Title: A brief guide to studying fear in developing rodents: Important considerations and common pitfalls

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Significance Statement

Substantial progress has been made in our understanding of learned fear, with important implications for the treatment of fear- and anxiety-related psychological disorders, including post-traumatic stress disorder, specific phobias, and social anxiety. However, the characterisation of fear learning during development has lagged behind studies of adults, despite a growing awareness that the majority of psychological disorders emerge during childhood or adolescence. In this primer, we outline important considerations and common mistakes in developmental studies of rats and mice. Using examples from the study of learned fear, we offer a number of practical suggestions to guide best practice in rodent studies of development.

Abstract

Development is a time of rapid change that sets the pathway to adult functioning across all aspects of physical and mental health. Developmental studies can therefore offer insight into the unique needs of individuals at different stages of normal development as well as the aetiology of various disease states. The aim of this overview is to provide an introduction to the practical implementation of developmental studies in rats and mice, with an emphasis on the study of learned fear. We first discuss how developmental factors may influence experimental outcomes for any study. This is followed by a discussion of methodological issues to consider when conducting studies of developing rodents, highlighting examples from the literature on learned fear. Throughout, we offer some recommendations to guide researchers on best practice in developmental studies.

Keywords: Development – Pavlovian fear conditioning – fear extinction – rodent studies – age-related changes – infancy – adolescence

Over recent decades substantial progress has been made in our understanding of learned fear, at both the behavioural and neural levels of analysis (McCullough, Morrison, & Ressler, 2016). One striking feature of this work has been the convergence between findings in humans and in rodents, highlighting the translational value of this line of research (Maren, Phan, & Liberzon, 2013; Milad & Quirk, 2012). However, there remain gaps in our knowledge, in part due to an ongoing focus on adult male subjects and participants. As the field continues to mature, there is growing recognition of the need to examine sex differences in learned fear (Li & Graham, 2017; Thibault, 2016). It is also the case that there is a relative dearth of research on learned fear across development, which we argue is fundamental to the progression of knowledge and treatment outcomes in this area.

Why study development?

It is a truism that children are not simply small adults. As will be discussed in the context of conditioned fear, our knowledge and understanding of adult functioning does not necessarily apply to young individuals. Development is characterised by rapid, often non-linear, changes in physiology and behaviour, including learned fear and the neural structures associated with such behaviour (Hunt & Campbell, 1997; McCutcheon & Marinelli, 2009; Semple, Blomgren, Gimlin, Ferriero, & Noble-Haeusslein, 2013). From a basic science perspective, these developmental differences – questions of what is different, when it is different, and why it is different – are of interest in and of themselves. From a societal and public health perspective, developmental studies are needed to effectively address medical and psychological questions of health and disease for children and adolescents. Periods of rapid change and growth render the developing individual vulnerable, but also offer windows of opportunity during which behavioural and neural profiles are more malleable and therefore more amenable to treatment (Lee et al., 2014). Essentially, developmental studies are useful as they help us to first define age-appropriate norms of behavioural and neurological

functioning and then to identify optimal environments and treatments that promote positive health outcomes within this most valued sector of society (see Figure 1 for a summary).

In saying this, the importance of developmental studies should not be viewed solely through the narrow lens of their application to those who are currently in the midst of development. All adults have a developmental history that informs their current functioning. Indeed, many psychological theories emphasise the formative nature of early-life experiences in the aetiology of psychopathology (e.g., Gross & Hen, 2004; Mineka & Zinbarg, 2006). In keeping with such theoretical accounts, epidemiological evidence suggests that childhood adversity (e.g., neglect, abuse, parental mental illness) increases the risk for adult psychopathology (Kessler et al., 2010), and that approximately half of all adult disorders emerge during childhood or adolescence (Jones, 2013). Aside from predicting pathological outcomes, the study of development can also provide insight into healthy neuropsychological processes as they occur across the lifespan. That is, characteristics normally observed only during development (e.g., rapid, non-pathological forgetting, or infantile amnesia) can offer unique opportunities to approach problems (e.g., the locus of memory) from new perspectives (Callaghan, Li, & Richardson, 2014).

