Berdel and colleagues (1997a) reported that the number of neurons increases in the rat lateral and basal nuclei up to P7. They also reported an increase in the volume of the rat lateral and basal nuclei during the first 2 weeks of postnatal life, which continued at a much slower rate until 180 days of age (Berdel et al., 1997b). Rubinow and Juraska (2009) reported a volumetric increase of the basal nucleus from P20 to P35, which was followed by a 15% increase until 19–22 months of age. Mizukami et al. (1983) reported that the volume of the medial nucleus increases from P1 to P5 and reaches an adult value at about P5 in females and P11 in males. These findings suggest that the postnatal maturation of the rat amygdala might extend for several weeks or months before reaching an adult-like state.

The aim of the present study was to provide quantitative neuroanatomical information about the postnatal maturation of the rat amygdala. We implemented design-based stereological techniques to characterize the cellular development of the Sprague-Dawley rat (*Rattus norvegicus*) amygdaloid complex from 1 week to 7 months of age. We aimed to determine when the volumes and cell numbers of its five main nuclei (i.e., lateral, basal, accessory basal, central, and medial) reach an adult-like state.

MATERIALS AND METHODS
Experimental animals
Twenty-eight Sprague-Dawley rats (*R. norvegicus*) were used for this study: four 1-week-olds, four 2-week-olds, four 3-week-olds (we did not determine the sex of individuals before 4 weeks of age), four 4-week-olds (two males, two females), four 2-month-olds (two males, two females), four 3-month-olds (two males, two females), and four 7-month-olds (two males, two females). Pregnant females as well as 3-month- and 7-month-old rats were obtained from commercial suppliers (Charles River, San Diego, CA; Harlan Laboratories, Venray, The Netherlands). Pregnant females were housed singly in standard laboratory cages with food and water ad libitum. Pups were housed in three different litters before killing. One-week-, 3-week-, 4-week-, 2-month-, 3-month- and 7-month-old rats came from two different litters housed at UC Davis. Two animals per litter, one male and one female in each age group, were removed from the litter and immediately anesthetized and perfused. Two-week-old rats and one 3-week-old rat came from a third litter housed at the University of Fribourg. All experimental procedures were approved by the Institutional Animal Care and Use Committee of UC Davis (protocol 10858) or the Fribourg Veterinary Commission (authorization FR6/10)

and were conducted in accordance with the U.S. National Institutes of Health guidelines for the use of animals in research.

Brain acquisition
At the time of killing, animals were deeply anesthetized with either an i.p. injection of 50 mg/kg pentobarbital (Davis) or a subcutaneous injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine (Fribourg) and perfused transcardially with 1% and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were postfixed for 6 hours in the same fixative, cryoprotected in 10% and 20% glycerol solutions in 0.1 M phosphate buffer (pH 7.4; for 24 and 72 hours respectively), frozen in powdered dry ice, and stored at −70°C until sectioning.

Coronal sections were cut using a freezing, sliding microtome (Microm HM 450) in four series at 40 μm. One series was collected in 10% formalin solution in 0.1 M PB (pH 7.4) and postfixed at 4°C for 4 weeks prior to Nissl staining with thionin.

Histological processing
The procedure for Nissl-stained sections followed our standard laboratory protocol described previously (Chareyron et al., 2011; Lavenex et al., 2009). Briefly, sections were taken from the 10% formaldehyde solution, thoroughly washed, mounted on gelatin-coated slides, and air dried overnight at 37°C. Sections were then defatted for 2 × 1 hour in a mixture of chloroform/ethanol (1:1, vol), partially rehydrated, and air dried overnight at 37°C. Sections were then fully rehydrated and stained for 40 seconds in a 0.25% thionin solution (Fisher Scientific, Waltham, MA; catalog No. T-409), dehydrated, and coverslipped with DPX (BDH Laboratories, Poole, United Kingdom).

Anatomical boundaries of the amygdala
The nomenclature and the basic description of the morphological characteristics of the rat amygdala nuclei have been described in detail previously (Cassell et al., 1986; Jolkkonen and Pitkänen, 1998; Krettek and Price, 1978; McDonald, 1982; Pitkänen, 2000; Pitkänen et al., 1995; Price et al., 1987; Savander et al., 1995, 1996). We used these descriptions to determine the boundaries of the five main nuclei (i.e., lateral, basal, accessory basal, central, and medial) of the rat amygdala (Fig. 1; Chareyron et al., 2011).

Stereological analyses
Volume measurements and neuron and glia counts were performed with StereoInvestigator 9.0 (MicroBrightField, Williston, VT). We estimated the volume of the brain (telencephalon and diencephalon), the volume of the
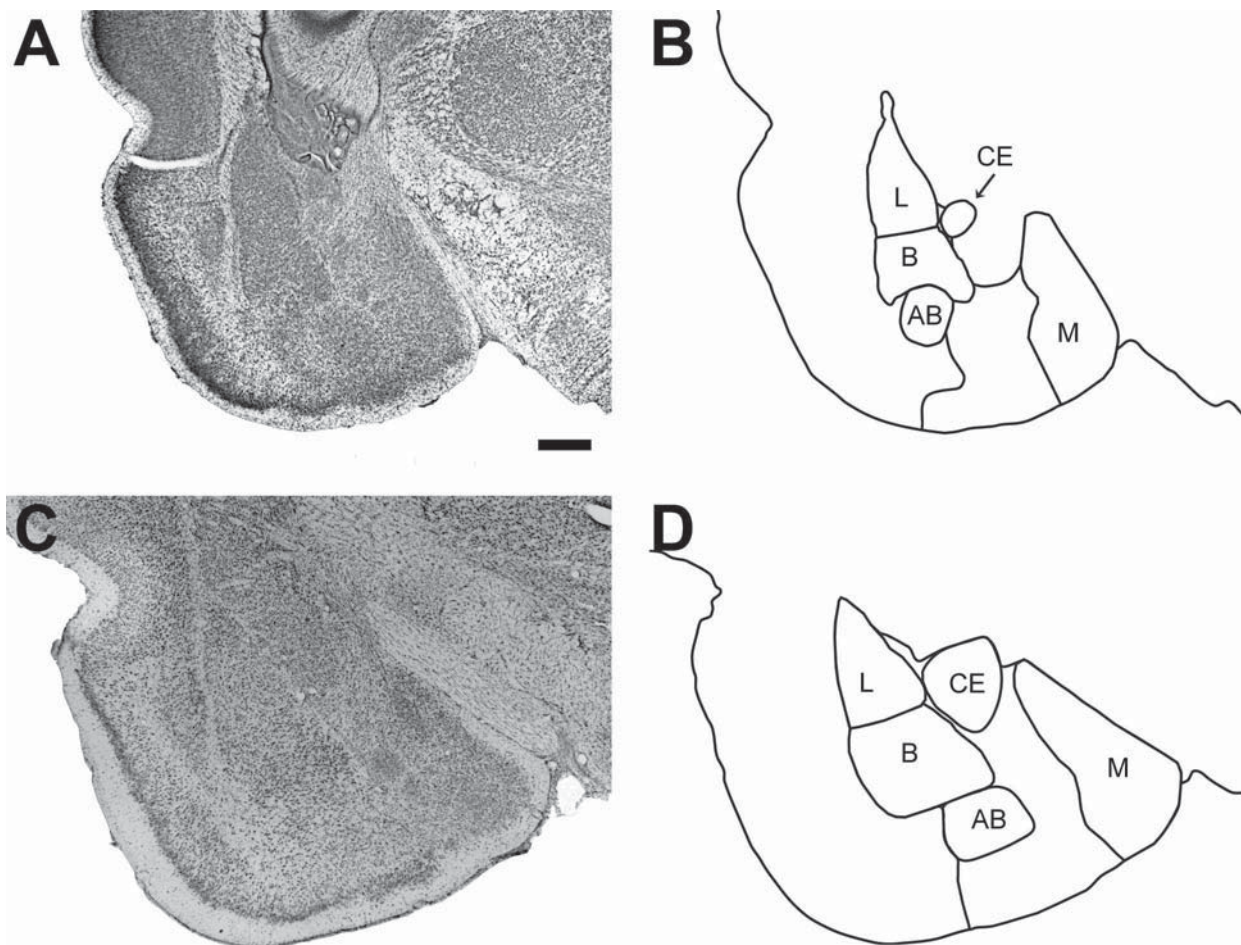
Figure 1. Low-magnification photomicrographs of a representative coronal section at a midstrocaudal level of the rat (*Rattus norvegicus*) amygdala, which illustrate the locations of the five main amygdala nuclei in a 1-week-old (**A,B**) and a 7-week-old (**C,D**) rat. L, lateral; B, basal; AB, accessory basal; CE, central; M, medial. Nonlabeled areas include the remaining nuclei of the amygdala (see Chareyron et al., 2011). Scale bar = 500 μm.

whole amygdala, and the volume of the main amygdala nuclei (lateral, basal, accessory basal, central, and medial) according to the Cavalieri principle on Nissl-stained sections cut at 40 μm (Gundersen and Jensen, 1987; Lavenex et al., 2000a,b; West and Gundersen, 1990). We used the section cutting thickness (40 μm) to calculate the volume. *Brain volume* refers to the volume of the telencephalon and diencephalon bilaterally (ventricles were excluded). Seventeen to thirty-two sections per animal (480 μm apart), with the first section selected randomly within the first three sections through the brain, were used for brain volume measurements. We estimated the volumes of individual amygdala nuclei in the left hemisphere for half of the animals and in the right hemisphere for the other half (balanced across sexes). On average, 23 sections per animal (160 μm apart) were used to measure the volume of the whole amygdala. About 13 sections per animal (160 μm apart) were used for volume

estimates of the main amygdala nuclei (for a complete list of amygdala nuclei see Chareyron et al., 2011).

Total numbers of neurons in the main amygdala nuclei were estimated by using the optical fractionator method (Gundersen, 1986; West et al., 1991). This design-based method allows an estimation of cell number that is independent of volume estimates. Neuron number was estimated in the right or in the left amygdala only, as for volume measurements. About nine sections per animal (160 μm apart, 320 μm for the medial nucleus with the first section selected randomly within the first two sections through the nucleus) were used for neuron counts (Table 1). We used a ×100 PlanFluor oil objective (N.A. 1.30) on a Nikon Eclipse 80i microscope (Nikon Instruments, Melville, NY) linked to PC-based StereoInvestigator 9.0. The sampling scheme was established to obtain individual estimates of neuron number with estimated coefficients of error: $CE = sqrt(CE^2[\Sigma Q] + CE^2[t]); CE[\Sigma Q] = sum [Q_i] +$

3

Amygdala nucleus	Average number of sections (range)	Distance between sections (µm)	Scan grid (µm)[1]	Counting frame (µm)	Disector height (µm)[2]	Guard zones (µm)[2]	Average section thickness (µm; range)[3]	Average number of cells counted (range)		
								Neurons	Oligodendrocytes	Astrocytes
Lateral	13 (9–17)	160	250 × 250	40 × 40	4–3	2–1.5	10.00 (6.07–12.72)	268 (174–346)	25 (0–80)	150 (77–252)
Basal	14 (12–17)	160	250 × 250	40 × 40	4–3	2–1.5	11.19 (6.32–14.30)	228 (141–352)	34 (1–111)	133 (69–226)
Accessory basal	7 (4–10)	160	200 × 200	40 × 40	4–3	2–1.5	10.48 (6.56–13.04)	194 (132–230)	18 (0–58)	103 (61–147)
Central	9 (6–11)	160	230 × 230	40 × 40	4–3	2–1.5	11.19 (6.83–14.12)	330 (205–438)	34 (0–123)	124 (68–185)
Medial	5(4–6)	320	250 × 250	40 × 40	4–3	2–1.5	10.28 (7.05–12.52)	217 (143–312)	10 (0–49)	91 (56–150)

[1]Scan grid was placed in random orientation.
[2]Disector height was set at 3 µm and guard zones were set at 1.5 µm for 1-week-old rats only.
[3]Section thickness was measured at every counting site.

