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Age matters

James Edgar McCutcheon and **Michela Marinelli**
Dept. of Cellular & Molecular Pharmacology, Rosalind Franklin University of Medicine and Science, The Chicago Medical School, 3333 Green Bay Rd, North Chicago, IL 60064

Abstract

The age of an experimental animal can be a critical variable, yet age matters are often overlooked within neuroscience. Many studies make use of young animals, without considering possible differences between immature and mature subjects. This is especially problematic when attempting to model traits or diseases that do not emerge until adulthood. In this commentary we discuss the reasons for this apparent bias in age of experimental animals, and illustrate the problem with a systematic review of published articles on long-term potentiation. Additionally, we review the developmental stages of a rat and discuss the difficulty in using the weight of an animal as a predictor of its age. Finally, we provide original data from our laboratory and review published data to emphasise that development is an ongoing process that does not end with puberty. Developmental changes can be quantitative in nature, involving gradual changes, rapid switches, or inverted U-shaped curves. Changes can also be qualitative. Thus, phenomena that appear to be unitary may be governed by different mechanisms at different ages. We conclude that selection of the age of the animals may be critically important in the design and interpretation of neurobiological studies.

Keywords

development; adult; LTP; hippocampus; dopamine

Introduction

Imagine if a pharmaceutical company registered a novel analgesic or antipsychotic drug intended for adult patients, and then announced that it would conduct its clinical trials in children. Without question, this proposal would be thought absurd, not only ethically but because the results might not be scientifically valid for adults. Clinical trials must recruit study subjects that represent the intended patient population.

Although this scenario seems extraordinary, it is in fact common in neuroscience to study young animals in which the brain has not fully developed. These studies can be informative and provide valuable information, but the age of animals needs to be considered as a factor that may influence the results. In addition, such young animals should not be erroneously defined as "adults". In this commentary we argue that as practitioners and consumers of science, it is necessary to pay close attention to the age of animals used in experiments. We review the stages of rodent maturation and discuss key principles arising from our own data and a review of the literature. We highlight that brain development continues well after puberty, that quantitative changes may occur gradually or rapidly, and that the mechanisms underlying certain neurobiological processes change as an animal matures.

Correspondence to: Michela Marinelli.

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Why and to what extent is age overlooked?

Neuroscientists have at their disposal numerous model systems with which to carry out their research. A key decision in designing an experiment is choosing the best model with which to tackle the question at hand. In simpler model systems such as cell lines or in those that more closely resemble neural systems, such as embryonic or postnatal neuronal cultures, the focus of the study can be reductionist, allowing consideration of aspects of complex systems in narrower terms. Such models are extremely useful for the study of cellular and molecular mechanisms, and the limitations of these simplified systems are recognized by most readers and authors. For example, those studying the biophysical properties of ion channels in cell lines generally recognize that they are not modelling the intact nervous system.

Over the last decade there has been a noticeable shift from a 'reductionist' to a 'systems' perspective and with this has come a substantial growth in the use of *ex vivo* brain slices - in which neuronal architecture is better preserved - for the study of synaptic and cellular biology. This trend has been facilitated by technological advances. For example, techniques such as patch-clamp electrophysiology and cellular imaging, once only possible in isolated cells, are now routinely practiced in tissue slices. However, such studies are performed using animals of various ages and it is seldom acknowledged that they are often limited to very young animals. This is important because findings in young animals do not necessarily extrapolate to adults. Although this limitation is not confined to slice physiology, it is here that the use of young animals is most evident.

To assess the extent to which animals of different ages are being used in neuroscience research, we performed a systematic review of the literature in which we tabulated the ages at which rodents were tested. We examined (i) studies on hippocampal long-term potentiation (LTP) in rodents and (ii) studies using the Morris water maze test of spatial memory (see supporting information for methodology and references). In rodents, hippocampal LTP has been extensively investigated as a mechanism for spatial memory (Morris *et al.*, 2003).

