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Protracted Maturation of Forebrain Afferent Connections of the Ventral Tegmental Area in the Rat

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Abstract

The mesocorticolimbic dopamine system has long attracted the interest of researchers concerned with the unique gamut of behavioral and mental health vulnerabilities associated with adolescence. Accordingly, the development of the mesocorticolimbic system has been studied extensively, but almost exclusively with regard to dopaminergic output, particularly in the nucleus accumbens and medial prefrontal cortex. To the contrary, the ontogeny of inputs to the ventral tegmental area (VTA), the source of mesocorticolimbic dopamine, has been neglected. This is not a trivial oversight, as the activity of VTA neurons, which reflects their capacity to transmit information about salient events, is sensitively modulated by inputs. Here, we assessed the development of VTA afferent connections using the β subunit of cholera toxin (Ct β) as a retrograde axonal tracer in adolescent (postnatal day 39) and early adult (8–9-week-old) rats. After intra-VTA injections of Ct β , adolescent and early adult animals exhibited qualitatively similar distributions of retrogradely labeled neurons in the sense that VTA-projecting neurons were present at all of the same rostrocaudal levels in all of the same structures in both age groups. However, quantitation of retrogradely labeled neurons revealed that adolescent brains, compared with early adult brains, had significantly fewer VTA-projecting neurons preferentially within an interconnected network of cortical and striatopallidal forebrain structures. These findings provide a novel perspective on the development of the mesocorticolimbic dopamine system and may have important implications for age-dependent specificity in the function of this system, particularly with regard to adolescent impulsivity and mental health vulnerabilities.

INDEXING TERMS

adolescence; basal forebrain; dopamine; development; medial prefrontal cortex; nucleus accumbens

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take full responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: LY, DSZ. Acquisition of data: LY, RAR, ZMS, KPP. Analysis and interpretation of data: LY, DSZ. Writing of manuscript: LY, DSZ.

In recent years, the mesocorticolimbic dopamine system has attracted increasing attention from researchers interested in the unique gamut of behavioral and mental health vulnerabilities associated with adolescence (for reviews see Spear, 2000; Ernst et al., 2009; O'Donnell, 2010; Wahlstrom et al., 2010a,b; Flores, 2011; Sturman and Moghaddam, 2011; Luciana et al., 2012). Adolescents are prone to impulsive and risky behaviors, and neuropsychiatric symptoms associated with schizophrenia and depression frequently emerge during adolescence (Spear, 2000; Kessler et al., 2007; Paus et al., 2008; Merikangas et al., 2009). Because the mesocorticolimbic dopamine system is critical to mechanisms of reward, learning, and cognition (Wise, 2004; Berridge, 2007; Salomone and Correa, 2012), and dysregulation of this system is associated with neuropsychiatric disorders (Koob and Volkow, 2010; Tost et al., 2010; Miyake et al., 2011; Chaudhury et al., 2013; Tye et al., 2013), it is of interest to understand how the mesocorticolimbic dopamine system develops during adolescence.

Accordingly, the development of the mesocorticolimbic dopamine system has been studied extensively, but almost exclusively with regard to its output via dopaminergic terminal fields, particularly in the nucleus accumbens (Acb) and medial prefrontal cortex (mPFC; Verney et al., 1987; Voorn et al., 1988; Kalsbeek et al., 1988; Murrin and Zeng, 1990; Leslie et al., 1991; Rao et al., 1991; Rosenberg and Lewis, 1994, 1995; Teicher et al., 1995; Benes et al., 1996, 2000; Andersen et al., 1997, 2000; Antonopoulos et al., 1997, 2002; Tarazi et al., 1998, 1999; Lambe et al., 2000; Tarazi and Baldessarini, 2000; Badanich et al., 2006; Tseng and O'Donnell, 2005, 2007; Benoit-Marand and O'Donnell, 2008; Huppe-Gourgues and O'Donnell, 2012a,b). In contrast, the ontogeny of inputs to the ventral tegmental area (VTA), the source of mesocorticolimbic dopamine, has been neglected. This oversight is not trivial, as the activity of VTA neurons, which reflects their capacity to transmit information about salient events (Kitai et al., 1999; Sombers et al., 2009; Owesson-White et al., 2012), is sensitively modulated by inputs (Murase et al., 1993; Takahata and Moghaddam, 2000; Grace et al., 2007; Lammel et al., 2012; Jennings et al., 2013). In the adult, the VTA receives afferent projections from manifold interconnected structures spread throughout the brainstem, basal forebrain, and cortex (Nauta and Domesick, 1978; Phillipson, 1979; Wallace et al., 1989; 1992; Carr and Sesack, 1999, 2000; Zahm et al., 2001a; Fadel and Deutch, 2002; Geisler and Zahm, 2005, 2006; Philpot et al., 2005; Olmechenko and Sesack, 2005, 2006, 2007, 2009, 2010; Balcita-Pedicino and Sesack, 2007; Geisler et al., 2007; Ferreira et al., 2008; Olmechenko et al., 2009; Zahm et al., 2011; Watabe-Uchida et al., 2012). The widespread distribution of so many sources of VTA innervation is consistent with a proximal relation of this region to essentially the entire brain (Geisler and Zahm, 2005) and an accompanying capacity to integrate diverse biologically salient and reward-relevant information to guide adaptive behavior (Floresco et al., 2001, 2003; Lodge and Grace, 2006; Grace et al., 2007). Quantitative or qualitative variations in the patterns of input to the VTA related to development could thus impose significant age-related variability of function on the mesocorticolimbic dopamine system. The present study was performed as a first-pass comparison of the organization of VTA afferents in adolescent and early adult rats.