$[(3 \times (\text{sum} [Q_i \times Q_i] - \text{sum} (Q_i)) - (4 \times \text{sum} [Q_i \times Q_{i+1}] + [Q_i \times Q_{i+2}])/12$; CE(t) = standard deviation (section thickness)/average (section thickness) about 0.10 (CE average [neurons] = 0.111). This sampling scheme was the same as that used in our previous study of the adult rat amygdala (Chareyron et al., 2011), except for the medial nucleus. Section thickness was measured at every counting site (Table 1).

The total number of glial cells in the main amygdala nuclei was estimated by using the optical fractionator method during neuron counting. Thus, the same sampling scheme used for neuron counts was used for glial cell counts (CE average [astrocytes] = 0.132, CE average [oligodendrocytes] = 0.379). We distinguished neurons, oligodendrocytes, and astrocytes based on morphological criteria identifiable in Nissl preparations (Chareyron et al., 2011; Fig. 2). We refer the reader to original publications (Fitting et al., 2008; Grady et al., 2003; Hamidi et al., 2004; Palackal et al., 1993) for detailed descriptions. Briefly, neurons are darkly stained and make up a single large nucleolus. Astrocytes are relatively smaller and exhibit pale staining of the nucleus. Oligodendrocytes are smaller than astrocytes and contain round, darkly staining nuclei that are densely packed with chromatin. Microglia were not counted but could be identified because they have the smallest nucleus, dark staining, and an irregular shape that is often rod-like, oval, or bent (Morris et al., 2008).

Photomicrographic production

Photomicrographs were taken with a Leica DFC490 digital camera on a Nikon Eclipse 80i microscope (Nikon Instruments, Tokyo, Japan). Artifacts located outside of the sections were removed, and levels were adjusted in Adobe Photoshop CS4, version 11.0 (Adobe, San Jose, CA), to improve contrast and clarity.

Statistical analysis

We performed ANOVAs with age as a factor on the estimates of the volume, neuron, astrocyte, and oligodendrocyte numbers, because these data were normally distributed. Post hoc analyses were performed with the Fisher PLSD test. Significance level was set at $P < 0.05$ for all analyses. We evaluated both left and right amygdala nuclei in a systematic manner (as described above) and found only one consistent lateralization effect: the number of neurons in the medial nucleus was higher in the right hemisphere across all ages ($F_{1,14} = 4.833$, $P = 0.045$; data not shown). We did not consider potential sex differences because of the relatively low number of individuals (only two males and two females per age group).

All sections used in this study were coded to allow blind analysis, and the code was broken only after completion of the analyses, with the exception of the cell counts for the
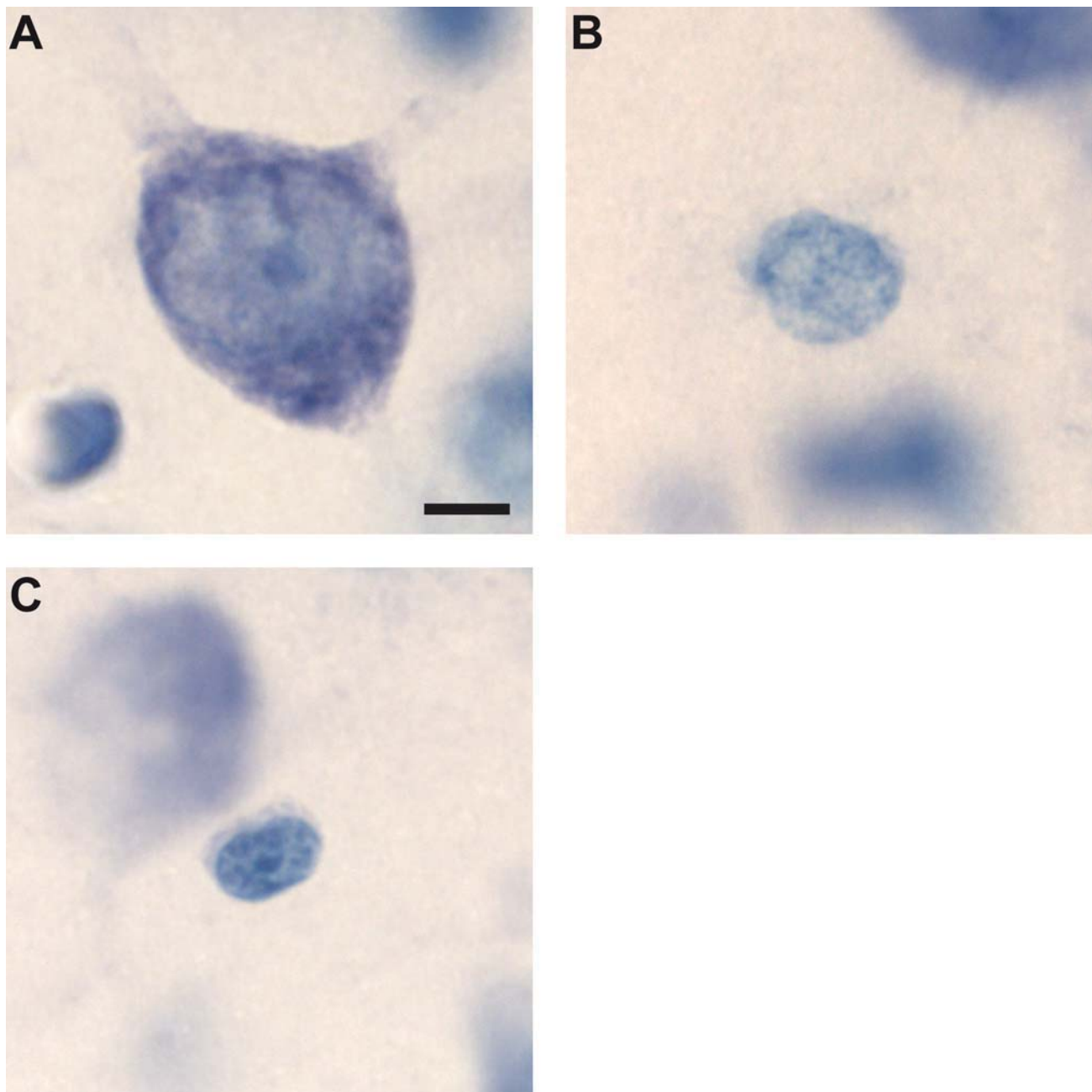
Figure 2. Classification and identification of different cell types in the basal nucleus of the 7-month-old rat amygdala, viewed with a ×100 objective in Nissl-stained, coronal sections cut at 40 μm. **A:** Neuron. **B:** Astrocyte. **C:** Oligodendrocyte. Scale bar = 5 μm.

7-month-old rats, which were performed first (after all volume estimates had been performed) and published in a separate article (Chareyron et al., 2011). All analyses were performed by the same experimenter (L.J.C.).

RESULTS
Volumes

The volumes of the rat brain, amygdala, and its five main nuclei at different postnatal ages are summarized in Table 2. Note that all these volumes were estimated following brain fixation by perfusion with 4% paraformaldehyde, using frozen coronal sections cut at 40 μm and Nissl-stained with thionin. The volume of the brain (telencephalon and diencephalon, bilaterally) differed between age groups ($F_{6,21}$ = 45.939, $P < 0.001$). At 1 week of age, brain volume was only 35% of the 7-month-olds' volume; it was 85% at 3 months. Brain volume increased continuously from 1 week to 7 months of age (1-week < 2-week < 3-week < 3-month < 7-month, all $P < 0.016$).

TABLE 2.

Volume and Cell Numbers of the Five Main Nuclei of the Rat Amygdala at Different Ages During Early Postnatal Development (±standard deviation) Along With Volumes of the Brain and Amygdala

Age	Lateral nucleus	Basal nucleus	Accessory basal nucleus	Central nucleus	Medial nucleus	Amygdala	Brain[1]
Volume (mm^3)							
1-Week	0.34 ± 0.03	0.53 ± 0.04	0.21 ± 0.02	0.52 ± 0.04	0.98 ± 0.10	5.32 ± 0.28	350 ± 13
2-Week	0.74 ± 0.04	0.83 ± 0.02	0.35 ± 0.02	0.71 ± 0.01	1.03 ± 0.14	7.00 ± 0.25	560 ± 42
3-Week	0.87 ± 0.06	0.97 ± 0.02	0.46 ± 0.02	0.77 ± 0.10	0.98 ± 0.08	7.65 ± 0.46	675 ± 39
4-Week	0.87 ± 0.05	0.97 ± 0.17	0.43 ± 0.05	0.67 ± 0.11	0.93 ± 0.11	7.83 ± 0.68	696 ± 41
2-Month	0.93 ± 0.03	0.97 ± 0.03	0.47 ± 0.03	0.70 ± 0.06	1.10 ± 0.13	8.50 ± 0.40	792 ± 40
3-Month	0.95 ± 0.11	0.98 ± 0.04	0.47 ± 0.01	0.78 ± 0.06	1.08 ± 0.18	8.89 ± 1.00	843 ± 87
7-Month	1.19 ± 0.10	1.20 ± 0.06	0.63 ± 0.07	1.00 ± 0.14	1.28 ± 0.23	10.62 ± 1.02	994 ± 110
Neuron number							
1-Week	99,758 ± 9,080	110,264 ± 9,101	45,114 ± 1,812	120,319 ± 2,496	190,709 ± 34,321		
2-Week	104,407 ± 16,232	91,594 ± 6,073	47,449 ± 2,958	127,691 ± 5,694	198,736 ± 3,996		
3-Week	115,465 ± 5,240	99,299 ± 9,887	55,809 ± 9,514	129,100 ± 19,215	165,402 ± 17,864		
4-Week	109,062 ± 5,415	101,139 ± 18,314	52,560 ± 7,899	126,623 ± 15,429	155,589 ± 27,612		
2-Month	97,912 ± 4,269	87,508 ± 7,146	54,771 ± 3,332	110,347 ± 9,365	174,983 ± 33,433		
3-Month	98,792 ± 18,352	92,608 ± 11,787	51,640 ± 6,160	119,822 ± 19,041	170,292 ± 29,373		
7-Month	117,486 ± 1,229	115,774 ± 13,793	57,017 ± 1,800	127,174 ± 10,434	187,038 ± 24,926		
Astrocyte number							
1-Week	49,175 ± 5,354	68,969 ± 10,171	25,967 ± 3,330	49,846 ± 5,848	83,697 ± 27,836		
2-Week	64,793 ± 9,781	70,030 ± 6,393	33,423 ± 939	54,096 ± 1,898	88,440 ± 10,405		
3-Week	82,722 ± 14,919	59,785 ± 6,753	33,125 ± 3,412	51,247 ± 11,005	80,742 ± 11,048		
4-Week	60,605 ± 9,275	54,369 ± 4,452	27,209 ± 4,212	39,580 ± 3,909	68,208 ± 15,300		
2-Month	54,077 ± 10,035	42,003 ± 6,667	24,908 ± 2,272	35,550 ± 6,436	64,614 ± 3,276		
3-Month	53,339 ± 14,519	46,484 ± 8,709	23,607 ± 2,373	39,889 ± 10,069	69,214 ± 7,753		
7-Month	45,668 ± 9,792	54,925 ± 12,645	22,491 ± 4,721	48,963 ± 3,110	65,995 ± 12,069		
Oligodendrocyte number							
1-Week	743 ± 335	1,043 ± 861	173 ± 223	720 ± 554	0 ± 0		
2-Week	1,063 ± 575	1,226 ± 617	1,095 ± 889	1,277 ± 709	711 ± 1,024		
3-Week	2,294 ± 2,014	2,733 ± 2,336	1,618 ± 1,124	2,918 ± 1,709	823 ± 670		
4-Week	10,491 ± 2,179	11,100 ± 3,008	5,418 ± 2,495	12,319 ± 6,798	1,703 ± 2,060		
2-Month	11,634 ± 6,525	23,362 ± 9,511	5,458 ± 2,182	16,829 ± 6,166	6,790 ± 2,931		
3-Month	16,201 ± 4,477	28,961 ± 4,886	9,465 ± 2,916	24,954 ± 8,881	12,527 ± 4,423		
7-Month	35,076 ± 3,661	51,596 ± 6,278	13,290 ± 2,543	37,727 ± 9,791	38,426 ± 5,608		