In our review on hippocampal LTP, we first examined studies that used *ex vivo* brain tissue preparations (see supporting information). Figure 1A shows that only a minority of studies (34%) used adult animals, while the majority (59%) used animals that had not yet reached adulthood (see next section for age definitions). The remaining 7% of studies did not specify an age or weight. Overall, the studies used a wide range of ages, from postnatal day 1 (P1) to P915 (i.e. 30 months). Furthermore, the age ranges at which animals were tested did not just vary between studies, but also within. Approximately 30% of experiments pooled animals whose ages varied by 3–4 weeks, often spanning critical age periods, such as puberty. We also found that 42% of studies defined animals as "adults", although the methods section or supplementary online material clearly indicated that the average age of the animals was not within an adult range. When we repeated this analysis using the lowest age in each study (see supporting information for detailed explanation) we found that 60% of studies incorrectly defined young animals as "adults". To highlight the problems in controlling for the developmental stage of rodents we looked at the various definitions of "adult" (or mature) in this cohort of papers and found that this varied across studies from a low of P24-25 to a high of P549 (18 months) (Larson *et al.*, 2005; Fukata *et al.*, 2006; Sim et al., 2006). The definition of "young adult" varied from a low of P21-22 to a high of P92 (3 months) (Xu et al., 2000; Meredith et al., 2003; Miyamoto et al., 2005; Sim et al., 2006; Lauterborn et al., 2007).

When we confined our analysis of *ex vivo* studies to experiments using voltage- or current-clamp slice electrophysiology (patch clamp or sharp electrodes) we found that 75% of

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studies were using young animals and only 20% examined adult animals; the rest (5%) did not specify the age of the experimental subjects (table 1).

Possible reasons for this bias towards young animals include improved cell visibility and identification in young tissue, primarily due to less advanced myelination. Although there are newer methods available to conduct such studies in older animals, they are more challenging. Interestingly though, the scarce use of adult animals was not just limited to voltage- and current-clamp studies; only 38% of experiments using field recordings in the tissue slice, and 45% of those using other techniques (e.g. western blots, immunohistochemistry, electron microscopy, etc.) examined adult animals (table 1). Such techniques do not rely on good individuation of cells, and could thus be performed easily in adult animals. Although a few of these *ex vivo* studies may have been specifically examining development and could therefore justify this choice of age, this is unlikely to be the case for the majority. Thus, studies of the same phenomenon (hippocampal LTP) in the live animal show quite a different age profile. As our next analysis shows (figure 1A and table 1), studies of hippocampal LTP using *in vivo* electrophysiology and/or behavioural tests, focused mostly on adult animals (65%); only 26% used young animals, and the rest (9%) did not report age. Likewise, studies using the Morris water maze to test spatial memory (figure 1B, table 1) were performed mainly on adult subjects (77%); only 17% of the studies were performed in young animals and in 5%, age was not specified. The lack of significant overlap between these different techniques makes it difficult to extrapolate between them and means that information gleaned from *ex vivo* work often does not directly complement studies of *in vivo* physiology and behaviour.

From birth to adulthood – the development of a lab rat

To determine when an animal is an adult, it is important to review the developmental stages the animal progresses through to reach adulthood. Both rats and mice show a similar developmental profile (figure 2). At P21, rodents are weaned i.e. separated from their mother, after which they begin to undergo sexual maturation (Sisk & Zehr, 2005). Sexual maturity is generally defined by vaginal opening (females) or balano-preputial separation (males). This point is reached in female rats at approximately P32-34 (Lewis *et al*., 2002) but in males, maturity occurs much later at around P45 to P48 (Lewis *et al*., 2002). However, the age of sexual maturity varies considerably between individuals, ranging from as young as P40 to as old as P76 in male rats (Lewis *et al*., 2002).

It is also important to note that sexual maturity itself does not mark the beginning of adulthood, but rather denotes the beginning of adolescence. Like humans, rats progress through a period of adolescence characterised by behaviours such as increased risk-taking and social play. These behaviours extend well beyond the pubertal period through the transition to adulthood (Spear, 2000), which begins after the eighth week of postnatal life (~P63).