In the current unit, we focus on the practicalities of conducting developmental studies with rats and mice. First, we discuss developmental factors that should, where possible, be taken into account for all studies with these species, regardless of the age of the test subjects. Following this, we discuss important considerations and common mistakes in developmental rodent studies. Throughout, we contextualise this information with concrete examples of how these considerations apply in the case of developmental studies of learned fear.

Developmental factors to consider in all studies.

As noted above, all adults have a history that encompasses their development. Given this, it is important to consider how developmental factors may influence current functioning. It is now recognised that early life experiences, including exposure to stress, infection, environmental microbes, or differing levels of parental care, can have long-lasting effects (Borre et al., 2014; Heindel et al., 2016; Kaffman & Meaney, 2007). For example, low levels of maternal care, repeated bouts of separation from the mother during early postnatal development (maternal separation stress), and germ-free rearing environments are all associated with heightened corticosterone responses to stress and altered anxiety-like behaviour in adulthood (Clarke et al., 2013; Francis, Diorio, Liu, & Meaney, 1999; Kalinichev, Easterling, Plotsky, & Holtzman, 2002). In fact, at least some of the effects of early-life manipulations are so strong that they can be observed across generations (Callaghan, Cowan, & Richardson, 2016; Francis et al., 1999; Franklin et al., 2010; for reviews, see Braun et al., in press; Cowan, Callaghan, Kan, & Richardson, 2016).

These findings strengthen the rationale for proper randomisation of experimental and control groups to ensure that extraneous developmental factors do not result in false positives or obscure group differences. For example, experimental and control groups should be obtained from the same breeding colonies (or, if multiple breeding colonies are used then animals from each colony must be randomly allocated to experimental groups), multiple cohorts should be tested, and groups should not be composed of animals from a limited number of litters. These developmental factors should be considered as part of a best practice random allocation process, which will incorporate procedures to ensure that the order of group allocation is also random (i.e., the first animal selected from a box/litter should not always be allocated to the same experimental group). Random sequence generators are one approach that has been suggested to assist in this and thereby minimise potential selection

bias (Hirst et al., 2014; see also the Animal Research: Reporting of *In Vivo* Experiments [ARRIVE] guidelines; Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

Litter effects. Individuals from the same litter are likely to have very similar early life experiences, on top of genetic similarities, that will shape their development and adult functioning, just as human siblings (especially twins) would be expected to exhibit overlapping characteristics. Therefore, data obtained from littermates should not be treated as independent observations as this will skew the representativeness of the sample and undermine the replicability of the study. This is particularly true when observations are made during development, and we emphasise here that the number of animals from a given litter allocated to each experimental group should be reported in all developmental studies.

We recommend that, wherever possible, only one animal (or one male and one female) from each litter be allocated to any given experimental group. To achieve this in practice, the researcher will likely need to have multiple experiments planned to ensure efficient use of the litter. This will not only allow allocation of littermates to separate experimental groups so as to eliminate litter effects, but is also in line with researchers' ethical responsibilities to reduce the number of animals culled from each litter. If more than one animal from a litter is included in any particular group, litter effects must be accounted for statistically (usually by averaging across individuals from the same litter and treating the average as a single data point, or by including litter as a nested factor in the analysis). The need to adjust for litter effects in statistical analyses has been identified as best practice by many before us (e.g., Abbey & Howard, 1973; Lazic & Essioux, 2013; Terranova & Laviola, 2005), and has even been incorporated into official OECD guidelines (OECD, 2007), yet many continue to ignore this advice, to the detriment of the field (Lazic & Essioux, 2013).