MATERIALS AND METHODS

Animals

All experiments were carried out in accordance with guidelines published in the National Institutes of Health *Guide for the care and use of laboratory animals* and were approved by the animal care committee at St. Louis University. Male Sprague Dawley rats (Harlan, Indianapolis, IN) were housed under a 12-hour light–dark cycle in groups of four to six until the time of tracer injections, after which they were housed individually. Food and water were provided ad libitum.

Amidst substantial variability in the literature regarding the ages that reflect adolescence and adulthood in the rat, a general pattern emerges in which adolescence is typically conceived as ranging from postnatal days (PND) 21 to 55 and early adulthood as beginning between PND55 and 60 (Fig. 1). With this consensus in mind, we injected tracer on $\text{PND}39 \pm 4$ days ($n = 5$), at which time rats weighed between 130 and 160 g (reflecting midadolescence), or between 8 and 9 weeks old ($n = 5$), at which time rats weighed between 270 and 310 g (reflecting early adulthood).

Tracer injections

Animals were injected intraperitoneally (i.p.) with a mixture of 45% ketamine (100 mg/ml), 35% xylazine (20 mg/ml), and 20% saline at a dose of 0.16 ml/100 g body weight. Several minutes later, they were placed into a Kopf stereotaxic instrument and injected in the VTA on one side of the brainstem with the β subunit of cholera toxin (Ct β ; List Biological Laboratories, Campbell, CA). Ct β (10%) was dissolved in 0.1 M Sorenson's phosphate buffer (SPB; pH 7.4) and injected iontophoretically from a 1.0-mm filament-containing borosilicate glass pipette pulled to a diameter of 20 μm . The injections were made over a period of 15 minutes using positive pulses of 3 μA (7 seconds on/7 seconds off). The pipettes were withdrawn 3 minutes after the current was stopped while applying a continuous negative current (1 μA) to minimize the leak of tracer into the pipette tract. Animals were given subcutaneous (s.c.) injections of carprofen (20 mg/kg) immediately after surgery and once daily for the next 3 days. Empirical optimization of stereotaxic coordinates for each age group was guided by the atlas of Paxinos and Watson (2007).

Brain fixation and immunocytochemistry

Three days after Ct β injections, the rats were deeply anesthetized with the ketamine-xylazine cocktail described above and perfused transaortically with 0.01 M SPB (pH 7.4) containing 0.9% sodium chloride and 2.5% sucrose, followed by 0.1 M SPB (pH 7.4) containing 4% paraformaldehyde and 2.5% sucrose. Upon removal from the skull, brains were postfixed in the same fixative for 4 hours, cryoprotected in 30% sucrose overnight, and sectioned at 50 μm on a freezing microtome. To accommodate possible differences in brain size and to allow precise matching of corresponding sections between age groups, five adjacent series of frontal sections from adult and three adjacent series from adolescent brains were collected. Thus, the entire brain was sampled at intervals of 250 μm in adults and 150 μm in adolescents.

One series of sections from each animal was immersed in 1% aqueous sodium borohydride for 15 minutes and thoroughly rinsed in 0.1 M SPB. Sections were then incubated overnight in 0.1 M SPB containing 0.1% Triton X-100 (SPB-t) and polyclonal primary antibody made in goat against Ct β (1:5,000; List Biological Laboratories). On the following day, sections were rinsed thoroughly in SPB-t and placed for 1 hour in SPB-t containing biotinylated secondary antibody raised in donkey against goat (1:200; Jackson Laboratories, West Grove, PA). After thorough rinsing, sections were placed for 1 hour in SPB-t containing avidin-biotin-peroxidase complex (ABC; 1:200; Vector Laboratories, Burlingame, CA), rinsed again in SPB, and incubated for 5–10 minutes in 0.05 M SPB (pH 7.4) containing 0.05% 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) and 0.003% hydrogen peroxide. This latter step generates an insoluble brown reaction product. The density and refractivity of the DAB reaction product were intensified by placing the sections first in 0.005% aqueous OsO₄ for 30 minutes, then in 0.1% aqueous thiocarbohydrazide (Sigma) for 10 minutes, and again in the OsO₄ solution for 10 minutes, with brief rinses between. Each series of sections was then mounted in rostrocaudal sequence onto gelatin-coated slides and coverslipped with Permount (Fisher, Pittsburgh, PA).