The body weight of an animal is sometimes considered an indicator of its age. However, weight is not an accurate surrogate marker for age. A comparison of the weights and ages from websites of two major vendors revealed that there is considerable variability both between vendors and between different colonies from the same vendor. In fact, Sprague Dawley rats are available in two sub-strains, one (originated by Charles River Laboratories, Wilmington, MA), which gains less weight than the other (originated by the Sprague Dawley Company, Madison, WI). Thus, a 300 g male Sprague Dawley rat can be between 57 and 70 days of age, depending on the vendor and colony (Charles River SD SAS, 67 days; Harlan SD, 57 days, Harlan CD, 70 days). Data from our own institution show that large variability exists even when rats are obtained from the same colony. As figure 3A

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shows, male rats weighing between 250 g and 274 g (a weight range commonly provided by vendors) differed in age by three weeks, from P49 (periadolescent) to P70 (young adulthood). In addition, male rats of the same exact age showed up to 100 g variation in body weight (figure 3B). Weight is, therefore, only an approximate marker of age.

Neural circuits change at different stages of postnatal development

A vast number of developmental processes are required to produce an adult animal from an embryo and many maturational changes occur during the early postnatal period. A number of these developmental changes are well known and extensively characterised. For example, the neurotransmitter γ-aminobutyric acid (GABA) switches from being depolarising to hyperpolarising during the first few postnatal days (Ben Ari *et al*., 1989; Rivera *et al*., 1999; Ben Ari, 2002). Likewise, NMDA receptor subunit composition changes gradually during the first few postnatal weeks (Monyer *et al*., 1994; Sheng *et al*., 1994; Zhong *et al*., 1995; Dumas, 2005a). Such changes radically alter the consequences of neurotransmitter release and receptor activation over early stages of development. However, even after these early stages, changes continue to occur and these are often less well characterised and recognised.

In the next sections we provide examples to show how such changes can impact experimental results.

Dopaminergic transmission – changes occurring during the periadolescent period

Some developmental changes are so abrupt that they render the adult system qualitatively different from its juvenile form. For example, electrophysiological studies in the prefrontal cortex (PFC) show that inhibitory fast-spiking GABAergic interneurons are unresponsive to dopamine D2 receptor activation when rats are prepubertal, aged P14-35 (Seamans *et al*., 2001; Gorelova *et al*., 2002; Tseng & O’Donnell, 2007). However, when rats reach P50 and start to approach adulthood, dopamine D2 receptor activation becomes excitatory in these neurons (figure 4; Tseng & O’Donnell, 2007). This is an example of a change occurring at a late time period (~P50-60), when many might have believed developmental processes had finished. In fact, those studies performed only at young ages erroneously concluded that dopamine D2 receptor activation does not modulate the activity of fast-spiking GABAergic interneurons in the PFC. This event is notable not only for its clear implications for adult neuromodulation of the PFC, but also because its sudden occurrence is indicative of a specific developmental shift, rather than a slower consequence of ageing.

Interestingly, another study of this system found that two populations of PFC interneurons develop dopamine D1 receptor-mediated excitation at different time points (Tseng & O’Donnell, 2007). Specifically, fast spiking interneurons show increased excitation to the application of a D1 dopamine receptor agonist before puberty whereas non-fast spiking interneurons only develop this response after P50. Thus, even within the same brain region, specific cell types can show different rates of development.

Dopamine in the PFC has been heavily implicated in schizophrenia, a disorder that typically develops during late adolescence and early adulthood. The significance of the late maturation of the PFC is heightened in this case; studying its role in schizophrenia using young animals would almost certainly result in misleading conclusions. Dopamine in the PFC is also involved in mediating a number of other traits and behaviours such as impulsivity and executive control and so the implications of these findings extend beyond schizophrenia research. In the examples above, early studies, performed only in young rats, led to the wrong assumptions about the role of dopamine in the PFC and this could have

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substantial, deleterious consequences on our understanding of disease processes and on drug development.