Litter size. One factor that contributes to litter effects and warrants further consideration is the size of the litter. The number of animals in a given litter has the potential

to influence the resources available to each individual and thereby influence the animals' growth and progression through developmental stages. Large litters will exhibit delayed growth as the individuals must compete for nursing time. In contrast, small litters will have access to an excess supply of breast milk, leading to overfeeding and weight gain. Both scenarios have long-term effects on metabolism, neuroinflammation, sexual maturation, anxiety-like behaviour, and cognitive performance (De Luca et al., 2016; Seitz, 1954). Essentially, large variation in litter sizes will increase variability in behavioural and neural outcomes. To reduce this source of variability, common practice is to cull litters to a consistent number of pups (e.g., 8 pups per litter) and cross-foster age-matched pups to increase numbers in smaller litters (Lohmiller & Swing, 2006). Although this is standard practice, and achieves the aim of equating growth curves across litters, it should be noted that it is unclear at present whether such cross-fostering has any systematic impacts on the cross-fostered pups or not. In any case, standardisation of litter sizes in this manner should be done as early in development as possible (i.e., at least within the first week of life) to minimise the likelihood of long-term effects on pup development.

Housing and husbandry. Finally, as noted above, the early rearing environment and levels of maternal care can have a substantial impact on later life outcomes. It should be noted that housing and husbandry practices can contribute to both of these factors. For example, the complete absence of handling or cage cleaning during early development can increase corticosterone responses to stress, similar to effects observed in animals exposed to early-life maternal separation stress procedure (Plotsky & Meaney, 1993). Care should therefore be taken to ensure that housing and husbandry conditions are kept constant across litters and during later development. This includes routine cleaning practices (typically once to twice per week) and environmental stimulation/enrichment (e.g., cage space, toys and chewing materials). The number of animals per cage might also be considered an enrichment

factor and should therefore be kept as constant as possible. In particular, housing animals in isolation should be avoided as it is a known stressor for rodents due to their inherent sociability (unless of course the objective is to induce stress; Beery & Kaufer, 2015; Sandi & Haller, 2015).

Considerations for studies of development.

As with all scientific studies, developmental studies require careful planning to ensure a robust study design. Here, we will briefly discuss some of the most important factors to consider when undertaking a study of infant, juvenile, or adolescent rodents (summarised in Table 1). Of course, this list is not exhaustive and there may be considerations that are specific to the experimental design or parameters, but we hope that this will provide a starting point for the experimenter who is new to studies of development.

A concrete example is used to illustrate each concept described here, focusing on the study of learned fear. For the purposes of this article, we will focus on Pavlovian fear conditioning (see Figure 2), which involves the paired presentation of a neutral sensory stimulus (e.g., a tone, light, or scent, which will become the conditioned stimulus; CS), with an inherently aversive stimulus (e.g., a small electrical shock – the unconditioned stimulus; US). After several pairings, an individual will begin to express fear to the previously neutral CS. This can be assessed by presenting the CS alone and measuring behavioural fear expression (the conditioned response; CR). We will also provide examples relating to the extinction of conditioned fear, which involves repeated or prolonged presentation of the CS alone such that conditioned responding is diminished (a process that forms the basis for clinical exposure therapy; Milad & Quirk, 2012).

Table 1. Summary of important considerations in developmental studies.