Antibody characterization

One primary antibody, anti-Ct β , was used in the study (Table 1). Immunoprocessed sections from brains lacking Ct β injections were devoid of reaction product. Brains that received injections of Ct β exhibited immunostaining only at the injection sites and in retrogradely labeled neurons and anterogradely labeled fibers.

Maps and photomicrographs

Sections selected for mapping represented corresponding rostrocaudal levels and contained the same sets of structures in both experimental groups. Retrograde labeling was plotted under brightfield optics with an Olympus BX51 microscope using $\times 10$ and $\times 20$ objectives and a dedicated hardware–software platform (Neurolucida; MBF Bioscience, Williston, VT). Maps of plotted sections were constructed in Adobe Illustrator CS2 (Adobe, San Jose, CA). Images were captured with a DVC 2000C-00-GE-MBF digital camera and adjusted for contrast and brightness in Adobe Photoshop CS2.

Quantitation of retrogradely labeled neurons

Brain structures were outlined by reference to the rat brain atlases of Paxinos and Watson (2007) and Paxinos et al. (1999); our own inhouse maps of basal forebrain immunoprocessed to show substance P, which emphasizes ventral pallidum, and nitric oxide synthase, which delineates several extended amygdala structures (Zahm et al., 2013); and maps of Fos labeling following psychostimulant treatment, which show the mesopontine rostromedial tegmental nucleus (RMTg; Zhou et al., 2009). Because afferent projections to the VTA are more robust ipsilateral to the injection (Geisler and Zahm, 2005), only ipsilateral structures were drawn, except in the case of midline structures (e.g., dorsal raphe). All outlined structures were present in at least two sections, except for the rostral pole of the Acb and nucleus of the solitary tract, each of which was present in a single section. Quantitation of Ct β -positive neurons in whole sections and individual structures was achieved with the aid of NeuroExplorer software (version 4.70.3). Labeled neurons in each

section and within all delineated structures were counted in all animals, and averages per section and structure for each age group were computed.

Group differences in total numbers of retrogradely labeled cells per section were analyzed using a two-way mixed ANOVA (age $\times$ brain section). Group differences in numbers of retrogradely labeled cells in individual structures were separately analyzed by using Bonferroni Student *t*-tests. For all statistical tests, the criterion for significance was set at 0.05. Fold differences in mean labeled neurons per structure between adolescent and adult rats were calculated using the following formula: (larger mean/smaller mean) – 1, such that structures with similar numbers of retrogradely labeled neurons in adolescent and adult rats gave fold differences approximating zero. Approximations of the effects of age on numbers of VTA-projecting neurons in a given structure were calculated by multiplying the lesser average of retrogradely labeled neurons by the fold difference.

To control for variability in injection size, data were standardized for adolescents and adults separately as follows: mean total labeled ($n = 5$)/total labeled (each case) $\times$ data for levels and structures (each case). Group differences in standardized values were analyzed as described above for the raw values.

Design-based estimates of numbers of neurons in the nucleus accumbens

Brains from adolescent ($n = 4$) and early adult ($n = 4$) rats were obtained and sectioned as described above. One series of sections from each animal was mounted onto gelatin-coated slides, defatted by immersion in 1:1 ethanol/chloroform overnight, rehydrated in decreasing concentrations of ethanol (100%, 95%, 90%, 80% 70%, 50%) for 10–15 minutes, and quickly rinsed in double-distilled water before being immersed in 0.1% cresyl violet solution for 10 minutes. After a brief rinse in double-distilled water, the sections were differentiated in ascending concentrations of ethanol for 10–15 minutes, cleared in xylene for 5 minutes, and cover-slipped with Permount.

Neurons in the Acb core and shell were counted using the optical fractionator probe of StereoInvestigator (MBF Bioscience) from sections spanning plates 11–25 of the rat brain atlas (Paxinos and Watson, 2007). Neurons were identified by diffuse staining throughout the soma and were easily differentiated from glial cells, which had very small, densely stained nuclei. A counting frame ($60 \times 60 \mu\text{m}$) was repeatedly superimposed in an unbiased manner on the Acb contour drawn on the right side of the brain, and counts were made at regular predetermined intervals ($x = 350 \mu\text{m}$, $y = 350 \mu\text{m}$) from a starting point determined randomly by StereoInvestigator. Counting was performed with the $\times 40$ objective by an investigator blind to the experimental groups. The average thickness of mounted sections was $19 \mu\text{m}$, and guard zones of $2 \mu\text{m}$ were used to avoid inconsistencies common to the cut surfaces of the sections. A coefficient of error below 0.05 was maintained in all animals, which minimized sampling variability. Group means were calculated based on the projected total numbers of neurons for each animal provided by StereoInvestigator. Acb volumes in adolescent and adult rats were calculated using the Cavalieri probe, and values for neuron density were obtained by dividing projected total numbers of neurons by the projected total volumes of the regions. Independent Bonferroni *t*-tests were used to assess group differences in neuron number, neuron density, and Acb volume.