The ontogeny of dopamine receptor expression is another example of a system that changes during adolescence and provides an example of a developmental change that occurs gradually with an inverted U-shaped trajectory. Several studies have shown that D1 and D2 dopamine receptors increase gradually after birth, and peak peripubertally, before receptor number declines in later life. Notably, for different brain regions this peak in receptor expression differs, reflecting the timing of maturation for each structure. For example, in the striatum, dopamine receptor levels peak between P28 and P40 (Noisin & Thomas, 1989; Andersen et al., 2000), before declining to stable levels during P60-120 (Andersen et al., 2000; Tarazi & Baldessarini 2000). In contrast, in the PFC, in which maturation is delayed, maximal receptor expression does not occur until about P60 and then declines to a stable level between P80-120. Of note, is that in studies where the authors considered rats to be adult at P45-60, analysis of receptor expression was not continued beyond this age, and thus the decline that occurs in true adulthood was missed (Sales *et al*., 1989; Chen & Weiss, 1991; Tarazi & Baldessarini, 2000).

Another example of a U-shaped trajectory comes from our data on dopamine cell firing in the midbrain. Figure 5 shows that dopamine cell activity changes over age. Activity is low after weaning, peaks at about P45, and declines thereafter. Adolescence, thus, seems to be a distinct developmental stage, with respect to dopamine cell firing and dopamine receptor expression. The biological relevance of this peak may be related to certain distinctive behavioural characteristics associated with adolescence in many species including humans, such as increased risk-taking, impulsivity, and social play. Without examining later time points, however, this peak would not have been observed. In addition, these studies demonstrate that drawing conclusions from only a limited number of time points that do not include the adolescent period would be misleading; i.e. it would have been assumed that firing rate is constant from the weanling period to adulthood. It is also important to note that, although cells in juvenile rats (P24-27) and adults (>P70) have similar firing rates, this does not mean that other characteristics of these cells are similar. To give just one example, dopamine neurons from juvenile rats are typically excited by nicotine, whereas in adults they are primarily inhibited (Marinelli, 2008).

These findings show that although some systems achieve features of their “adult” phenotype at ages preceding true adulthood, others do not. They also emphasise that maturational changes do not necessarily follow a simple linear course characterised by progressive increases or decreases. In addition to the PFC and midbrain, age-related changes in neuronal activity and reactivity to dopamine also occur in other brain areas, such as the arcuate nucleus and the striatum (Coyle *et al.*, 1985; Napier *et al.*, 1985; Arbogast & Voogt, 1991; Andersen *et al.*, 2000). Hence, there is compelling evidence that throughout the brain dopamine circuits are not mature in young animals, and so any study of this system that uses young animals should be interpreted with caution.

Developmental changes in the induction and expression of LTP

LTP is one of the most studied phenomena in neuroscience and so provides a good illustrative case for this commentary. The above literature review (figure 1A) shows that studies of LTP use animals of many different ages ranging from before, during, and after puberty. LTP at different ages is likely to serve different functions and may well arise from different mechanisms (for review see Dumas, 2005b). Despite this, as shown in figure 1A, few studies of LTP have considered age to be a relevant factor. However, the few papers that have looked across different ages have identified important developmental differences.

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Yasuda *et al.* (2003) examined changes in the way LTP is regulated in the first four postnatal weeks. Consistent with studies showing the essential role of calcium/calmodulin-dependent protein kinase II (CaMKII) for the induction of LTP (Lisman *et al*., 2002), Yasuda *et al*. (2003) found that CaMKII inhibition did indeed block LTP in hippocampal slices from three-week old animals (P21). In younger (P17-18) animals, however, inhibition of CaMKII had a far weaker effect and in P7-8 rats there was no effect at all. In contrast, inhibition of protein kinase A (PKA) had no effect on LTP in animals >P27 but blocked LTP in young animals (P7-8). Lu *et al.* (2007) extended this work showing that a second critical period for PKA emerges after P49, concluding that PKA is required for LTP induction at both early (<P27) and late (>P49) time points but not in between. These careful studies showed that what appears to be a unitary phenomenon at all ages – synaptic strengthening induced by the same pairing protocol – actually relies on different molecular mechanisms at different ages. Whether these particular characteristics change further as animals go further through adulthood, remains to be tested.