[1]Refers to the volume of the telencephalon and diencephalon bilaterally (ventricles were excluded).
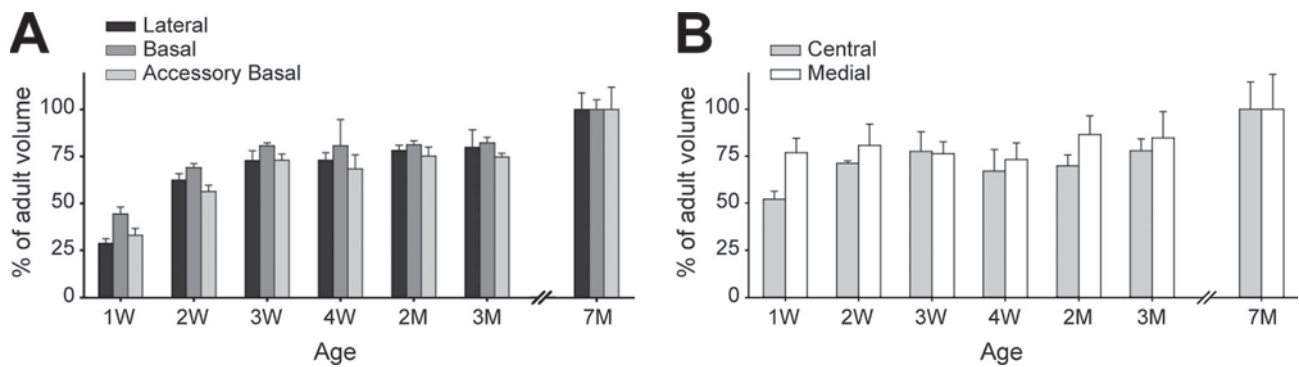
Figure 3. Volumes of the main nuclei of the rat amygdala at different ages during early postnatal development (expressed as a percentage of the volume of the structure observed in 7-month-old rats; average $\pm$ SD). **A:** Lateral, basal and accessory basal nuclei. **B:** Central and medial nuclei. 1W, 1-week-olds; 2W, 2-week-olds; 3W, 3-week-olds; 4W, 4-week-olds; 2M, 2-month-olds; 3M, 3-month-olds; 7M, 7-month-olds.

The volume of the whole amygdala also differed between age groups ($F_{6,21} = 25.075$, $P < 0.001$). At 1 week of age, amygdala volume was 50% of the 7-month-olds' volume; it was 84% at 3 months. Amygdala volume increased continuously from 1 week to 7 months of age (1-week < 3-week < 3-month < 7-month, all $P < 0.015$).

The volumes of the lateral, basal, and accessory basal nuclei exhibited similar developmental patterns, whereas those of the central and medial nuclei differed (Table 2). At 1 week of age, the volume of the lateral nucleus was 29% of the volume observed at 7 months (Fig. 3A); it was about 62% at 2 weeks and 73% at 3 weeks of age ($F_{6,21} = 57.691$, $P < 0.001$; 1-week < 2-week < 3-week < 7-month-olds, $P < 0.017$). At 1 week of age, the volume of the basal nucleus was 44% of the volume observed at 7 months (Fig. 3A); it was about 69% at 2 weeks and 81% at 3 weeks of age ($F_{6,21} = 32.146$, $P < 0.001$; 1-week < 2-week < 3-week < 7-month-olds, $P < 0.013$). Similarly, at 1 week of age, the volume of the accessory basal nucleus was 33% of the 7-month-olds' volume (Fig. 3A); it was about 56% at 2 weeks and 73% at 3 weeks of age ($F_{6,21} = 45.766$, $P < 0.001$; 1-week < 2-week < 3-week < 7-months, all $P < 0.001$).

In contrast, the volume of the central nucleus was 52% of the 7-month-olds' volume at 1 week of age and 71% at 2 weeks of age (Fig. 3B; $F_{6,21} = 10,946$, $P < 0.001$; 1-week < 2-week < 7-week-olds, all $P < 0.006$). Finally, although the volume of the medial nucleus increased from 77% of its adult value at 1 week of age, the differences between age groups failed to reach statistical significance with only four rats per group (Fig. 3B; $F_{6,21} = 2.423$, $P = 0.061$).

Neuron numbers

There were no differences in the numbers of neurons estimated at different postnatal ages in four of the main amygdala nuclei in rats (Table 2): lateral nucleus (Fig. 4A;

$F_{6,21} = 2.395$, $P = 0.064$), accessory basal nucleus (Fig. 4C; $F_{6,21} = 2.490$, $P = 0.056$), central nucleus (Fig. 4D; $F_{6,21} = 1.035$, $P = 0.431$), and medial nucleus (Fig. 4E; $F_{6,21} = 1.341$, $P = 0.284$). In contrast, the number of neurons appeared to differ between age groups in the basal nucleus (Fig. 4B; $F_{6,21} = 3.185$, $P = 0.022$). Surprisingly, neuron numbers were greater in the 1-week- and 7-month-old groups compared with the 2-week-, 2-month-, and 3-month-old groups (all $P < 0.043$). In contrast, there was no statistical difference in neuron number in the basal nucleus among 1-week-old, 3-week-old, 4-week-old, and 7-month-old rats. In addition, the total number of neurons in the five main amygdala nuclei (i.e., the sum of all nuclei) did not change postnatally (Fig. 4F; $F_{6,21} = 1.542$, $P = 0.213$). These reported differences in the basal nucleus are thus likely spurious results, resulting from particularities of individual rats in the 2-week-, 2-month-, and 3-month-old groups, and do not reflect meaningful changes. In sum, we believe that there were no consistent developmental changes in neuron number in any of the five main nuclei of the rat amygdala.

Astrocyte numbers

There were differences in the numbers of astrocytes estimated at different postnatal ages in four of the main amygdala nuclei in rats (Table 2): lateral nucleus (Fig. 5A; $F_{6,21} = 5.156$, $P = 0.002$), basal nucleus (Fig. 5B; $F_{6,21} = 6.351$, $P < 0.001$), accessory basal nucleus (Fig. 5C; $F_{6,21} = 7.253$, $P < 0.001$), and central nucleus (Fig. 5D; $F_{6,21} = 4.333$, $P = 0.005$). In contrast, the number of astrocytes did not differ in the medial nucleus (Fig. 5E; $F_{6,21} = 1.781$, $P = 0.152$). In each amygdala nucleus, astrocyte number was greater before 3 weeks than after 2 months of age. This difference was statistically significant when data from the five main nuclei were pooled (Fig. 5F; $F_{6,21} = 8.361$, $P < 0.001$; 1-2-3 weeks > 2-3-7 months, all $P < 0.038$).
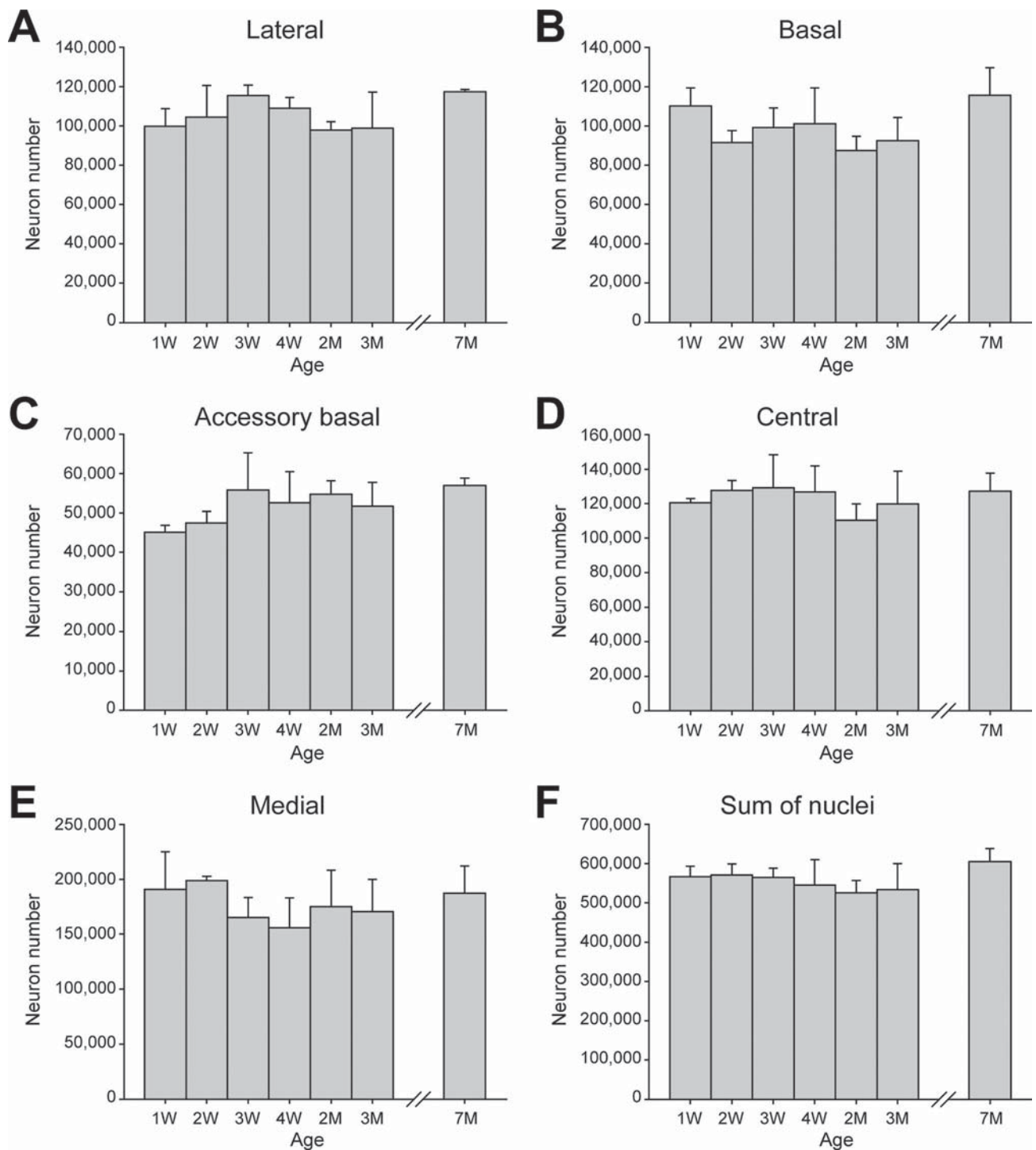
7

Figure 4. Numbers of neurons in the five main nuclei of the rat amygdala at different ages during early postnatal development. **A:** Lateral. **B:** Basal. **C:** Accessory basal. **D:** Central. **E:** Medial. **F:** Sum of the five nuclei. Error bars: ± SD. 1W, 1-week-olds; 2W, 2-week-olds; 3W, 3-week-olds; 4W, 4-week-olds; 2M, 2-month-olds; 3M, 3-month-olds; 7M, 7-month-olds.