RESULTS

Tracer injections

Tracer deposits occupied nearly the entire mediolateral extent and rostrocaudal axis of the VTA on one side of the brain in both adolescent and adult animals. All cases exhibited minimal rostral and caudal spread of tracer into adjacent parts of the lateral hypothalamus and RMTg, respectively. Significant spread of tracer into the interpeduncular nucleus was observed in one adult case, and spread of tracer into the substantia nigra was observed in a second adult case. No systematic differences in the size or shape of tracer deposits were observed between adolescent and adult cases. However, the deposits appeared to be lighter in color and less dense in adolescent compared with adult brains (Fig. 2).

Retrograde labeling

Retrogradely labeled neurons were identified by the presence of DAB deposits in neuronal somata. Although local anterograde labeling and retrograde labeling of somata and proximal dendrites had a darker, denser quality in some regions of adult compared with adolescent brains (e.g., Acb; Fig. 3), VTA-projecting neurons were present at all of the same rostrocaudal levels (Figs. 4, 5) in all of the same structures (Fig. 6) in both age groups. Sparse to moderate retrograde labeling was present in the cerebral cortex, including cingulate, prelimbic, infralimbic, and dorsal peduncular areas. Robust retrograde labeling was present in the basal forebrain, particularly in the Acb, ventral pallidum, lateral septum, and preoptic area. Sparse retrograde labeling was observed in amygdala and some hypothalamic nuclei, whereas retrograde labeling in the lateral hypothalamus was robust. Sparse to moderate retrograde labeling was observed in midbrain and brainstem structures, including the periaqueductal gray and dorsal raphe.

Quantitation of the raw (unstandardized) data revealed that adolescent compared with adult brains had significantly fewer VTA-projecting neurons at several forebrain levels (Fig. 5A; levels 3–11 and 14, representing structures contained within plates 9–37 and 44 of the Paxinos and Watson (2007) rat brain atlas; significant main effect of age: $F_{1,8} = 21.69$, $P < 0.01$; significant main effect of section level: $F_{34,272} = 76.51$, $P < 0.0001$; significant age $\times$ section level interaction: $F_{34,272} = 10.60$, $P < 0.0001$; Bonferroni post hoc tests: section 3: $t_{280} = 4.34$, $P < 0.001$; section 4: $t_{280} = 5.70$, $P < 0.0001$; section 5: $t_{280} = 6.15$, $P < 0.0001$; section 6: $t_{280} = 6.33$, $P < 0.0001$; section 7: $t_{280} = 7.05$, $P < 0.0001$; section 8: $t_{280} = 7.10$, $P < 0.0001$; section 9: $t_{280} = 6.32$, $P < 0.0001$; section 10: $t_{280} = 7.16$, $P < 0.0001$; section 11: $t_{280} = 9.73$, $P < 0.0001$; section 14: $t_{280} = 3.81$, $P < 0.01$). In contrast, the numbers of retrogradely labeled neurons were similar in adolescent and adult animals at all levels of the brain on the side contralateral to the injection site. Consistent with a previous report (Geisler and Zahm, 2005), a greater proportion of contralateral retrogradely labeled neurons was present at certain caudal compared with rostral levels (Fig. 5B; levels 17 and 21–29; main effect of age: $F_{1,8} = 0.008$, $P = 0.93$; significant main effect of section level: $F_{34,272} = 23.66$, $P < 0.0001$; age $\times$ section level interaction: $F_{34,272} = 0.56$, $P = 0.98$; Bonferroni post hoc tests in comparison to section 1: section 17: $t_{272} = 4.25$, $P < 0.05$; section 21: $t_{272} = 5.50$, $P < 0.0001$; section 22: $t_{272} = 7.02$, $P < 0.0001$; section 23: $t_{272} = 9.44$, $P < 0.0001$; section 24: $t_{272} = 11.56$, $P < 0.0001$; section 25: $t_{272} = 10.2$, $P < 0.0001$; section 26: $t_{272} =$

9.68, $P < 0.0001$; section 27: $t_{272} = 7.5$, $P < 0.0001$; section 28: $t_{272} = 4.97$, $P < 0.01$; section 29: $t_{272} = 4.0$, $P < 0.05$). Diminishing numbers of retrogradely labeled neurons were observed on both sides of the brain at far caudal levels (levels 30–35; Fig 5A, B).

Cortical structures that contained significantly fewer retrogradely labeled neurons in adolescent compared with adult rats included the tenia tecta (Fig. 6; $t_8 = 4.2$, $P < 0.01$; age effect of 215 neurons) and ventral orbital ($t_8 = 2.7$, $P < 0.05$; age effect of 200 neurons), cingulate ($t_8 = 7.7$, $P < 0.0001$; age effect of 621 neurons), prelimbic ($t_8 = 3.6$, $P < 0.01$; age effect of 384 neurons), infralimbic ($t_8 = 4.6$, $P < 0.01$; age effect of 230 neurons), and dorsal peduncular ($t_8 = 3.6$, $P < 0.01$; age effect of 432 neurons) areas. Fewer VTA-projecting neurons in adolescent compared with adult rats was also observed in the insular ($t_8 = 2.3$, $P < 0.05$; age effect of 383 neurons) and claustrum/endopiriform ($t_8 = 3.1$, $P < 0.05$; age effect of 280 neurons) areas.