Lu *et al.* (2007) also demonstrated that a requirement for calcium-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors for LTP induction - currently a controversial topic in the field - is also age-dependent. Specifically, these receptors were shown to be required in 2 week old (P14) and 8 week old (P56) mice but, crucially, not in 3 week old mice (P21). This age-dependency may explain why some authors have found calcium-permeable AMPA receptors to be critical for LTP induction whereas others have not. Lu *et al.* (2007) point out that two critical experiments with contradictory findings (Plant *et al.*, 2006; Adesnik & Nicoll, 2007) may have been conducted with animals at different ages, spanning the time between when calcium-permeable AMPA receptors are required (P14) and when they are not (P21). Jensen *et al.* (2003) also showed a developmental shift in the requirement for putative calcium-permeable AMPA receptors for the induction of LTP.

The efficacy of different pairing protocols at inducing LTP during development was also examined across ages. Meredith *et al.* (2003) found that the effectiveness of pairing pre-synaptic stimulation with single post-synaptic action potentials (30 pairings) at CA3-CA1 synapses gradually decreases with advancing age until, in the "adult", a paired burst of post-synaptic activity is required to elicit LTP (they considered their animals adults at P30-45, hence the quote marks). By manipulating GABA-A receptor-mediated inhibition the authors were able to reestablish the effectiveness of single spikes in the adult and so suggested that differences across age may reflect maturation of the GABA system. Whether further changes continue to occur after P45 remains unknown.

Age continues to be important factor in neurobiological studies of adult animals as well. LTP has been examined in mature animals to study age-related memory decline. As adult animals grow older, there is an increase in the induction threshold and potentiation becomes less stable (Barnes, 1979), and there is growing literature on how synaptic plasticity changes during both adulthood (Lynch *et al.*, 2006) and old age (Foster, 1999). These studies provide another rich source of data about age-related changes in neural function, even after the animal has reached adulthood.

Development of spinal sensory processing

The examples presented above relate mostly to higher cortical functions, such as memory and cognition. Similar age-related factors may influence functions that involve subcortical neural structures. For example, although the neural circuitry for pain signalling in the spinal cord emerges at birth or shortly after, the type of afferent input to the spinal cord dorsal horn differs substantially between P23 rats and P60 rats (Park *et al.*, 1999). Similarly, the expression of glutamatergic and GABAergic receptors in the spinal cord differs between

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immature and mature rats (Pattinson & Fitzgerald, 2004). Whether such reorganization and maturation in spinal cord dorsal horn participates in age-related differences in pain sensitivity (Pattinson & Fitzgerald, 2004; Howard et al., 2005) or if parallel changes are of greater import remains under investigation.

Summary and conclusions

Neuroscience studies, in particular those involving *ex vivo* electrophysiology, are performed on animals of all ages, mostly young animals. In addition, definitions of adulthood vary widely across studies, with the most concerning trend being the categorization of young rodents as adults. We have presented a number of examples that illustrate several reasons why it is essential to avoid this error, and why it is important to consider age as a critical experimental variable.

First, developmental changes continue far beyond the first few postnatal weeks. Therefore, accurate characterisation of the true adult phenotype requires the study of adult animals.

Second, developmental changes occur with different patterns or trajectories. For example, certain characteristics change gradually over time while others show much more sudden shifts. Additionally, certain phenotypes like dopamine receptor expression and dopamine cell firing rate show an inverted U-shaped trajectory, the peak of which could potentially define a critical period for a certain behaviour or phenomenon (Paus et al., 2008).