Factor to consider	**Common mistakes**	**Recommendations**
Number of litters	Use of multiple pups from the same litter in the same experimental group as independent observations, restricting generalisability and reproducibility of results.	Maximum of one male and one female per litter allocated to each experimental group. Ideally, researchers should run multiple experiments concurrently to allow efficient use of pups. If this is not possible, multiple observations from the same litter should be averaged and treated as a single data point.
Litter size	Large variation in litter size, leading to individuals of ostensibly the same age being at different developmental stages (i.e., individuals from small litters will develop faster due to increased availability of resources).	Maintain even litter sizes (e.g., 8 pups per litter) using a combination of culling and cross-fostering as close to birth as possible.
Housing and husbandry	Inconsistency in housing conditions or husbandry routines, which can alter stress responses and other behaviours.	Maintain consistency in environmental conditions, including availability of social and non-social stimuli, and regular cage cleaning. Rodents are social animals, so should be housed with a minimum 2 animals per cage.
Timing of observations	Failure to account for rapid developmental transitions. Inappropriate labelling of 'infant', 'juvenile', and 'adolescent' rats.	Be as precise as possible in timing of experimental observations. We recommend testing all individuals within ± 1 day of the chosen age, especially in infancy. See Table 2 for a rough guide to age ranges for different developmental stages in rats.
Outcome measures	Use of inappropriate measures or inappropriate interpretation of measures.	Choose a measure of fear that is expressed in the chosen age range (see Figure 3). Note that neural pathways supporting fear expression also change across development.
Experiment duration	Long experiments that encompass multiple developmental stages. Long training or testing sessions causing distress in the dam and pups, with disruption to feeding patterns and thermoregulation when pups are very young.	Where possible, minimise length of experimental protocols to a few days and avoid lengthy periods of separation from the dam prior to weaning (ideally <15 min). If pups are <P10, maintain body temperature using a heat-pad during long separations.

Species & strain selection	Failure to adjust timing of observations based on species- and strain-specific variation in developmental trajectories.	Developmental transitions will generally occur earlier in mice than in rats. Refer to the literature for the specific outcome measure in the chosen strain and if this information is not available conduct pilot studies based on timing of other developmental transitions.
Sex differences	Assumption that males and females will respond in equivalent ways prior to puberty, even though developmental manipulations (e.g., early life stress) are known to have different long-term effects on male and female rodents.	When possible sex differences are of interest, both male and female animals should be tested, in line with recent policy updates from the National Institutes of Health (2015) to consider sex as a biological variable.
Apparatus & equipment	Failure to adjust equipment for developing individuals, preventing accurate assessment of performance.	Scale equipment to suit developmental stage. When equipment is introduced to the home cage (e.g., headpieces attached to pups), monitor the reaction of the dam to avoid damage to the equipment and harm to the dam or litter.
Drug dosage	Assumption that adult dosages will be appropriate for use in infants, leading to inaccurate assessment of drug effects or potential harm to the individual.	Refer to the literature for the chosen drug; dose may need to be increased or decreased depending on the specific pharmacokinetics for the chosen developmental stage. Where this information is unavailable, carefully monitored dose-response pilots should be conducted to ensure safety and efficacy.
Experimental design	Assumption that the same parameters will assess outcomes validly across development.	Depending on the experimental hypothesis, it may be necessary to choose either parameters or performance to be held constant. E.g., to conduct a valid assessment of long-term fear retention across development, it will be necessary to increase the number of CS-US pairings for younger animals in order to equate initial learning.

Timing of observations. The rapid changes that occur during development also necessitate precise timing of experimental observations. This is important to ensure that the age of the experimental subjects fits within the developmental stage of interest. Unfortunately, many studies mislabel the stage of development of experimental animals (McCutcheon & Marinelli, 2009). In Table 2 we provide a rough guide to developmental stages in rats. Similar age ranges are used to describe developmental stages in mice (Brust,

Schindler, & Lewejohann; 2015), although mice tend to mature slightly faster than rats and the distinction between infant and juvenile stages is not made as often for mice. A useful rule of thumb in distinguishing between the "infant" and "juvenile" stages of development is the normal age of weaning for that species. That is, if an animal is younger than the normal age of weaning (P21/22 in rats) then it would usually be referred to as an "infant" while those older than that would be referred to as a "juvenile". Note that there remains some controversy over the definition of adolescence. In Table 2, we have given a relatively broad definition of adolescence, which in some studies will be further broken down into early (or peri-), mid, and late adolescence.

Table 2. Estimated age ranges for different developmental stages in rats.