Differences between adolescent and adult groups were also prominent in the basal forebrain. Adolescent rats had significantly fewer VTA-projecting neurons in the Acb, including its rostral pole ($t_8 = 2.5$, $P < 0.05$; age effect of 179 neurons), core ($t_8 = 3.9$, $P < 0.01$; age effect of 1118 neurons), and shell ($t_8 = 7.3$, $P < 0.0001$; age effect of 1,872 neurons) subterritories. Fewer VTA-projecting neurons in the adolescent compared with the adult were also observed in the caudomedial extremity of the olfactory tubercle, which the atlas of Paxinos and Watson (2007) identifies as the navicular nucleus ($t_8 = 3.8$, $P < 0.01$; age effect of 165 neurons), and ventral pallidum ($t_8 = 4.3$, $P < 0.01$; age effect of 1,192 neurons), lateral preoptic area ($t_8 = 4.5$, $P < 0.01$; age effect of 589 neurons), sublenticular extended amygdala ($t_8 = 4.1$, $P < 0.01$; age effect of 328 neurons), lateral hypothalamus ($t_8 = 3.3$, $P < 0.01$; age effect of 439 neurons), and zona incerta ($t_8 = 3.0$, $P < 0.05$; age effect of 157 neurons). Adolescent rats also exhibited statistically fewer VTA-projecting neurons in the basomedial (accessory basal) amygdaloid nucleus ($t_8 = 3.7$, $P < 0.01$; age effect of 152 neurons), medial amygdaloid nucleus ($t_8 = 3.5$, $P < 0.01$; age effect of 109 neurons), and central amygdaloid nucleus ($t_8 = 2.6$, $P < 0.05$; age effect of 318 neurons). No difference in numbers of VTA-projecting neurons between adolescent and adult rats was observed in any other structures examined, including the far-caudal nucleus of the solitary tract ($t_8 = 0.7$, $P = 0.53$; age effect of two neurons). Statistical analyses conducted on standardized data (see Materials and Methods; data not shown) yielded results identical to those acquired from the raw data except for two rostrocaudal levels that became statistically significant at the level of the lateral hypothalamus (Fig. 5A).

Numbers of neurons in the Acb of adolescent and adult rats

Comparison of adolescent and adult brains using design-based stereology (Table 2) yielded no differences in total number of Acb neurons ($t_6 = 0.43$, $P = 0.77$), density of Acb neurons ($t_6 = 2.0$, $P = 0.07$), or Acb volume ($t_6 = 1.6$, $P = 0.17$).

DISCUSSION

The key finding of this study is that adolescent rats differed quantitatively from adults in the complement of inputs to the VTA. Continuing a trend observed in prepubescent animals (Zahm et al., 2001b), adolescent rats had fewer VTA-projecting neurons mainly in ventral

striatal territories, including the Acb and striatal districts of the medial olfactory tubercle (including Paxinos and Watson's [2007] navicular nucleus). There were also fewer retrogradely labeled neurons in structures to which the ventral striatum projects, such as the ventral pallidum, lateral preoptic area, and lateral hypothalamus, and in cortical areas that project to ventral striatum, including dorsal peduncular, infralimbic, prelimbic, and cingulate areas. The central nucleus of the amygdala and sublenticular extended amygdala also had fewer neurons in adolescent rats, as did the basomedial amygdala. The observation that there are fewer VTA-projecting neurons in adolescent compared with adult rats mainly in a select group of interconnected forebrain structures suggests possible ontogenetic linkage between these regions. In this regard, it is noteworthy that the innervation of the rat Acb core by the medial prefrontal cortex (mPFC) also continues to mature until adulthood (Brenhouse et al., 2008). Fewer retrogradely labeled neurons in adolescent rats were also present in some structures that are negligibly connected with the VTA, such as the zona incerta and medial amygdaloid nucleus (Watabe-Uchida et al., 2012; Zahm et al., 2011). Differences in numbers of VTA-projecting neurons presumably affect the composition of the corticoventral striatopallidal-VTA circuitry and thus would be expected to distinguish associated neural mechanisms and accompanying behavior between adolescent and adult rats (see below).

Methodological considerations

It seems unlikely that the differences reported here reflect methodological biases, insofar as in most structures numbers of VTA-projecting neurons were statistically indistinguishable in adolescent and adult brains. Interestingly, retrograde labeling had a darker, denser quality in adult brains, which may reflect a continuing maturation through adolescence of the dendritic and local axonal arbors of VTA-projecting neurons or of proteins involved in axonal transport (Fig. 3, insets). This, together with possible continuing maturation of axonal terminations in the VTA, is a subject for additional study (Yetnikoff and Zahm, in preparation). It is not likely that there was less efficacious retrograde transport of tracer to far-rostral structures in the adolescent group, e.g., because of possible developmental differences in expression of axonal motor proteins, insofar as differences in numbers of retrogradely labeled neurons were not observed between groups in structures located far caudally in the brainstem, such as the nucleus of the solitary tract. In support of this, it has recently been shown using the retrograde tracer Fluorogold that mPFC-amygdala projecting neurons are *more numerous* during adolescence than in adulthood (Cressman et al., 2010).