Third, phenomena that appear to be unitary in their form may be governed by different mechanisms at different ages. Here, we discussed work on LTP that showed a differing cohort of molecular effectors and rules for induction of synaptic potentiation at different ages.

The major reason that people have used younger animals for *ex vivo* electrophysiology studies is that, historically, many experiments have been difficult to perform in older animals. Now, however, techniques for using older animals in patch-clamp recordings are readily available and should not be considered overly daunting. Simple protocol adaptations, such as modifying visualisation methods, make recordings from adult animals attainable (Moyer & Brown, 1998; Moyer & Brown, 2002). In addition to cortex, hippocampus, and spinal cord, scientists have become proficient in obtaining patch-clamp recordings in adults from other structures including the striatum (Martin *et al.*, 2005), midbrain (Fagen *et al.*, 2007; Chen et al., 2008), and amygdala (Tye *et al.*, 2008). An additional benefit of using older animals is that it is possible to collect data from animals that have been subjected to repeated manipulations, such as drug administrations or learning tasks. However, care should be taken that manipulations are performed in adult animals, because there is abundant evidence that the brain reacts differently to perturbations across ages (Andersen *et al.*, 2000; Andersen, 2003; Bolaños *et al.*, 2003; Black *et al.*, 2006).

Because age-related changes have now been observed in numerous systems, we believe that it is essential for investigators to identify the developmental stage of their experimental subjects and to give due consideration to whether it is likely that their results can be generalised to adult ages. Although we have focused largely on rats, the same age-related issues apply to studies using different species including primates and mice. Thus, for each species, it is critical to be aware of the developmental period that subjects occupy. Such considerations are most important when the phenomenon to be modelled is known to be age-dependent. One of the best examples involves the link between PFC function and schizophrenia, a disease that does not emerge until late adolescence or early adulthood (Paus *et al.*, 2008). Other traits and pathologies are age-dependent, for example, diseases

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associated with ageing such as Parkinson's or Alzheimer's Diseases, and these too may require animal models of appropriate age to be of relevance.

In conclusion then, stark developmental changes appear to be the rule rather than the exception. In all the examples considered herein, when investigators have conducted thorough investigations over long developmental time spans they have either overturned false assumptions in the field (e.g the essential role of CaMKII in LTP or the insensitivity of PFC interneurons to D2 dopamine receptor activation) or they have clarified discrepancies in the subject (e.g. whether calcium permeable AMPA receptors are required for LTP). Thus, giving proper consideration to matters of age has the potential to radically improve our understanding of brain function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

GABA γ-aminobutyric acid

LTP long term potentiation

P postnatal day

PKA protein kinase A

PFC prefrontal cortex

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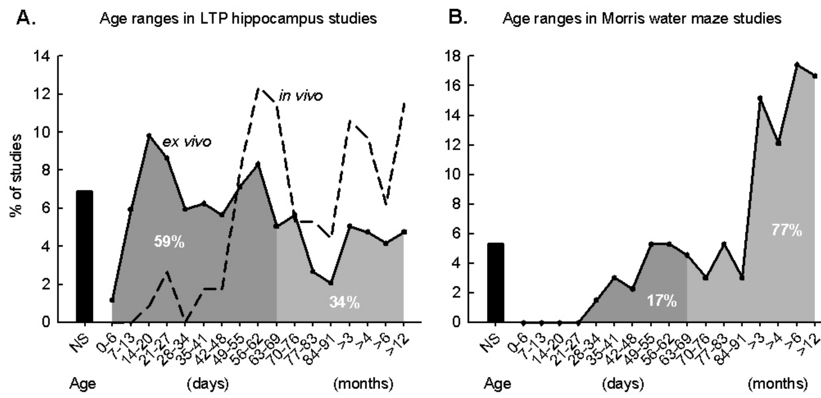

Figure 1.