Developmental stage	**Approximate age range**
Perinatal	Up to P7
Infant	P7 – P21
Juvenile	P22 – P28
Adolescent	P30 – P55
Adult	P60+

Stages based on Semple et al. (2013) and Sengupta (2013). P = postnatal day.

In comparison to studies of adults, the age of individuals also needs to be equated more strictly. Whereas adult animals may be considered equivalent within a fairly large timeframe ranging up to weeks, such intervals will have a dramatic influence on an individual's response during development. It is recommended that all individuals are tested within 1 day of the chosen age (e.g., P17 ± 1 day) because, particularly during infancy, stark qualitative transitions from one type of responding to another can occur in a single day. For example, very early in development, on postnatal day (P) 9 or earlier, infant rats will exhibit a paradoxical approach response to an odour CS paired with shock (Sullivan, Landers, Yeaman, & Wilson, 2000). From P10 onwards, however, rats will exhibit avoidance of the odour CS in this same task (Sullivan et al., 2000; for review see Debiec & Sullivan, 2017).

Other species-typical conditioned fear responses, such as freezing, bradycardia (i.e., slowing of the heart-rate), and potentiated startle, emerge in a sensory- and response-specific sequence (see Figure 3; for a review, see Hunt & Campbell, 1997). Specifically, CS-elicited freezing is typically exhibited at a younger age than bradycardia, which is exhibited at a younger age than fear potentiated startle. The availability of these responses is also dependent on the characteristics of the CS, with behavioural expression of learned fear typically emerging first to olfactory, then auditory, and finally to visual cues (Hunt & Campbell, 1997). Importantly, the expression of these responses is determined by the animal's age at acquisition, rather than its age at test. That is, an animal that has the capacity to express fear in a particular manner will only do so if that response was also available to the individual at the time of training. Thus, a 25-day-old rat that is capable of expressing fear to a visual CS through both freezing and fear potentiated startle will only exhibit the freezing response, and not fear potentiated startle, if it was trained on P18 (Barnet & Hunt, 2006; also see Richardson & Fan, 2002).

Once a behavioural fear response to a CS is learned, there are also developmental differences in the retention and extinction of the conditioned response. Specifically, infant animals tend to exhibit rapid forgetting of learned fear associations (and other experiences) in comparison to adults, a phenomenon known as *infantile amnesia* that is observed across species (Campbell & Spear, 1972; Josselyn & Frankland, 2012). With regards to extinction of conditioned fear, infant rats are less prone to fear relapse after extinction. That is, whereas adult animals will exhibit recovery of an extinguished fear response under certain conditions (e.g., in novel contexts, after a reminder treatment or exposure to a stressor; Bouton, 2002), infants are less likely to display these relapse behaviours (i.e., they exhibit relapse-resistant extinction; Kim & Richardson, 2010). In contrast, the juvenile rat will exhibit a more adult-like pattern of relapse-prone extinction (see Figure 4 for a summary of developmental

differences in extinction retention and relapse). The curvilinear nature of development becomes most apparent in adolescence, where the evidence suggests that adolescents are primed to learn negative associations, in comparison to individuals at other stages of development (Den & Richardson, 2013: Hunt, Burk, & Barnet, 2016), as well as being impaired in extinguishing such associations (Figure 4; McCallum, Kim, & Richardson, 2010; Pattwell et al., 2012; for review see Baker, Den, Graham & Richardson, 2014).