We also considered the possibility that fewer VTA-projecting neurons occupying certain structures in adolescent compared with adult brains might simply reflect fewer neurons in these structures in adolescence. We controlled for this in the Acb, where one of the greatest age effects in VTA-projecting neurons was observed, by making design-based estimates of numbers of neurons in the adolescent and adult Acb. No age difference was observed. In a relevant study, Markham et al. (2007) revealed *greater* numbers of neurons in the ventral mPFC (corresponding to prelimbic and infralimbic cortices in the present study) of adolescent compared with adult rats. Our results are thus most simply explained as reflecting a delay in the establishment of VTA afferent connections arising mainly from a group of interconnected forebrain structures.

Indeed, Rinaman et al. (2000) have shown that Ct β can be taken up by axons that have yet to establish synaptic connections, suggesting that our results likely reflect a delayed arrival of VTA projections. The mechanisms that underlie these findings are open to speculation. Arrival of the final complement of afferents in the VTA could be linked to a hormonal surge that occurs during the transition to puberty (Spear, 2000; Tena-Sempere, 2012) and could depend on the expression in adults of intracellular androgen receptors in certain populations of VTA-projecting neurons (Aubele and Kritzer, 2012). Another possibility is that the maturation of dopaminergic activity within VTA afferent structures stimulates a final surge of axonal growth and innervation of the VTA, insofar as how dopamine modulates Acb and mPFC neurons changes between adolescence and adulthood (Tseng and O'Donnell, 2005, 2007; Benoit-Marand and O'Donnell, 2008; Huppe-Gourgues and O'Donnell, 2012a, b).

Retrograde labeling methods are invariably subject to potential confounds, such as uptake of tracer by fibers-of-passage and spread of injected tracer to unintended structures. We have discussed these complex problems extensively in some recent papers (Geisler and Zahm, 2005; Jhou et al., 2009; Zahm et al., 2011), concluding, at least for injections into brainstem structures, that purely spurious retrograde labeling is rare. Labeling that seems likely to be an artifact, however, was observed in the brainstem trigeminal complex in the present study. The impression conveyed by Figure 5 that labeling at level 29 is greater contralateral than ipsilateral to the Ct β injection site may well be due to uptake by damaged fibers of the medial lemniscus.

Functional considerations

The significance of the protracted maturation of VTA afferent connections lies in the consensus view that, whereas midbrain dopaminergic neurons exhibit pacemaker-like activity in the absence of afferent input (Grace and Onn, 1989), in vivo tonic dopaminergic firing is controlled by a highly organized and widespread system of inputs of various transmitter identities. Afferent volleys generated in response to internal and external stimuli elicit burst firing (Schultz, 1998; Carr and Sesack, 2000; Floresco et al., 2001, 2003; Sesack et al., 2003; Geisler and Zahm, 2005; Lodge and Grace, 2006; Grace et al., 2007; Lammel et al., 2012; Jennings et al., 2013). Accordingly, VTA dopamine neurons respond to novel, salient, and reward-predicting stimuli with bursting and to reward omission or aversive stimuli mainly with inhibition, although VTA neurons may fire in response to aversive stimuli under certain circumstances (Schultz, 1998; Bromberg-Martin et al., 2010; Ungless et al., 2011; Cohen et al., 2012; McCutcheon et al., 2012b; Salomone and Correa, 2012). Burst firing of VTA dopamine neurons correlates with increased synaptic concentrations of dopamine in the Acb (Heien and Wightman, 2006; Sombers et al., 2009), which in turn has been related to the “burning in” of adaptive responses (Bozarth and Wise, 1981; Wise and Schwartz, 1981; Spyraiki et al., 1982; Wise, 2004), allocation of effort to achieve appetitive goals (Salomone and Correa, 2012), and attribution of incentive salience to reward-related stimuli (Berridge, 2007). Within this functional context, projections from various afferent structures exert cooperative and competing influences to regulate the activity of VTA dopamine neurons and behavior (Grace et al., 2007; Lammel et al., 2012; Jennings et al., 2013) by targeting dopamine neurons directly or indirectly via local interneurons (Carr and Sesack, 2000; Sesack and Carr, 2002; Xia et al., 2011). If behavioral inhibition in the service

of selecting adaptive actions is an overarching function of ventral striatopallidum (Taha and Fields, 2006; Krause et al., 2010; Nicola, 2007; Carlezon and Thomas, 2009), with an additional contribution to the suppression of impulsive actions made by the mPFC (Cardinal, 2006; Pattij and Vanderschuren, 2008; Dalley et al., 2011), projection to the VTA by fewer neurons in these regions during adolescence compared with adulthood could contribute to the lower reported capacity of adolescent rats to suppress impulsive, risky responses (Burton and Fletcher, 2012).