Oligodendrocyte numbers

The numbers of oligodendrocytes differed between age groups in the five main amygdala nuclei (Table 2; Fig. 6). For the lateral nucleus (Fig. 6A; $F_{6,21} = 48.550$, $P < 0.001$), 1-week-old rats had fewer cells than 4-week-olds, which had fewer cells than 3-month-olds, which had fewer cells than 7-month-olds (all $P < 0.032$). In the basal nucleus (Fig. 6B; $F_{6,21} = 58.464$, $P < 0.001$), 1-week-old rats had fewer cells than 4-week-olds, which had fewer cells than 3-month-olds, which had fewer cells than
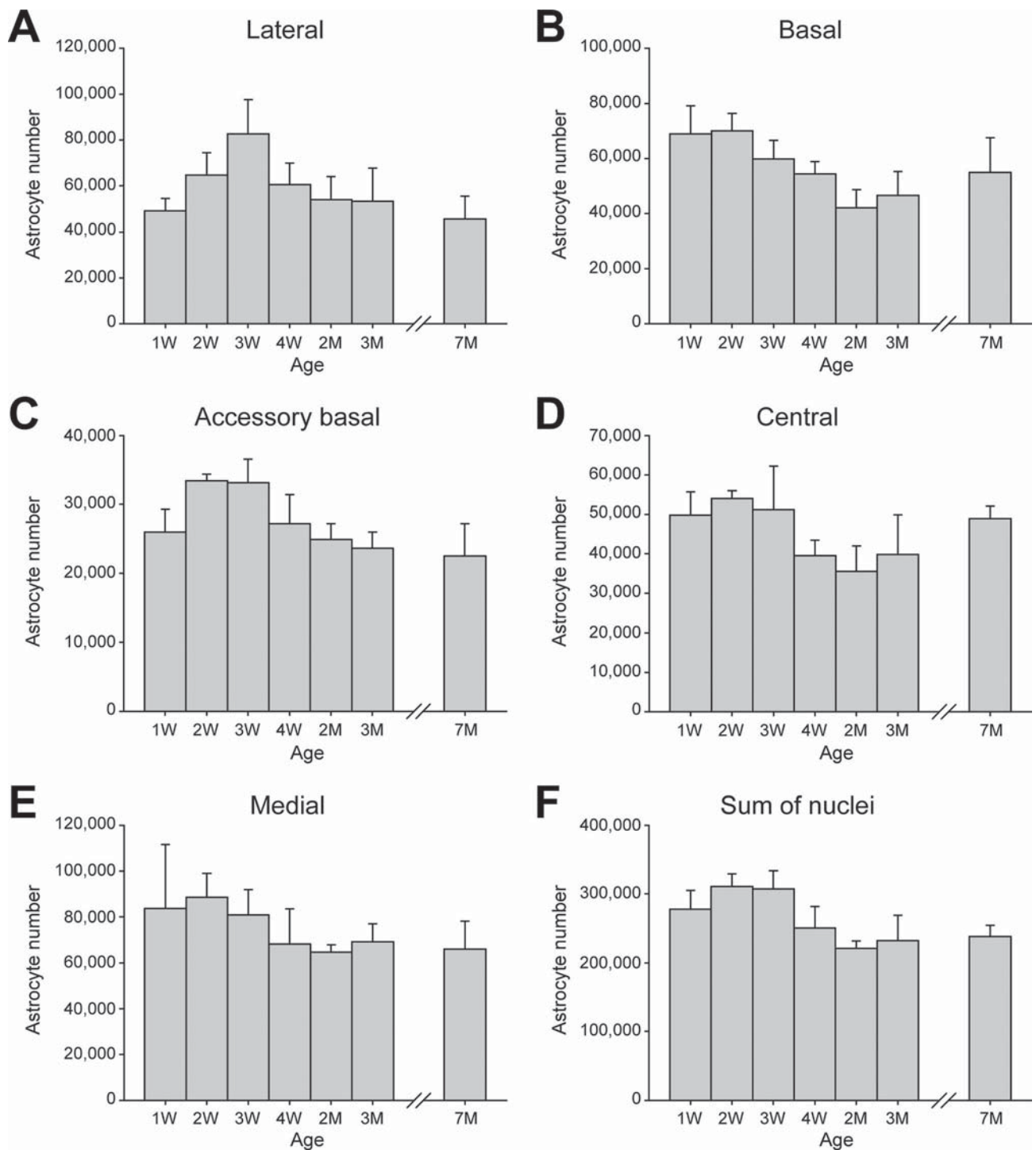
Figure 5. Numbers of astrocytes in the five main nuclei of the rat amygdala at different ages during early postnatal development. **A:** Lateral. **B:** Basal. **C:** Accessory basal. **D:** Central. **E:** Medial. **F:** Sum of the five nuclei. Error bars: ± SD. 1W, 1-week-olds; 2W, 2-week-olds; 3W, 3-week-olds; 4W, 4-week-olds; 2M, 2-month-olds; 3M, 3-month-olds; 7M, 7-month-olds.

7-month-olds (all $P < 0.01$). For the accessory basal nucleus (Fig. 6C; $F_{6,21} = 23.070$, $P < 0.001$), 1-week-old rats had fewer cells than 4-week-olds, which had fewer cells than 3-month-olds, which had fewer cells than 7-month-olds (all $P < 0.014$). For the central nucleus (Fig. 6D; $F_{6,21} = 20.513$, $P < 0.001$), 1-week-old rats had fewer cells than 4-week-olds, which had fewer cells than 3-month-olds, which had fewer cells than 7-month-olds (all $P < 0.015$). Finally, in the medial nucleus (Fig. 6E; $F_{6,21} = 82.291$, $P < 0.001$), 1-week-old rats had fewer cells than 2-month-olds, which had fewer cells than 3-month-olds, which had fewer cells than 7-month-olds (all $P < 0.016$).
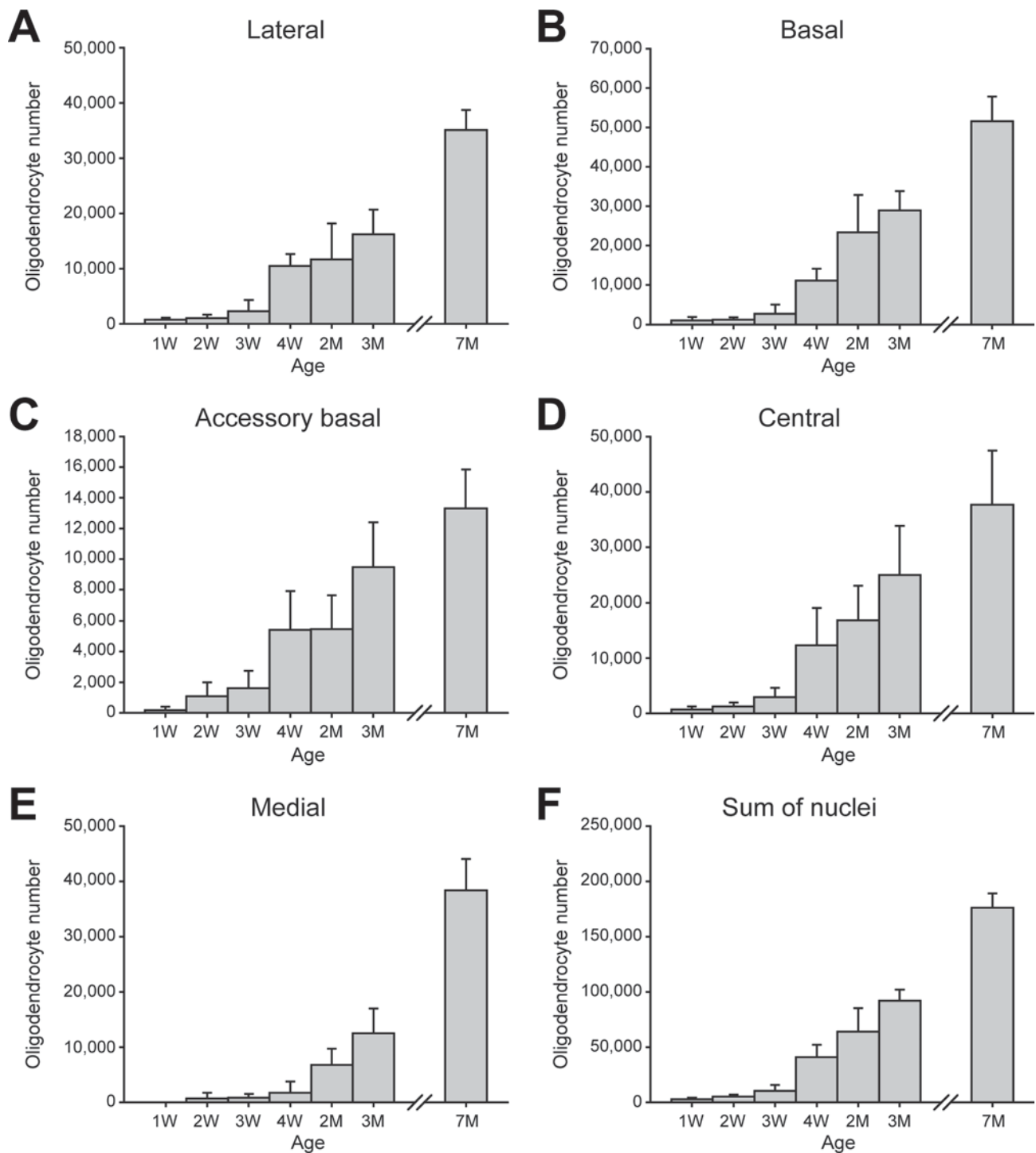
9

Figure 6. Numbers of oligodendrocytes in the five main nuclei of the rat amygdala at different ages during early postnatal development. **A:** Lateral. **B:** Basal. **C:** Accessory basal. **D:** Central. **E:** Medial. **F:** Sum of nuclei. Error bars: ± SD. 1W, 1-week-olds; 2W, 2-week-olds; 3W, 3-week-olds; 4W, 4-week-olds; 2M, 2-month-olds; 3M, 3-month-olds; 7M, 7-month-olds.

Accordingly, the total number of oligodendrocytes in these five main amygdala nuclei differed among age groups (Fig. 6F; $F_{6,21} = 122.481$, $P < 0.001$); 1-week-old rats had fewer cells than 4-week-olds, which had fewer cells than 2-month-olds, which had fewer cells than 3-month-olds, which had fewer cells than 7-month-olds (all $P < 0.01$).

Volume and oligodendrocyte numbers

During the first 3 weeks of postnatal life, the main nuclei of the rat amygdala exhibited increases in volume that were not related to any changes in cell (i.e., neurons, astrocytes, or oligodendrocytes) numbers evaluated in the current study. This indicates that the increased
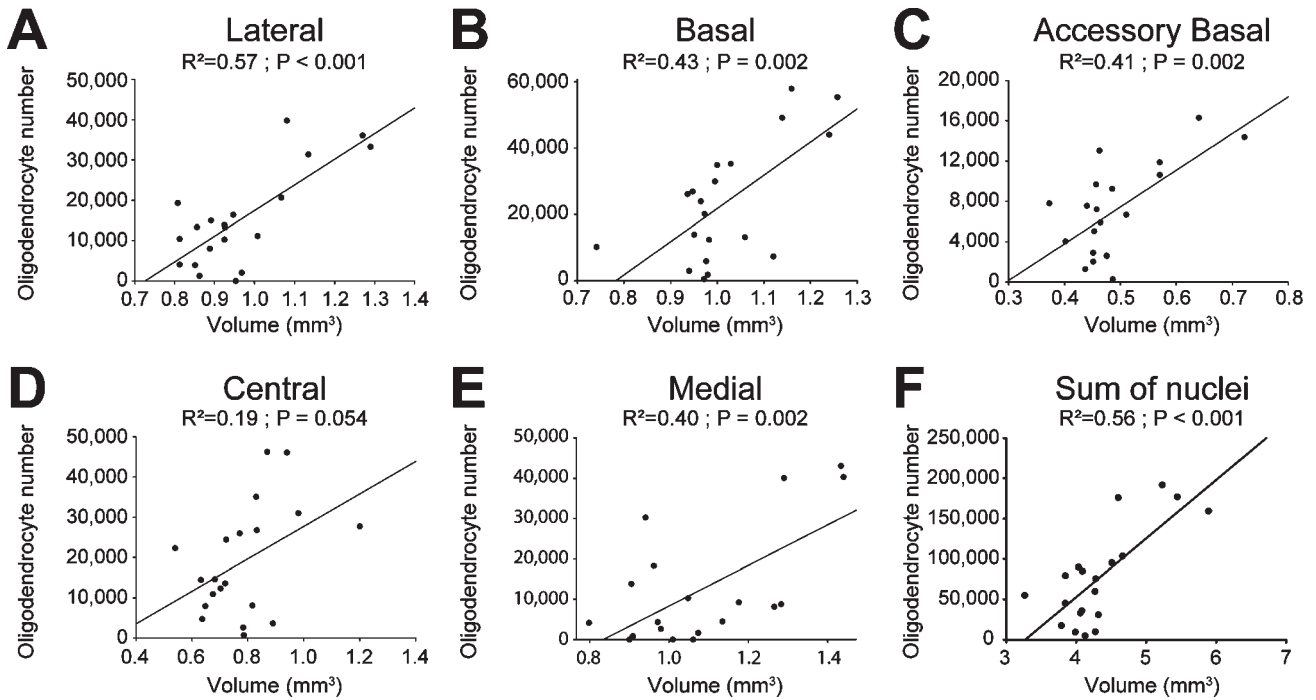
Figure 7. Relationship between oligodendrocyte number and volume of the main amygdala nuclei of rats from 3 weeks to 7 months of age. **A:** Lateral (volume = 0.827 + 0.00000902 × oligodendrocyte number; R^2 = 0.57, $F_{1,18}$ = 24.178, $P < 0.001$). **B:** Basal (volume = 0.916 + 0.00000434 × oligodendrocyte number; R^2 = 0.43, $F_{1,18}$ = 13.869, P = 0.002). **C:** Accessory basal (volume = 0.409 + 0.0000114 × oligodendrocyte number; R^2 = 0.41, $F_{1,18}$ = 12.822, P = 0.002). **D:** Central (volume = 0.693 + 0.00000475 × oligodendrocyte number; R^2 = 0.19, $F_{1,18}$ = 4.262, P = 0.054). **E:** Medial (volume = 0.977 + 0.00000811 × oligodendrocyte number; R^2 = 0.40, $F_{1,18}$ = 12.396, P = 0.002). **F:** Sum of nuclei (volume = 3.735 + 0.00000772 × oligodendrocyte number; R^2 = 0.56, $F_{1,18}$ = 22.976, $P < 0.001$).