Outcome measures. Given that certain behaviours change over the course of development and others are not expressed at all prior to a certain age, the timing of experimental observations will ultimately affect the range of outcome measures that are available to the experimenter. For instance, it would be inappropriate to use potentiation of startle to assess fear learning prior to P23. Also, even when behaviour appears similar across age groups, the same behavioural output may represent different underlying patterns of neural activation (Chan et al., 2011; Kim, Hamlin, & Richardson, 2009; Kritman, Lahoud, & Maroun, 2017; Li, Kim, & Richardson, 2012a). For example, the prefrontal cortex, a region known to regulate expression of conditioned fear and extinction in adults (Quirk & Mueller, 2008), matures relatively late in postnatal development (Semple et al., 2013). Although rats can exhibit both conditioned fear and extinction of conditioned fear from infancy, these behaviours do not become dependent on the prefrontal cortex (PFC) until the juvenile period, between P23-25 (Kim et al., 2009; Li et al., 2012a). Furthermore, as was the case for behavioural expression of learned fear, the animal's age at acquisition determines the later recruitment of neural networks (Li, Kim, & Richardson, 2012b). Specifically, infant rats conditioned on P17 do not exhibit activation of the PFC during fear expression, even when the test takes place at P23, after this behaviour is typically observed to be PFC-dependent (Li et al., 2012b). Thus, effective fear conditioning and extinction should not necessarily be interpreted as measures of prefrontal cortex output during development.

Experiment duration. Another factor that should be considered in designing both outcome measures and experimental procedures for studies of development is the duration of the protocol. Long treatment periods or training protocols that occur over many days will likely encompass multiple developmental stages, and may therefore be inappropriate for answering questions about differential expression of outcomes across development. For example, the advent of optogenetic technologies has enabled the investigation of the role of specific neuronal populations in behavioural outcomes with a high degree of temporal specificity (Zhang et al, 2010). However, the most common approaches to optogenetics (i.e., injection of the viral vectors lentivirus or adeno-associated virus into the target brain region) require 2-3 weeks after injection to achieve the required level of gene expression (Zhang et al., 2010), reducing their utility in studies of development. As the technology improves, faster infection rates may be achievable and even commonplace (e.g., the herpes simplex virus requires only 1-2 weeks to achieve sufficient expression; Zhang et al., 2010), which will make optogenetic studies during development more feasible.

The length of the individual training or testing sessions is also important during development. Extended periods of separation from the dam prior to weaning, and particularly in the first two weeks of life, will cause stress for both the infant and the dam. Indeed, 3 hours of daily maternal separation is a procedure that is commonly used in rats to model negative psychological (e.g., depression and anxiety-like behaviour) and physiological outcomes (e.g., hormonal response to stress, gastrointestinal symptoms like irritable bowel syndrome; O'Mahony, Hyland, Dinan, & Cryan, 2011). In addition to the psychological stress of separation, very young rodents will also suffer disruption to feeding patterns and thermoregulation during long periods of separation. Particularly prior to gaining fur, the rodent's capacity to self-regulate body temperature is reduced and infants are therefore susceptible to both hypo- and hyperthermia (Gordon, 1993). Typically, the nest temperature

is maintained between 35 – 37 ºC by the shared body heat of the dam and littermates (Gordon, 1993). If a pup, especially one younger than P10, is to be separated from the litter and kept in a room-temperature environment for extended testing sessions, a heat pad or other insulation (e.g., container kept in a temperature-controlled water bath) should be used to prevent hypothermia, but it is also imperative to prevent overheating as young rats are particularly vulnerable to hyperthermia-induced seizures (Gordon, 1993).

Species and strain selection. It is important to be aware of the developmental transitions and norms that occur for your chosen experimental species or strain. Perhaps the most stark contrasts in this respect are between altricial species, such as mice, rats, and primates (including humans), which are born relatively immature and have an extended period of postnatal development, in comparison to precocial species, such as guinea pigs, which are born relatively mature and exhibit less postnatal development (e.g., low rates of postnatal neurogenesis; Josselyn & Frankland, 2012). One striking illustration of these differences can be found in the expression of infantile amnesia, which is observed across altricial species (as described earlier; Campbell & Spear, 1972; Josselyn & Frankland, 2012), but is notably absent in the precocious guinea pig, which exhibits similar rates of memory and forgetting across the lifespan (Akers et al, 2014; Campbell, Misanin, White, & Lytle, 1974). Different developmental trajectories are also observed between altricial rodents (i.e., mice typically mature faster than rats), and even between strains of rats or mice (e.g., Molenhuis, de Visser, Bruining, & Kas, 2014). For this reason, the appropriate timing of experimental observations may differ between strains and species and should be adjusted accordingly.