Results of a systematic review examining age distribution of rodents used in neuroscience experiments. **A**, Age distribution of subjects used in studies on hippocampal LTP using *ex vivo* (solid line, n=336) and *in vivo* (dashed line, n=112) preparations. **B**, Age distribution of studies using Morris water maze (n=132). In both graphs, the black bar represents the number of studies in which no age or weight was specified (NS) and the shaded areas represent pre-adulthood (dark grey) and adulthood (light grey). See supporting information for detailed description of how the systematic review was performed. Figures demonstrate that the age profile of subjects differs considerably depending on technique used. As such, for *ex vivo* preparations, the majority of studies are carried out in animals before they have reached adulthood whereas the converse is true for *in vivo* LTP studies and Morris water maze behavioural studies (see main text for more details).

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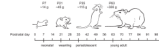

Figure 2.
Different developmental stages of a rat. The body weight of a rat changes greatly during the first two postnatal months. Male rats weigh approximately 14 g at P7, 45 g at weaning (P21), 115 g as adolescents (P35) and 300 g by young adulthood (>P63). As shown by figure 1A, the majority of *ex vivo* studies are carried out during this period of rapid growth, rather than in fully grown animals. Illustration by Dr. Claire Cannon.

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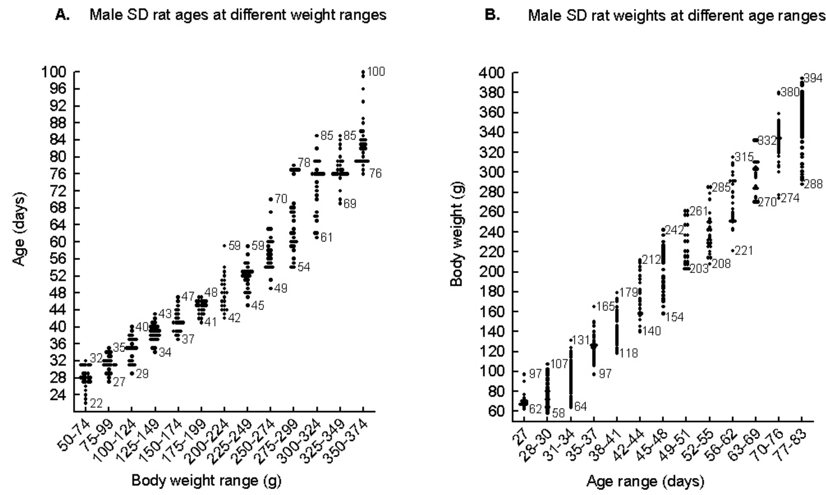

Figure 3.

Body weights and ages of male Sprague-Dawley rats received in our institution over the past four years. **A**, rats are divided in weight-ranges, corresponding to those provided by vendors. Within each weight-range, animals vary considerably in age. **B**, rats are divided by age-groups, in bins of 3-4 days. Within each age-group, rats differ greatly in body weight. Each point represents 5% of the measured population from the same dataset (n= 904 measurements for A, where body weight range ends at 374g, and n=869 measurements for B, where age range ends at P83). Numbers indicate the minimum and maximum age/weight recorded for each range. See supporting information for detailed methodology.

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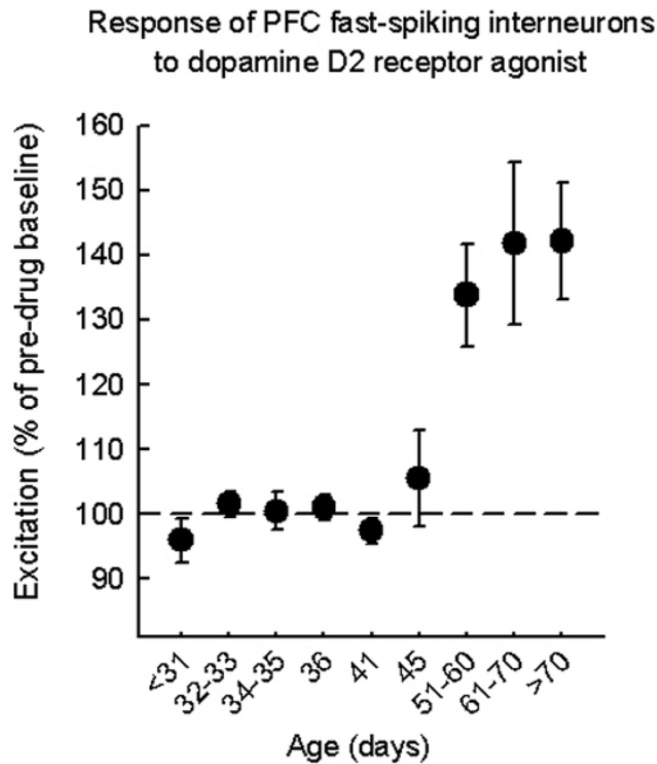