volume was due to an increase in neuropil volume. Between 3 weeks and 7 months of age, increases in the volumes of individual amygdala nuclei were not correlated with the numbers of neurons or astrocytes. In contrast, after 3 weeks of age, we found a significant linear relationship between volume and oligodendrocyte number for the lateral, basal, accessory basal, and medial nuclei: lateral (volume = 0.827 + 0.00000902 × oligodendrocyte number; R^2 = 0.57, $F_{1,18}$ = 24.178, $P < 0.001$; Fig. 7A), basal (volume = 0.916 + 0.00000434 × oligodendrocyte number; R^2 = 0.43, $F_{1,18}$ = 13.869, P = 0.002; Fig. 7B), accessory basal (volume = 0.409 + 0.0000114 × oligodendrocyte number; R^2 = 0.41, $F_{1,18}$ = 12.822, P = 0.002; Fig. 7C), and medial (volume = 0.977 + 0.00000811 × oligodendrocyte number; R^2 = 0.40, $F_{1,18}$ = 12.396, P = 0.002; Fig. 7E). This relationship failed to reach statistical significance for the central nucleus (volume = 0.693 + 0.00000475 × oligodendrocyte number; R^2 = 0.19, $F_{1,18}$ = 4.262, P = 0.054; Fig. 7D). Accordingly, the volume of the whole amygdala correlated with the total number of oligodendrocytes (volume = 3.735 + 0.00000772 × oligodendrocyte number; R^2 = 0.56, $F_{1,18}$ = 22.976, $P < 0.001$; Fig. 7F). Overall,

these data suggest that increases in volume after 3 weeks of postnatal life are linked, at least in part, to increases in oligodendrocyte numbers and myelination of fibers within the amygdala. Other changes, including the maturation of dendritic arborization, also likely contribute to these postnatal volumetric changes.

DISCUSSION

The goal of the present study was to provide quantitative information about the postnatal cellular maturation of the rat amygdala. Our major findings are as follows: 1) distinct amygdala nuclei exhibited different developmental profiles; 2) neuron number did not vary after birth in the main amygdala nuclei; 3) astrocyte number decreased between 3 weeks and 3 months of age; and 4) oligodendrocyte number increased from 1 week to beyond 3 months of age. We compare our findings with previous results in rats as well as with our previous systematic investigation carried out in monkeys (Chareyron et al., 2012). We also discuss our findings in relation to functional studies on the ontogeny of fear behaviors in the rat.

Volumetric changes
Brain

The increase in rat brain volume that we observed from 1 week to 7 months of age is consistent with previous findings (Bandeira et al., 2009; Berdel et al., 1997b; Brizzee et al., 1964; Sullivan et al., 2006; Yates and Juraska, 2007). As discussed previously (see Chareyron et al., 2012), age-related differential shrinkage that might affect brain tissue during processing is unlikely to explain the specific changes in volume and cell numbers observed in the postnatally developing rat amygdala. Briefly, frozen section shrinkage is minor in the x- and y-planes compared with shrinkage in the z-plane (Carlo and Stevens, 2011). Although the z-plane shrinkage of processed sections was inversely proportional to age (83% reduction of the section thickness in 1-week-olds and 70% in 7-month-olds), our volume estimates were not impacted by this effect, because we used the cutting section interval (4 × 40 µm for the amygdala and 12 × 40 µm for the brain) to estimate volumes. Cells counts were not impacted by differential shrinkage either; the optical fractionator technique provides estimates of cell numbers that are independent of volume estimates.

Our volumetric data on the postnatal development of the rat brain are comparable to the brain weight data reported for Wistar rats by Bandeira et al. (2009). We found a 93% increase in brain volume from 1 week to 3 weeks of age, and they reported a 90% increase in brain weight during the same developmental period. We found that brain volume increases 21% from 4 weeks to 3 months of age, whereas Bandeira et al. (2009) reported that brain weight increases 21% from P32 to P81. We found a further 18% increase in brain volume from 3 to 7 months of age; a similarly late, 9% increase in brain volume from P90 to P180 was previously reported by Berdel and colleagues (1997b). By using magnetic resonance imaging, Sullivan and colleagues (2006) found a 10% increase in total brain volume from P88 to P284 in alcohol-preferring Wistar rats. Yates and Juraska (2007) also reported a 4% increase in brain weight of Long-Evans rats from 4 to 12–13 months, followed by another 5% increase until 18–26 months. A similar, 16% increase in brain volume was reported for C57BL/6J mice from 4 to 24 months of age (von Kienlin et al., 2005). Finally, Brizzee and colleagues (1964) estimated that the weight of the rat brain increases until P200, and then remains relatively stable until 2 years of age. Although there are clear difficulties in directly relating developmental changes in volume with brain weight changes (insofar as there might be some discrepancies in the actual growth rates based on these parameters), the different stages of postnatal development elucidated in our systematic study are consistent with previously published reports: very rapid brain growth up to 3 weeks of age, moderate growth from 3 weeks to 3 months of age, and slow but continuous growth until 7 months of age.

Amygdala

The postnatal volumetric increases of the main nuclei of the rat amygdala that we observed are also consistent with previous findings (Berdel et al., 1997b; Mizukami et al., 1983). We found a 156% and 83% increase in volume of the lateral and basal nuclei, respectively, from 1 to 3 weeks of age, whereas Berdel and colleagues (1997b) reported a 150% and 116% increase of these nuclei during the same developmental period. We found a 37% and 24% increase in the lateral and basal nuclei, respectively, from 3 weeks to 7 months of age, whereas Berdel and colleagues (1997b) reported a 8% and 21% increase in these nuclei from 3 weeks to 6 months. The lack of volumetric change in the medial nucleus is consistent with previous data from Mizukami and colleagues (1983), who reported that the medial nucleus increases in volume between P1 and P5 and reaches adult values at P5 in females and P11 in males.

Despite some differences in absolute values reported by us and by Berdel and colleagues, which could be due to differences in calibration or differences in the delineation of the nuclei, the overall pattern of postnatal development of the basolateral amygdala is consistent between our study and the studies of both Berdel and colleagues (1997b) and Mizukami and colleagues (1983). In contrast, Rubinow and Juraska (2009) reported a significant increase in volume of the basal nucleus from P20 to P35, no changes from P35 to P90, and a 15% increase from P90 to 19–22 months of age. The reliability of our quantitative data in mature, 7-month-old rats has been demonstrated and discussed previously (Chareyron et al., 2011).

In sum, our current findings, together with previous findings in rats (Berdel et al., 1997b; Mizukami et al., 1983) and monkeys (Chareyron et al., 2012), indicate that the volume of the rat amygdala follows a developmental pattern similar to that of the whole brain, with a rapid growth up to 3 weeks of age, followed by slower but continuous growth until at least 7 months of age.

Neuron number

We found no reliable changes in neuron number from 1 week to 7 postnatal months in the five main nuclei of the rat amygdala. Our findings are thus consistent with previous reports. Bayer (1980) showed that neurogenesis is completed before birth in the rat amygdala. Berdel and colleagues (1997a) reported that the number of neurons in the lateral and basal nuclei might increase until P7 but remains stable thereafter. It should be noted, however,

that Berdel and colleagues did not use modern stereological techniques to estimate neuron number, and their results might have been influenced by differential shrinkage (see above). This could explain their reported increase in neuron number up to P7 and the discrepancy in estimates of total neuron numbers at later ages between their study and ours. The purported changes in neuron numbers in the "basolateral" nucleus of the rat amygdala after 1 month of age reported by Rubinow and Juraska (2009) are also inconsistent. This could be due to biological variability and unknown bias in experimental sampling or result from bias in volumes and cell density measurements. Indeed, in contrast to the data obtained in the present study, cell number estimates presented by Rubinow and Juraska (2009) were dependent on the volume of the structure and cell density and were thus highly sensitive to potential differences in tissue processing and shrinkage. Considering the results of these studies, current experimental evidence indicates that the total number of neurons does not vary after birth in the main nuclei of the rat amygdala.

Glia number

To our knowledge, there are no published data on the numbers of astrocytes or oligodendrocytes in the postnatally developing rat amygdala. Nevertheless, a decrease in astrocyte number in the amygdala from 3 weeks to 2 months of age is consistent with findings in other brain regions. Astrocyte death has been observed in the rat cerebellum during the first 2 weeks of life (Krueger et al., 1995) and in the mouse cortex between birth and P8 (Soriano et al., 1993). For the postnatal visual cortex of the cat, Müller (1992) reported that large, glial fibrillary acidic protein (GFAP)-positive cells thought to be radial glial cells present at P7 have disappeared by the fifth postnatal week. A decrease of astrocytic processes and gene expression has also been reported for the monkey (Lavenex et al., 2011) and rat (Nixdorf-Bergweiler et al., 1994) hippocampus. In contrast, other studies reported no postnatal changes in astrocyte numbers in the auditory (Vaughan and Peters, 1974) or visual (Parnavelas et al., 1983) cortex. In our study, a decrease in astrocyte number reached statistical significance only when all amygdala nuclei were grouped. It is possible that astrocytes are initially required to support axons and are no longer needed once oligodendrocytes differentiate and begin to ensheathe the axons (Krueger et al., 1995). Accordingly, we found that astrocyte number decreases at the same time as oligodendrocyte number increases in the rat amygdala, at about 4 weeks of age. In addition, however, and as suggested previously (Lavenex et al., 2011), a decrease in astrocyte number might also reflect a decrease in astrocytic coverage of the synapse, which

contributes to increased synaptic selectivity advantageous for learning.

Comparison with the development of the monkey amygdala

The patterns of postnatal development of the main rat amygdala nuclei are strikingly similar to those we previously described for monkeys (Fig. 8; Chareyron et al., 2012). The lateral, basal, and accessory basal nuclei exhibit a large increase in volume from 1 week to 3 weeks of age in rats and from birth to 3 months of age in monkeys. In contrast, in both rats and monkeys, the medial nucleus is volumetrically mature very early and exhibits only a slight linear increase from birth until young adulthood. The central nucleus is the only amygdala nucleus exhibiting different postnatal developmental profiles in rats and monkeys. However, it is possible that differences in overall maturity levels at birth (i.e., monkeys being more mature at birth) could also explain the seeming discrepancy in the postnatal development of the central nucleus between rats and monkeys: Specifically, the early exponential growth of the rat central nucleus between 1 and 2 weeks of age might occur before birth in monkeys.