Sex differences. As has been the case for rodent studies of adulthood (Thibaut, 2016), the majority of developmental studies have focused exclusively on males. Although this may be somewhat more defensible prior to the onset of puberty and the associated increase in sex

hormones, a convincing argument can be mounted to study both sexes even during early development. For example, there is evidence of sex-specific effects of early-life manipulations in rodents, with male rats exhibiting more profound physiological and behavioural abnormalities following either early-life maternal separation stress or germ-free rearing (e.g., Clarke et al., 2013; Diehl et al., 2007). With respect to the translational and clinical implications of developmental studies, sex differences in psychological and neurodevelopmental disorders often emerge during development. For anxiety and depression, sex differences emerge around puberty (disproportionately affecting girls and women), but sex differences in autism, disruptive behaviour disorders, and attention-deficit hyperactivity disorder all emerge during childhood (affecting more boys and men; Thibault, 2016). We are not suggesting that all studies include both sexes and multiple stages of development as to do so would be unfeasible and unwieldly. Nonetheless, it is important to acknowledge the potential impact of sex and developmental stage on the behavioural/physiological outcomes of interest and to encourage the investigation of these variables, either within or across experiments.

Apparatus and equipment. Due to the obvious size differences between infant and adult rodents, equipment that has been built for use with adults will often not be appropriate for use with developing individuals. For example, in the classic test of spatial memory, the Morris Water Maze, the performance of rat pups is dramatically altered by both the size of the pool (Carman & Mactutus, 2001) and the visibility of spatial cues (Carman, Booze, Snow, & Mactutus, 2003). In other words, adjustments to the equipment are needed to accurately assess the spatial abilities of young animals in this task (and the task will not be at all appropriate for use in very young rodents that have not yet opened their eyes and have difficulty locomoting even on dry surfaces; see Albani, McHail, & Dumas, 2014 for a review of appropriate measurement of hippocampal-dependent behaviours during development). In

the case of fear conditioning, training is generally conducted in chambers with a grid floor used to administer a mildly aversive foot-shock. However, grids built for adult rats will be too large to accommodate an infant rat, whose paws will slip through large gaps between grids, meaning their bodies rather than paws will be exposed to the shock. Therefore, more tightly spaced grids (e.g., 5-7 mm apart) should be used when conditioning infant rats as opposed to adult rats (grids spaced 10-13 mm apart).

Aside from scaling equipment to suit the physical proportions of the animal, there are also some, perhaps less obvious, considerations that should be taken into account when selecting experimental apparatus for use with developing rodents. For example, if equipment is to be attached to the animal (e.g., cannulae, electrophysiology tetrodes), the effects of this on the individual, the dam, and littermates need to be carefully monitored. Large headpieces, although they have been successfully used in the young rat (e.g., Ng & Freeman, 2012), may prevent a pup from nursing, affecting its health or causing the dam to reject the pup. Dams may also attempt to remove large attachments, especially if they interfere with normal licking and grooming, which may result in damage to the equipment or harm to the offspring. In addition, if there is a delay between implantation and testing, this may cause problems due to the physical growth that will occur in the intervening interval.