Figure 4.

Age-dependent responses to dopamine D2 receptor stimulation in prefrontal cortex fast-spiking interneurons. In *ex vivo* cortical slices, cells from pre-pubertal rats do not respond to quinpirole (1 μm), a D2 agonist whereas in adult rats quinpirole excites these cells. Higher doses of quinpirole (2–5 μm) were also without effect in young animals (data not shown). Excitation was defined as an increase in the number of spikes eliclited in response to depolarising current injection. Points are mean ± S.E.M. (n=3–6). Data previously reported in Tseng & O'Donnell (2007).

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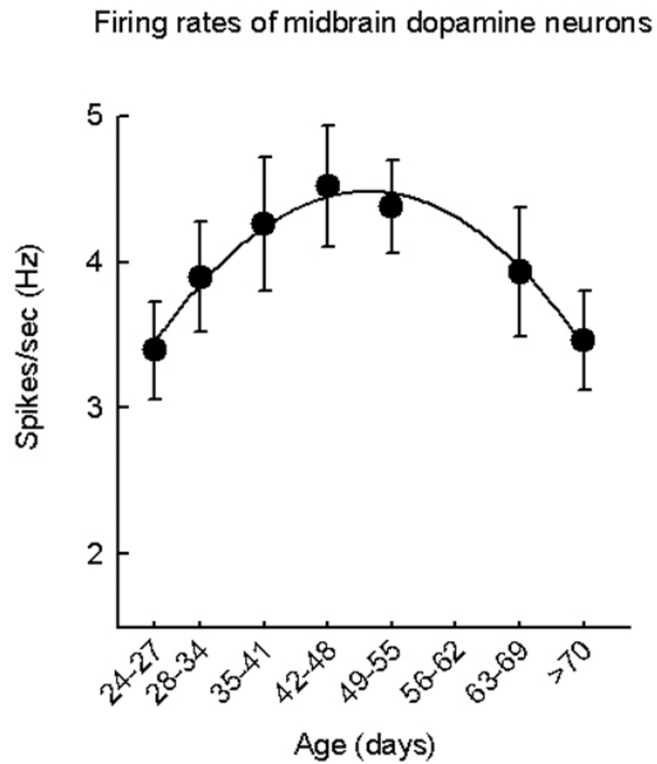

Figure 5.

Age-dependent changes on firing rate of midbrain dopamine neurons. Dopamine neuron activity is low shortly after weaning, it increases during the adolescent period and peaks around P45, and it decreases thereafter. Dopamine neuron activity was assessed with *in vivo* extracellular recordings in chloral hydrate-anesthetized animals, as detailed in Marinelli & White (2000). Points represent mean ± S.E.M. (n=12–26). Data obtained by M. Marinelli for the purpose of this review.

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Table 1

Studies on hippocampal LTP and Morris water maze (Morris WM) analysed with respect to experimental approaches and ages at which animals were tested.

	LTP: *ex vivo*			LTP: *in vivo*	Morris WM
	V/C Clamp	Field	Various		
Young	75%	55%	42%	26%	17%
Adult	20%	38%	45%	65%	77%
NS	5%	7%	13%	9%	5%
n	117	207	98	112	132