The number of neurons did not change postnatally in the main nuclei of the amygdala in either rats or monkeys, with the exception of an increase in mature neurons in the monkey paralaminar nucleus (Chareyron et al., 2012), a nucleus not found in rats (but see deCampo and Fudge, 2012; Price et al., 1987). In contrast, the number of oligodendrocytes increased continuously in all amygdala nuclei from early postnatal ages until young adulthood in both rats and monkeys. The number of astrocytes in the whole amygdala decreased postnatally in rats but not in monkeys. This discrepancy might be due to the differential development of the rodent and primate brains at birth (Rice and Barone, 2000; Watson et al., 2006). Indeed, the peak of overall brain growth, which occurs before birth in monkeys, occurs postnatally in rats (Dobbing and Sands, 1979). More specifically, the peak of neurogenesis in the amygdala occurs during the first half of gestation in monkeys (E40, total gestation is 165 days; Kordower et al., 1992), whereas it occurs during the last stages of gestation in rats (E15, total gestation is 21 days; Bayer, 1980). This earlier maturation of the primate brain is also reflected by the relatively early increase in oligodendrocyte number at 3 months of age in the monkey amygdala, whereas oligodendrocyte number begins to increase significantly at the end of the first postnatal month in rats. It is thus possible that a decrease in astrocyte number, similar to that observed postnatally in rats, occurs prenatally in the monkey amygdala (Fig. 8).

In sum, despite species differences in overall brain maturity at birth, the main nuclei of the rat and monkey
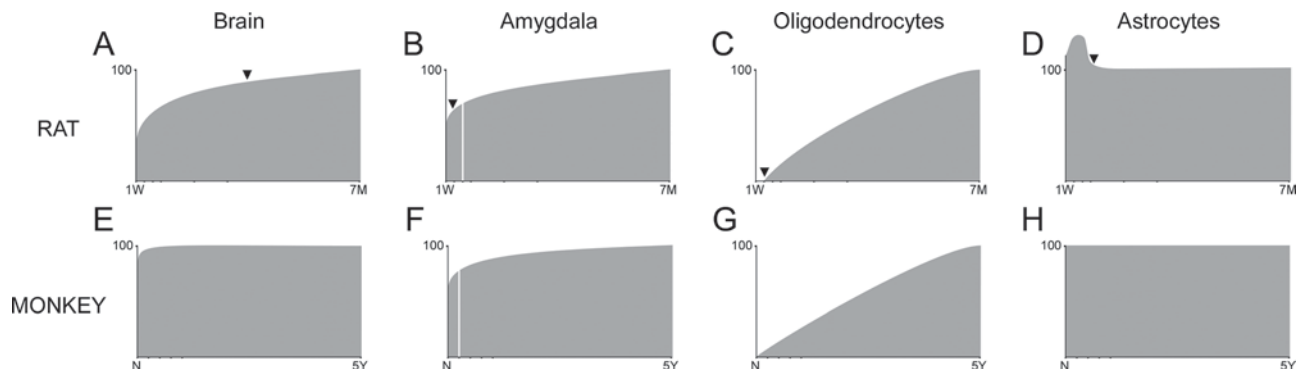
Figure 8. Schematic representation of the postnatal development of the brain and amygdala in rats and monkeys. **A:** Rat brain volume. **B:** Rat amygdala volume. **C:** Oligodendrocyte number in the rat amygdala. **D:** Astrocyte number in the rat amygdala. **E:** Monkey brain volume. **F:** Monkey amygdala volume. **G:** Oligodendrocyte number in the monkey amygdala. **H:** Astrocyte number in the monkey amygdala. 1W, 1-week-olds; 7M, 7-month-olds; N, newborns; 5Y, 5-year-olds. For each parameter, the shaded area represents the percentage of adult value (i.e., 7 months in rat and 5 years in monkey). Arrowheads are placed on the rat curves to indicate the age at which this parameter corresponds to the newborn monkey level. Note that the level of maturity at birth varies between the different parameters. White lines in amygdala volume graphs represent the end of the first phase of postnatal development characterized by neuropil volume increase and the beginning of the second phase characterized by oligodendrocyte number increase.

amygdala exhibit similar patterns of morphological and cellular development during early postnatal life. This suggests that the postnatal development of the human amygdala might follow similar patterns.

Differential maturation of distinct amygdala circuits

Similar to what we previously found in monkeys (Chareyron et al., 2012), our current data in rats identified different profiles of postnatal development that might reflect the maturation of distinct cellular processes and amygdala circuits. The first stage was characterized by the dramatic enlargement of the lateral, basal, and accessory basal nuclei from 1 week to 3 weeks of age. The second stage was characterized by the continuous increase in size of all the main amygdala nuclei beyond 3 months of age.

First stage: neuropil expansion

In the absence of detectable changes in cell numbers (including neurons, astrocytes, and oligodendrocytes) between 1 week and 3 weeks of age, the dramatic enlargement of the lateral, basal, and accessory basal nuclei of the rat amygdala likely reflects an increase in neuropil volume. Indeed, the neuropil, which is defined as the portion of neural tissue composed mostly of small dendrites and spines and fine glial processes, unmyelinated axons, and axon terminals (Bourgeois et al., 1994; Bourgeois and Rakic, 1993), represents the largest fraction of these nuclei. Neuronal somas occupy only about 10% of the volume of the amygdala in mature individuals

(Chareyron et al., 2011). Combined, the volume of these three nuclei exhibited a 112% increase from 1 to 3 weeks of age. It is thus plausible that neuropil expansion might underlie these observed volumetric changes.

An increase in neuropil volume might, in turn, reflect an increase in the connectivity of these nuclei. The lateral, basal, and accessory basal nuclei are highly interconnected with the neocortex (McDonald, 1998; Pitkänen, 2000), and their volumetric expansion from 1 to 3 weeks of age effectively parallels the volumetric expansion of the rest of the brain (amygdala/brain ratio: 1 week = 0.015, 2 weeks = 0.013, 3 weeks = 0.011, 4 weeks = 0.011, 2 months = 0.011, 3 months = 0.011, 7 months = 0.011). In rats, major changes in the interconnections between the basal nucleus of the amygdala and the prefrontal cortex occur between P7 and P19 (Bouwmeester et al., 2002a,b). Although fibers from the basal nucleus are already present in the rat prefrontal cortex at P3, their density increases progressively until P120 (Cunningham et al., 2002; Verwer et al., 1996). In cats, an increase in total dendritic length in the basal nucleus of the amygdala has been observed during the first postnatal month (Wakefield and Levine, 1985). Similarly, electrophysiological studies suggest major changes in intra-amygdala functional connectivity: Tetanic stimulation of the lateral nucleus does not produce long-term potentiation in the basal nucleus in amygdala slice preparations of P7–10 rat pups, whereas it does in P11–19 slices (Thompson et al., 2008). In addition, the amygdala is resistant to seizure induced by kindling stimulation in 10- and 14-day-old rats, but not in rats 21 days old and older (Gilbert and Cain, 1981; Terasawa and Timiras, 1968). Altogether,

these observations suggest that neuropil expansion might be related to changes in amygdala intraconnectivity and interconnectivity with neocortical areas and underlie the volumetric increases of the lateral, basal, and accessory basal nuclei from 1 week to 3 weeks of age. This hypothesis will require additional experimental investigation.

In contrast to the deep nuclei, the central nucleus exhibits a relatively small increase in volume (37%) from 1 week to 2 weeks of age, and the medial nucleus does not exhibit any significant increase in volume. These two nuclei are connected mainly with the thalamus, hypothalamus, and brainstem (Pitkänen, 2000). In contrast to cortical connections, the interconnections between the amygdala and the thalamic regions, substantia innominata, or accumbens nuclei do not change between P7 and P19 in rats, suggesting that these connections are established by birth or very shortly after (Bouwmeester et al., 2002a,b). The density of connections between the posteriodorsal part of the medial nucleus (Mpd) of the amygdala and the bed nucleus of the stria terminalis (BNST) increases mainly before P10 and has already reached an adult-like pattern by P15 (Cooke and Simerly, 2005). The absence of changes in volume of the medial nucleus from 1 to 2 weeks of age could be explained by the fact that the Mpd represents only ∼50% of the medial nucleus volume (Cooke et al., 2000), and volume changes induced by the reorganization of this unique type of interconnections with the BNST might be too marginal to be observed. However, it is well established that the BNST is a major target of central amygdala projection neurons (Cassell et al., 1986; Zahm et al., 1999), and, although not yet investigated, such connectional reorganization between the central amygdala and the BNST could potentially explain the increase in volume of the central nucleus from 1 to 2 weeks of age. In sum, the subtle volumetric changes of the rat central and medial nuclei during the first postnatal weeks might reflect the minimal structural (and potentially functional) refinement of the interconnections between these nuclei and the rest of the brain after 1 week of age.

Second stage: myelination

We observed a continuous increase in the size of all amygdala nuclei from 3 weeks to 7 months of age, which coincided with an increase in the number of oligodendrocytes. This suggests that the myelination of amygdala circuits may underlie, at least in part, the late and continuous enlargement of amygdala nuclei after 3 weeks of age. Indeed, oligodendrocytes produce the myelin sheath that surrounds axons (Nave, 2010), and an increase in oligodendrocyte number has been linked to an increase in axon myelination during development in the monkey visual system (O'Kusky and Colonnier, 1982). Ono et al.

(2008) demonstrated that the concentration of glycolipid galactosylceramide, a myelin constituent, increases linearly between P21 and P54 in the basal nucleus of the mouse amygdala. Their results also indicated that myelin formation occurs later in the amygdala than in the prefrontal cortex and hippocampus (Ono et al., 2008). It thus stands to reason that the late increase in oligodendrocyte number in the amygdala might accompany delayed myelination of this structure.

However, the contribution of oligodendrocyte processes to the volumetric changes observed after 3 weeks of age in the rat was not uniform across distinct amygdala nuclei. For the lateral, basal, accessory basal, and medial nuclei, statistical analyses indicated that oligodendrocyte numbers were only partially related to the volumetric changes, and, in the central nucleus, the correlation was strikingly low. This observation contrasts with the highly significant correlation between oligodendrocyte number and volume of the monkey central nucleus during postnatal development (Chareyron et al., 2012). In the absence of obvious species differences in the cellular composition (such as glia/neuron ratio or oligodendrocyte and astrocyte density) of the central nucleus compared with other amygdala nuclei, it is possible that the neuropil of the central nucleus undergoes greater postnatal refinement in rats than in monkeys. For example, other factors such as changes in dendritic arborization, astrocytic processes refinement, and astrocyte elimination might also contribute to volumetric changes of the amygdala nuclei after 3 weeks of age in the rat. Indeed, we found a decrease in the number of astrocytes in the rat amygdala after 3 weeks of age. Other studies have suggested a decrease in neuropil volume in the medial amygdala during puberty in the rat (Zehr et al., 2006). Similarly, a tract-tracing study, using both retrograde and anterograde tracers, has shown that mPFC projections to the basal and accessory basal nuclei decrease between P25 and P90 in rats (Cressman et al., 2010).

In sum, despite subtle differences between individual amygdala nuclei, increases in oligodendrocyte number and myelination likely contribute significantly to the continuous increase in overall amygdala volume observed until young adulthood. This hypothesis will require additional experimental investigation.