That VTA dopamine neurons, and, in turn, behavioral activation, may be influenced differently by ventral basal ganglia inputs during adolescence and adulthood gains support from recent findings indicating that VTA dopamine neurons are more active in adolescent compared with adult rats. γ -Aminobutyric acid type A (GABA_A) receptor-mediated spontaneous inhibitory postsynaptic potentials are fewer in putative VTA dopamine neurons during adolescence compared with adulthood, and the frequency of baseline firing of VTA dopamine neurons is greater and burst firing duration longer during adolescence (McCutcheon et al., 2012a). Also, intra-VTA administration of a GABA_A receptor antagonist increases dopamine levels in the Acb to a lesser extent during adolescence compared with adulthood (Natividad et al., 2010). Having acknowledged these points, however, we should stress that, to our knowledge, there is as yet no objective evidence indicating that the essence of having fewer VTA-projecting neurons in ventral basal ganglia structures in adolescent vs. adult rats is an actual basis for these observations. Afferents of the VTA terminate within a complex mix of interconnected (Omelchenko and Sesack, 2009; Dobi et al., 2010) dopaminergic, GABAergic, and glutamatergic neurons (Swanson, 1982; Yamaguchi et al., 2007; Nair-Roberts et al., 2008). Developmental maturation of neuronal firing would depend on the relative maturations of specific relationships between particular inputs and postsynaptic targets.

Quantitative differences in VTA afferent connections between adolescence and adulthood also might have implications for the induction of enduring plasticity in mesocorticolimbic dopaminergic function by motivationally relevant stimuli. For example, behavioral and neurochemical sensitization to stimulant drugs of abuse is thought to be mediated, at least in part, by drug-induced synaptic plasticity in select VTA dopamine neurons with distinct afferent connections (Carr and Sesack, 2000; Lammel et al., 2011). Insofar as adult VTA-projecting mPFC glutamatergic neurons play a critical role in stimulant drug-induced behavioral and neurochemical sensitization (Wolf, 1998), having fewer forebrain VTA-projecting neurons in adolescent vs. adult animals may subtly alter the mechanisms by which drugs of abuse induce such plasticity, as previously suggested (Yetnikoff et al., 2011, 2013).

CONCLUSIONS

Adolescents are prone to impulsive and risky behaviors (Romer, 2010; Gladwin et al., 2011), and neuropsychiatric symptoms associated with schizophrenia and depression frequently emerge during adolescence (Spear, 2000; Kessler et al., 2007; Paus et al., 2008; Merikangas et al., 2009), often in association with earlier prodromal changes (Corcoran et al., 2011; Devylder et al., 2013). Although the precise mechanisms that underlie the

uniquely vulnerable nature of adolescent behavior are not known (see, e.g., Andersen and Navalta, 2004; Wong et al., 2013), the responsible maturational processes are thought to reside in specific neural circuits (Paus et al., 2008; Tau and Peterson, 2009; Brenhouse and Andersen, 2011; Blakemore and Robbins, 2012) that particularly affect mesocorticolimbic dopaminergic function (Ernst et al., 2009; Wahlstrom et al., 2010; Luciana et al., 2012). We have demonstrated in the rodent a protracted maturation of VTA afferents arising almost exclusively in the forebrain. This provides a new perspective on the development of the mesocorticolimbic dopamine system by emphasizing the afferent side of the story. The possibility that an analogous effect exists in humans is supported by several recent studies (Paus et al., 1999; Sowell et al., 1999; Urošević et al., 2012).

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Figure 1.

Diagram showing the ages used to define adolescence (broken lines) and early adulthood (straight lines) in a sample of recently published papers. Horizontal lines represent the age spans assessed in each referenced study. Shaded vertical bars represent the adolescent (light gray) and early adult (dark gray) ages used in the current study. PND, postnatal day. [1] Albert et al., 2013; [2] Baldini et al., 2013; [3] Crawford et al., 2013; [4] Fleming et al., 2013; [5] Du and Grace, 2013; [6] Karlsson et al., 2013; [7] Saul et al., 2013; [8] Schneider, 2013; [9] Suo et al., 2013; [10] Zitman and Richter-Levin, 2013; [11] Schulz et al., 2013a; [12] Schulz et al., 2013b; [13] Sherrill et al., 2010; [14] Suri et al., 2013; [15] Zoratto et al., 2013; [16] Bhardwaj et al., 2012; [17] Clinton et al., 2012; [18] Dalton et al., 2012; [19] Damborsky et al., 2012; [20] Vazquez-Roque et al., 2012; [21] Klug and van den Buuse, 2012; [22] McFadden et al., 2012; [23] Nielsen et al., 2012; [24] Pocivavsek et al., 2012; [25] Rosenfeld and Weller, 2012; [26] Shipman and Nicoll, 2012; [27] Van Hasselt et al., 2012; [28] Vetreno and Crews, 2012; [29] Avital et al., 2011; [30] Crawford et al., 2011; [31] Huppe-Gourgues and O'Donnell, 2011a; [32] Huppe-Gourgues and O'Donnell, 2011b; [33] Schwarz et al., 2011; [34] Trezza et al., 2011; [35] Turner et al., 2011; [36] Wright et al., 2011; [37] Carboni et al., 2010; [38] Pian et al., 2010; [39] Philpot et al., 2009; [40] Schramm-Sapya et al., 2009; [41] Andersen et al., 2008; [42] Benoit-Marand and O'Donnell, 2008; [43] Tseng and O'Donnell 2007; [44] Badanich et al., 2006; [45]

Andersen, 2005; [46] Tseng and O'Donnell, 2005; [47] Andersen 2004; [48] Badanich and Kirstein, 2004; [49] Pistis et al., 2004; [50] Spear, 2004.