Drug dosage. In studies of adults, drug doses are typically titrated according to the weight of the animal. This is also necessary in studies of developing animals. However, it is important to note that pharmacokinetics and pharmacodynamics tend to differ in complex, drug-specific ways across development, giving rise to the field of developmental pharmacology (Spear & Brake, 1983; van den Anker, Schwab, & Kearns, 2011). This means that the effective and safe doses for specific drugs will differ according to the animal's developmental stage. For example, two drugs used to induce a potentiated startle response in adult rats, strychnine and corticotrophin-releasing hormone (CRH), have been shown to have

similar effects on potentiated startle in infants, but only when the doses were adjusted in opposite directions (Weber & Richardson, 2001). That is, whereas a dose of 1-2 mg/kg of strychnine is required to induce potentiation of startle in adults, a dose of only 0.25 mg/kg was required in infants (i.e., one quarter of the adult dosage). In fact, strychnine doses above 0.25 mg/kg have been reported to have convulsive or lethal effects during infancy (Kubova & Mares, 1995). In contrast, the intracerebroventricular dose of CRH required to induce potentiated startle was four times higher in infants than in adults (1.0 µg for adults, 4.0 µg for infants). Overall, calculation of developmental drug doses based on standard adult dosages will not necessarily produce the desired effects, and in fact may be detrimental or even toxic to the infant or adolescent. Therefore, a literature review to determine age-specific drug dosages is always recommended and, where this information is unavailable, pilot studies to establish dose-response curves in the chosen developmental stage should be conducted with careful monitoring of animal welfare.

Experimental design. If the objective of the experiment is to compare performance across developmental stages then it will be important to consider whether it is of higher priority to equate the experimental parameters or to equate some measure of performance. For example, in assessing infantile amnesia, it may be necessary to use different training parameters in infants and adults to equate the initial levels of behavioural expression of learned fear. Learning is typically slower in developing animals, meaning that more conditioning trials are required to elicit equivalent levels of behavioural fear expression in infants (e.g., Li et al., 2012a, 2012b). Without equating this initial fear expression, it would be difficult to distinguish whether the more rapid rate of forgetting in the infant was due to "weaker" initial learning rather than a true developmental difference in fear retention. In the end, it may be worthwhile to use parameter-equating and performance-equating approaches in parallel to provide the most definitive answers to questions of developmental differences in

behavioural or neural function. Regardless of the approach chosen, it is essential for scientific rigour and transparency to describe the experimental design and other relevant factors in as much detail as possible to promote efforts to reproduce findings (see also ARRIVE guidelines; Kilkenny et al., 2010).

Conclusion.

This primer is intended to serve as a guide to help researchers navigate the particular challenges of developmental research. We have offered a number of suggestions to avoid methodological problems and improve reliability through sound experimental design. Although many of these recommendations have been made in the past and largely ignored, we hope that this paper will act as a timely reminder to combat the current issues of reproducibility in scientific research. Furthermore, we hope to encourage new researchers to delve into the exciting field of developmental research. This is an important pursuit because, when conducted appropriately, developmental studies can add valuable insights to our understanding of physiological and psychological problems as they occur across the lifespan.

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Figure Legends

Figure 1. The investigation of developing individuals is an often neglected but highly important area of study, with the potential to expand basic scientific knowledge and enhance clinical practice.

Figure 2. Pavlovian fear conditioning and extinction. During fear conditioning, the conditioned stimulus (CS; e.g., a white noise) is paired with an aversive, unconditioned stimulus (US; e.g., a foot-shock) so that the animal exhibits fear to the CS alone. During extinction, the CS is presented alone so that the animal's fear response diminishes. Elements of this figure adapted from Cowan et al., (in press).

Figure 3. Approximate age of emergence for a range of fear-related behaviours in rats. Note: CS = Conditioned stimulus; FPS = fear potentiation of startle.

Figure 4. Developmental differences in fear responding after extinction training. When test occurs in the same context as extinction training, adolescent individuals exhibit a deficit in extinction retention (i.e., heightened fear expression) compared to other developmental stages. In regards to renewal of extinguished fear, a developmental transition from relapse-resistant to relapse-prone extinction occurs between the infant and juvenile stages such that only infants exhibit low levels of fear expression in a context that differs from the extinction context. Data redrawn from Baker, McNally, & Richardson (2013), Kim & Richardson (2007), McCallum et al. (2010).