Functional considerations

Our quantitative data on the postnatal development of the rat amygdala identified different periods of postnatal development of distinct amygdala nuclei and cellular components, which we find are concomitant with the ontogeny of different fear and emotional behaviors as described in the literature. We hypothesize that the early maturation of the central nucleus could support the early

expression of simple fear reactions, such as fleeing and freezing behavior in response to individual stimuli. Indeed, before showing a slow linear volumetric increase until 7 months of age, the central nucleus exhibits early exponential growth between 1 and 2 weeks of age. Functional studies have shown that flight reactions are not present in 5-day-old rat pups, are still weak at 10 days of age, but are largely mature at 15 days of age (Collier et al., 1979). Freezing in response to a conditioned stimulus appears at P10 in rats (Sullivan, 2001). Interestingly, both flight and freezing reactions are mediated by the central amygdala projections to the periaqueductal gray (LeDoux, 2007). Early in development, the central nucleus could sustain simple fear reactions either through the activation of the immature basolateral complex of the amygdala (i.e., including the lateral, basal, and accessory basal nuclei; Sullivan et al., 2000) or through parallel circuits involving direct inputs from sensory and nociceptive afferents to the central nucleus (Balleine and Killcross, 2006).

The late maturation of the lateral, basal, and accessory basal nuclei is consistent with the relatively late involvement of these nuclei in the regulation of more complex fear behaviors. For example, at P7–8, a 0.5-mA or a 1.2-mA shock or LiCl injection induces odor preference or avoidance without activation of the basolateral complex of the amygdala as revealed by 2-deoxyglucose uptake (Raineki et al., 2009; Shionoya et al., 2006). At P12–13, the three conditions induce odor avoidance, but the basolateral complex of the amygdala is activated only when the odor is paired with a 0.5-mA shock but not when a stronger, 1.2-mA shock or LiCl injection is administered. In contrast, at P23–24, the basolateral complex is activated under all three conditions (Raineki et al., 2009; Shionoya et al., 2006). Similarly, at P14, exposure to a male rat induces *c-fos* expression in the medial nucleus of the amygdala, but not in the lateral nucleus, whereas, at P18, *c-fos* expression is observed in both the medial and the lateral nuclei (Chen et al., 2006; but see Wiedenmayer and Barr, 2001a). This suggests that there are several neural pathways for threat-induced fear responses, which exhibit different developmental time courses, and that the lateral, basal, and accessory basal nuclei of the amygdala do not become involved in these pathways until sometime during the second postnatal week. The basolateral complex could then begin to integrate contextual information sometime during the third week of life to drive contextual fear responses. Both the amygdala and the hippocampus are involved in contextual conditioning (Anagnostaras et al., 2001; LeDoux, 2000b; Matus-Amat et al., 2007; Wiltgen et al., 2006). It has been shown that conditioning to a context emerges between 17 and 24 days of age in rats (Foster and Burman, 2010; Raineki

et al., 2010; Rudy, 1993). Interestingly, however, whereas the hippocampus is not activated during such context conditioning in 21-day-old rats, it is activated in 24-day-olds (Raineki et al., 2010). The emergence of contextual conditioning is thus likely related to the maturation of both the hippocampus, which also exhibits a delayed developmental profile (Bekenstein and Lothman, 1991; Jabès et al., 2010, 2011; Nurse and Lacaille, 1999; Swann et al., 1990), and the basolateral complex of the amygdala, which likely is insufficiently mature to integrate such contextual information until the beginning of the third week of life.

Finally, the late myelination-related increase in volume of the amygdala nuclei might contribute to the improvement of more appropriate fear responses. We found a continuous increase in oligodendrocyte numbers in all amygdala nuclei from 4 weeks to 7 months of age, a period that coincides with significant changes in defensive behaviors. Indeed, 50-day-old rats are more attentive and display longer freezing, at a greater distance from the threat, than 20-day-old rats (Bronstein and Hirsch, 1976). In contrast, although 4-week-old mice exhibit more rapid and more robust acquisition of a conditioned association between an auditory stimulus and a foot shock compared with 6–8-week-old mice (Hefner and Holmes, 2007), other studies have shown that 4-week-old mice also display excessive generalization to auditory conditioned stimuli compared with 9–10-week-old mice (Ito et al., 2009). Altogether, these observations in mice suggest that aversive associations might be less specific in juveniles than in adults, and, although connectional refinement might occur in the amygdala during this late development (Pan et al., 2009), improvement of threat detection and defensive responses might also be linked to the maturation of other brain areas (e.g., prefrontal and sensory cortex) connected to the amygdala.

CONCLUSIONS

Our stereological study provides reliable, quantitative information on the cellular changes underlying the normal structural development of the main nuclei of the rat amygdala. We have identified two stages of postnatal development of the rat amygdala comparable to those that we previously described for monkeys. The first stage is characterized by a large increase in the volumes of the lateral, basal, and accessory basal nuclei from 1 to 3 weeks of age in the absence of any detectable changes in cell numbers, thus suggesting an increase in neuropil volume. The second stage is characterized by a slow and continuous increase in size of most amygdala nuclei from 3 weeks of age to young adulthood, which is accompanied by an increase in oligodendrocyte number. This

stage likely reflects the postnatal myelination of amygdala circuits.

ACKNOWLEDGMENTS

LITERATURE CITED

Amaral DG, Bauman MD, Capitanio JP, Lavenex P, Mason WA, Mauldin-Jourdain ML, Mendoza SP. 2003. The amygdala: is it an essential component of the neural network for social cognition? Neuropsychologia 41:517–522.

Anagnostaras SG, Gale GD, Fanselow MS. 2001. Hippocampus and contextual fear conditioning: recent controversies and advances. Hippocampus 11:8–17.

Balleine BW, Killcross S. 2006. Parallel incentive processing: an integrated view of amygdala function. Trends Neurosci 29:272–279.

Bandeira F, Lent R, Herculano-Houzel S. 2009. Changing numbers of neuronal and non-neuronal cells underlie postnatal brain growth in the rat. Proc Natl Acad Sci U S A 106: 14108–14113.

Bayer SA. 1980. Quantitative ^{3}H-thymidine radiographic analyses of neurogenesis in the rat amygdala. J Comp Neurol 194:845–875.

Bekenstein JW, Lothman EW. 1991. A comparison of the ontogeny of excitatory and inhibitory neurotransmission in the CA1 region and dentate gyrus of the rat hippocampal formation. Brain Res Dev Brain Res 63:237–243.

Berdel B, Morys J, Maciejewska B. 1997a. Neuronal changes in the basolateral complex during development of the amygdala of the rat. Int J Dev Neurosci 15:755–765.

Berdel B, Morys J, Maciejewska B, Dziewiatkowski J. 1997b. Volume and topographical changes of the basolateral complex during the development of the rat's amygdaloid body. Fol Morphol 56:1–11.

Blozovski D, Cudennec A. 1980. Passive avoidance learning in the young rat. Dev Psychobiol 13:513–518.

Bourgeois JP, Rakic P. 1993. Changes of synaptic density in the primary visual cortex of the macaque monkey from fetal to adult stage. J Neurosci 13:2801–2820.

Bourgeois JP, Goldman-Rakic PS, Rakic P. 1994. Synaptogenesis in the prefrontal cortex of rhesus monkeys. Cereb Cortex 4:78–96.

Bouwmeester H, Smits K, Van Ree JM. 2002a. Neonatal development of projections to the basolateral amygdala from prefrontal and thalamic structures in rat. J Comp Neurol 450:241–255.

Bouwmeester H, Wolterink G, van Ree JM. 2002b. Neonatal development of projections from the basolateral amygdala to prefrontal, striatal, and thalamic structures in the rat. J Comp Neurol 442:239–249.

Brizzee KR, Vogt J, Kharetchko X. 1964. Postnatal changes in glia/neuron index with a comparison of methods of cell enumeration in the white rat. Prog Brain Res 4:136–149.

Bronstein PM, Hirsch SM. 1976. Ontogeny of defensive reactions in Norway rats. J Comp Physiol Psychol 90:620–629.

Carlo CN, Stevens CF. 2011. Analysis of differential shrinkage in frozen brain sections and its implications for the use of guard zones in stereology. J Comp Neurol 519:2803–2810.

Cassell MD, Gray TS, Kiss JZ. 1986. Neuronal architecture in the rat central nucleus of the amygdala: a cytological, hodological, and immunocytochemical study. J Comp Neurol 246:478–499.

Chareyron LJ, Banta Lavenex P, Amaral DG, Lavenex P. 2011. Stereological analysis of the rat and monkey amygdala. J Comp Neurol 519:3218–3239.

Chareyron LJ, Banta Lavenex P, Amaral DG, Lavenex P. 2012. Postnatal development of the amygdala: a stereological study in macaque monkeys. J Comp Neurol 520: 1965–1984.

Chen SW, Shemyakin A, Wiedenmayer CP. 2006. The role of the amygdala and olfaction in unconditioned fear in developing rats. J Neurosci 26:233–240.

Collier AC, Mast J, Meyer DR, Jacobs C. 1979. Approach–avoidance conflict in preweanling rats: a developmental study. Anim Learn Behav 7:514–520.

Cooke BM, Simerly RB. 2005. Ontogeny of bidirectional connections between the medial nucleus of the amygdala and the principal bed nucleus of the stria terminalis in the rat. J Comp Neurol 489:42–58.

Cooke BM, Chowanadisai W, Breedlove SM. 2000. Post-weaning social isolation of male rats reduces the volume of the medial amygdala and leads to deficits in adult sexual behavior. Behav Brain Res 117:107–113.

Cressman VL, Balaban J, Steinfeld S, Shemyakin A, Graham P, Parisot N, Moore H. 2010. Prefrontal cortical inputs to the basal amygdala undergo pruning during late adolescence in the rat. J Comp Neurol 518:2693–2709.

Cunningham MG, Bhattacharyya S, Benes FM. 2002. Amygdalo-cortical sprouting continues into early adulthood: implications for the development of normal and abnormal function during adolescence. J Comp Neurol 453:116–130.

deCampo DM, Fudge JL. 2012. Where and what is the paralaminar nucleus? A review on a unique and frequently overlooked area of the primate amygdala. Neurosci Biobehav Rev 36:520–535.

Dobbing J, Sands J. 1979. Comparative aspects of the brain growth spurt. Early Hum Dev 3:79–83.

Fitting S, Booze RM, Hasselrot U, Mactutus CF. 2008. Differential long-term neurotoxicity of HIV-1 proteins in the rat hippocampal formation: a design-based stereological study. Hippocampus 18:135–147.

Foster JA, Burman MA. 2010. Evidence for hippocampus-dependent contextual learning at postnatal day 17 in the rat. Learn Mem 17:259–266.

Gilbert ME, Cain DP. 1981. A developmental study of kindling in the rat. Brain Res 254:321–328.

Grady MS, Charleston JS, Maris D, Witgen BM, Lifshitz J. 2003. Neuronal and glial cell number in the hippocampus after experimental traumatic brain injury: analysis by stereological estimation. J Neurotrauma 20:929–941.

Gundersen HJ. 1986. Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J Microsc 143:3–45.

Gundersen HJ, Jensen EB. 1987. The efficiency of systematic sampling in stereology and its prediction. J Microsc 147:229–263.

Hamidi M, Drevets WC, Price JL. 2004. Glial reduction in amygdala in major depressive disorder is due to oligodendrocytes. Biol Psychiatry 55:563–569.

Hefner K, Holmes A. 2007. Ontogeny of fear-, anxiety- and depression-related behavior across adolescence in C57BL/6J mice. Behav Brain Res 176:210–215.

Hubbard DT, Blanchard DC, Yang M, Markham CM, Gervacio A, Chun IL, Blanchard RJ. 2004. Development of defensive behavior and conditioning to cat odor in the rat. Physiol Behav 80:525–530.

Ito W, Pan BX, Yang C, Thakur S, Morozov A. 2009. Enhanced generalization of auditory conditioned fear in juvenile mice. Learn Mem 16:187–192.

Jabès A, Banta Lavenex P, Amaral DG, Lavenex P. 2010. Quantitative analysis of postnatal neurogenesis and neuron number in the macaque monkey dentate gyrus. EurJ Neurosci 31:273–285.