Figure 2.

A: Representative examples of Ct β injection sites at rostral (top) and caudal (bottom) levels of the VTA in adolescent (left) and adult (right) rats. **B:** Diagrams of Ct β injection sites from all of the subjects are shown at several levels along the rostrocaudal extent of the VTA. Scale bar = 400 μ m.

Figure 3.

Micrographs illustrating retrograde labeling in the Acb in adolescent (**A**) and adult (**C**) rats. **B** and **D** are enlargements of the boxed areas in **A** and **C**, respectively. **Insets** in **A** and **C** are enlargements of the endopiriform cortex (indicated by arrows). **Insets** in **B** and **D** are enlargements of boxed areas. Note that anterograde and retrograde labeling in the Acb is somewhat darker and more robust in adults compared with adolescents. In contrast, while retrograde labeling of cell bodies in the endopiriform cortex is qualitatively similar in the two age groups, dendritic labeling appears more robust in the adult. Scale bar = 200 μ m in **D** (applies to **B**, **D**); 1 mm for **A**, **C**; 50 μ m for insets.

Figure 4.

Representative examples of corresponding sections plotted in adolescent (**A**) and adult (**B**) rats. Note the apparent difference between adolescent and adult animals in numbers of retrogradely labeled neurons in the accumbens and cingulate cortex and the variability between groups in the retrograde labeling in the lateral septum, which is reflected in a seemingly large but nonsignificant age effect in Figure 6. Cg, cingulate cortex; Acb, nucleus accumbens; LS, lateral septum.

Figure 5.

Adolescent rats exhibited fewer VTA-projecting neurons than adult rats selectively in the forebrain. Mean $\pm$ SEM retrogradely labeled neurons per section across rostrocaudal levels on ipsilateral (**A**) and contralateral (**B**) sides relative to the injection in adolescent (open squares) and adult (solid squares) rats. Arrows indicate the first most rostral level in which several brain structures were observed; numbers in parentheses indicate number of sections extending caudalward in which those structures were observed. V Orb, ventral orbital cortex (cx); PrL, prelimbic cx; DP, dorsal peduncular cx; IL, infralimbic cx; Cg, cingulate cx; Acb, nucleus accumbens; VP, ventral pallidum; LPO, lateral preoptic area; LH, lateral hypothalamus; LHb, lateral habenula; VTA, ventral tegmental area; DR, dorsal raphe; PPTg, pedunculopontine tegmental nucleus; N Sol, nucleus of the solitary tract. ** $P < .01$, *** $P < .001$, **** $P < .0001$, † $P < .05$ after standardization.

Figure 6. Effect of age on numbers of VTA-projecting neurons within individual brain structures. Means $\pm$ SEM of retrogradely labeled neurons within brain structures along the rostrocaudal axis of the brain are shown at left. Magnitudes of the age effect in numbers of retrogradely labeled neurons are shown at right. Greater numbers of neurons in adolescents deflect leftward and in adults rightward. Magnitude of the age effect was calculated by multiplying the smaller group average of retrogradely labeled neurons for a given structure by the fold

difference (see Materials and Methods) between adult and adolescent rats for that structure. Adol, adolescent.

TABLE 1

Primary Antibodies

Antiserum	Immunogen	Source (catalog No.)	Working dilution	Specificity
Goat polyclonal anticholera toxin β subunit (Ct β)	Cholera toxin β subunit (cholera toxin itself)	List Biological Laboratories, Campbell, CA (703)	1:5,000	In our hands, immunoprocessed sections from brains lacking Ct β injections were devoid of reaction product; in brains that received injections of Ct β , immunostaining was observed only at the injection sites, in retrogradely labeled neurons, and anterogradely labeled fibers

TABLE 2

Stereology-Based Estimates of Numbers of Neurons, Volumes Measured, and Neuron Density (Means $\pm$ SEM) in the Acb of Adolescent and Adult Rats

	Adolescent	Adult
Neuron numbers	500,334 $\pm$ 79,832	515,539 $\pm$ 46,252
Measured volume (μm^3)	$3.5 \times 10^9 \pm 6.9 \times 10^7$	$4.1 \times 10^9 \pm 3.6 \times 10^8$
Neurons/measured volume	$1.42 \times 10^6 \pm 6.8 \times 10^4$	$1.27 \times 10^6 \pm 2.9 \times 10^4$