Jabès A, Banta Lavenex P, Amaral DG, Lavenex P. 2011. Postnatal development of the hippocampal formation: a stereological study in macaque monkeys. J Comp Neurol 519:1051–1070.

Jolkkonen E, Pitkänen A. 1998. Intrinsic connections of the rat amygdaloid complex: projections originating in the central nucleus. J Comp Neurol 395:53–72.

Kim JH, Richardson R. 2007. A developmental dissociation in reinstatement of an extinguished fear response in rats. Neurobiol Learn Mem 88:48–57.

Kordower JH, Piecinski P, Rakic P. 1992. Neurogenesis of the amygdaloid nuclear complex in the rhesus monkey. Brain Res Dev Brain Res 68:9–15.

Krettek JE, Price JL. 1978. A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. J Comp Neurol 178:255–280.

Krueger BK, Burne JF, Raff MC. 1995. Evidence for large-scale astrocyte death in the developing cerebellum. J Neurosci 15:3366–3374.

Lavenex P, Steele MA, Jacobs LF. 2000a. The seasonal pattern of cell proliferation and neuron number in the dentate gyrus of wild adult eastern gray squirrels. Eur J Neurosci 12:643–648.

Lavenex P, Steele MA, Jacobs LF. 2000b. Sex differences, but no seasonal variations in the hippocampus of food-caching squirrels: a stereological study. J Comp Neurol 425:152–166.

Lavenex P, Banta Lavenex P, Bennett JL, Amaral DG. 2009. Postmortem changes in the neuroanatomical characteristics of the primate brain: hippocampal formation. J Comp Neurol 512:27–51.

Lavenex P, Sugden SG, Davis RR, Gregg JP, Banta Lavenex P. 2011. Developmental regulation of gene expression and astrocytic processes may explain selective hippocampal vulnerability. Hippocampus 21:142–149.

LeDoux J. 2000a. The amygdala and emotion: a view through fear. In: Aggleton J, editor. The amygdala: the functional analysis of the amygdala.New York: Wiley-Liss. p 289–310.

LeDoux JE. 2000b. Emotion circuits in the brain. Annu Rev Neurosci 23:155–184.

LeDoux J. 2007. The amygdala. Curr Biol 17:R868–R874.

Matus-Amat P, Higgins EA, Sprunger D, Wright-Hardesty K, Rudy JW. 2007. The role of dorsal hippocampus and basolateral amygdala NMDA receptors in the acquisition and retrieval of context and contextual fear memories. Behav Neurosci 121:721–731.

McDonald AJ. 1982. Cytoarchitecture of the central amygdaloid nucleus of the rat. J Comp Neurol 208:401–418.

McDonald AJ. 1998. Cortical pathways to the mammalian amygdala. Prog Neurobiol 55:257–332.

Mizukami S, Nishizuka M, Arai Y. 1983. Sexual difference in nuclear volume and its ontogeny in the rat amygdala. Exp Neurol 79:569–575.

Moriceau S, Roth TL, Okotoghaide T, Sullivan RM. 2004. Corticosterone controls the developmental emergence of fear and amygdala function to predator odors in infant rat pups. Int J Dev Neurosci 22:415–422.

Morris JA, Jordan CL, Breedlove SM. 2008. Sexual dimorphism in neuronal number of the posterodorsal medial amygdala is independent of circulating androgens and regional volume in adult rats. J Comp Neurol 506:851–859.

Muller CM. 1992. Astrocytes in cat visual cortex studied by GFAP and S-100 immunocytochemistry during postnatal development. J Comp Neurol 317:309–323.

Nave KA. 2010. Myelination and support of axonal integrity by glia. Nature 468:244–252.

Nixdorf-Bergweiler BE, Albrecht D, Heinemann U. 1994. Developmental changes in the number, size, and orientation of GFAP-positive cells in the CA1 region of rat hippocampus. Glia 12:180–195.

Nurse S, Lacaille JC. 1999. Late maturation of $GABA_B$ synaptic transmission in area CA1 of the rat hippocampus. Neuropharmacology 38:1733–1742.

O'Kusky J, Colonnier M. 1982. Postnatal changes in the number of astrocytes, oligodendrocytes, and microglia in the visual cortex (area 17) of the macaque monkey: a stereological analysis in normal and monocularly deprived animals. J Comp Neurol 210:307–315.

Ono M, Kikusui T, Sasaki N, Ichikawa M, Mori Y, Murakami-Murofushi K. 2008. Early weaning induces anxiety and precocious myelination in the anterior part of the basolateral amygdala of male Balb/c mice. Neuroscience 156:1103–1110.

Palackal T, Neuringer M, Sturman J. 1993. Laminar analysis of the number of neurons, astrocytes, oligodendrocytes and microglia in the visual cortex (area 17) of 6- and 12-month-old rhesus monkeys fed a human infant soy-protein formula with or without taurine supplementation from birth. Dev Neurosci 15:54–67.

Pan BX, Ito W, Morozov A. 2009. Divergence between thalamic and cortical inputs to lateral amygdala during juvenile–adult transition in mice. Biol Psychiatry 66:964–971.

Parnavelas JG, Luder R, Pollard SG, Sullivan K, Lieberman AR. 1983. A qualitative and quantitative ultrastructural study of glial cells in the developing visual cortex of the rat. Philos Trans R Soc Lond B Biol Sci 301:55–84.

Pitkänen A. 2000. Connectivity of the rat amygdaloid complex. In: Aggleton J, editor. The amygdala: the functional analysis of the amygdala.New York: Wiley-Liss. p 31–115.

Pitkänen A, Stefanacci L, Farb CR, Go GG, LeDoux JE, Amaral DG. 1995. Intrinsic connections of the rat amygdaloid complex: projections originating in the lateral nucleus. J Comp Neurol 356:288–310.

Prather MD, Lavenex P, Mauldin-Jourdain ML, Mason WA, Capitanio JP, Mendoza SP, Amaral DG. 2001. Increased social fear and decreased fear of objects in monkeys with neonatal amygdala lesions. Neuroscience 106:653–658.

Price JL, Russchen FT, Amaral DG. 1987. The limbic region. II: The amygdaloid complex. In: Björklund A, Hökfelt T, Swanson LW, editors. Handbook of chemical neuroanatomy, vol 5, integrated systems of the CNS, part I. Amsterdam: Elsevier. p 279–388.

Raineki C, Shionoya K, Sander K, Sullivan RM. 2009. Ontogeny of odor-LiCl vs. odor-shock learning: similar behaviors but divergent ages of functional amygdala emergence. Learn Mem 16:114–121.

Raineki C, Holman PJ, Debiec J, Bugg M, Beasley A, Sullivan RM. 2010. Functional emergence of the hippocampus in context fear learning in infant rats. Hippocampus 20:1037–1046.

Rice D, Barone S Jr. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108(Suppl 3):511–533.

Rosen JB. 2004. The neurobiology of conditioned and unconditioned fear: a neurobehavioral system analysis of the amygdala. Behav Cogn Neurosci Rev 3:23–41.

Rubinow MJ, Juraska JM. 2009. Neuron and glia numbers in the basolateral nucleus of the amygdala from preweaning

through old age in male and female rats: a stereological study. J Comp Neurol 512:717–725.

Rudy JW. 1993. Contextual conditioning and auditory cue conditioning dissociate during development. Behav Neurosci 107:887–891.

Savander V, Go CG, LeDoux JE, Pitkänen A. 1995. Intrinsic connections of the rat amygdaloid complex: projections originating in the basal nucleus. J Comp Neurol 361: 345–368.

Savander V, Go CG, Ledoux JE, Pitkänen A. 1996. Intrinsic connections of the rat amygdaloid complex: projections originating in the accessory basal nucleus. J Comp Neurol 374:291–313.

Shionoya K, Moriceau S, Lunday L, Miner C, Roth TL, Sullivan RM. 2006. Development switch in neural circuitry underlying odor-malaise learning. Learn Mem 13:801–808.

Soriano E, Del Rio JA, Auladell C. 1993. Characterization of the phenotype and birthdates of pyknotic dead cells in the nervous system by a combination of DNA staining and immunohistochemistry for 5′-bromodeoxyuridine and neural antigens. J Histochem Cytochem 41:819–827.

Stanton ME. 2000. Multiple memory systems, development and conditioning. Behav Brain Res 110:25–37.

Sullivan EV, Adalsteinsson E, Sood R, Mayer D, Bell R, McBride W, Li TK, Pfefferbaum A. 2006. Longitudinal brain magnetic resonance imaging study of the alcohol-preferring rat. Part I: adult brain growth. Alcohol Clin Exp Res 30: 1234–1247.

Sullivan RM. 2001. Unique characteristics of neonatal classical conditioning: the role of the amygdala and locus coeruleus. Integr Physiol Behav Sci 36:293–307.

Sullivan RM, Landers M, Yeaman B, Wilson DA. 2000. Good memories of bad events in infancy. Nature 407:38–39.

Swann JW, Smith KL, Brady RJ. 1990. Neural networks and synaptic transmission in immature hippocampus. Adv Exp Med Biol 268:161–171.

Takahashi LK. 1992. Developmental expression of defensive responses during exposure to conspecific adults in preweanling rats (Rattus norvegicus). J Comp Psychol 106: 69–77.

Terasawa E, Timiras PS. 1968. Electrophysiological study of the limbic system in the rat at onset of puberty. Am J Physiol 215:1462–1467.

Thompson JV, Sullivan RM, Wilson DA. 2008. Developmental emergence of fear learning corresponds with changes in amygdala synaptic plasticity. Brain Res 1200:58–65.

Vaughan DW, Peters A. 1974. Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. J Neurocytol 3:405–429.

Verwer RW, Van Vulpen EH, Van Uum JF. 1996. Postnatal development of amygdaloid projections to the prefrontal cortex in the rat studied with retrograde and anterograde tracers. J Comp Neurol 376:75–96.

von Kienlin M, Kunnecke B, Metzger F, Steiner G, Richards JG, Ozmen L, Jacobsen H, Loetscher H. 2005. Altered metabolic profile in the frontal cortex of PS2APP transgenic mice, monitored throughout their life span. Neurobiol Dis 18:32–39.

Wakefield CL, Levine MS. 1985. Early postnatal development of basolateral amygdala in kitten: a Golgi morphometric analysis. Brain Res Bull 14:159–167.

Watson RE, Desesso JM, Hurtt ME, Cappon GD. 2006. Postnatal growth and morphological development of the brain: a species comparison. Birth Defects Res B Dev Reprod Toxicol 77:471–484.

West MJ, Gundersen HJ. 1990. Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296:1–22.

West MJ, Slomianka L, Gundersen HJ. 1991. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231:482–497.

Wiedenmayer CP. 2009. Plasticity of defensive behavior and fear in early development. Neurosci Biobehav Rev 33: 432–441.

Wiedenmayer CP, Barr GA. 1998. Ontogeny of defensive behavior and analgesia in rat pups exposed to an adult male rat. Physiol Behav 63:261–269.

Wiedenmayer CP, Barr GA. 2001a. Developmental changes in c-fos expression to an age-specific social stressor in infant rats. Behav Brain Res 126:147–157.

Wiedenmayer CP, Barr GA. 2001b. Developmental changes in responsivity to threat are stimulus-specific in rats. Dev Psychobiol 39:1–7.

Wiltgen BJ, Sanders MJ, Anagnostaras SG, Sage JR, Fanselow MS. 2006. Context fear learning in the absence of the hippocampus. J Neurosci 26:5484–5491.

Yates MA, Juraska JM. 2007. Increases in size and myelination of the rat corpus callosum during adulthood are maintained into old age. Brain Res 1142:13–18.

Zahm DS, Jensen SL, Williams ES, Martin JR 3rd. 1999. Direct comparison of projections from the central amygdaloid region and nucleus accumbens shell. Eur J Neurosci 11: 1119–1126.

Zehr JL, Todd BJ, Schulz KM, McCarthy MM, Sisk CL. 2006. Dendritic pruning of the medial amygdala during pubertal development of the male Syrian hamster. J Neurobiol 66: 578–590.