Ethological and methodological considerations in the use of newborn rodents in biomedical research

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Summary. - The use of newborn or immature animals in biomedical research poses certain challenges, in that the ecological niche of immature animals differs from that of adults, and the stimuli provided to immature animals can have profound physiological and behavioural effects through adulthood, even at the cerebral level. In particular, the newborn’s behaviour and physiology are regulated by olfactory, thermal, and tactile stimulation supplied by the mother in the nest environment. Thus any disturbance to the mother-offspring relationship in the first two weeks of life can profoundly change the physiological and psychological state of the young. For this reason, research experiments must be adapted to the specific characteristics of the immature animal, to improve not only the quality of the data obtained but also the welfare of the animals. To this end, the present work provides some ethological and methodological considerations in the maintenance, handling, and testing of newborn rats and mice in biomedical research.

Key words: neonate rodents, behavioural development, mother-infant bond, olfactory learning, hypothermia, animal welfare.

Riassunto (Considerazioni etologiche e metodologiche sull’impiego di roditori neonati nella ricerca biomedica). - In importanti settori della ricerca biomedica i soggetti sperimentali sono roditori neonati o in corso di sviluppo. L’impiego di soggetti immaturi richiede allo sperimentatore la scelta di procedure adatte alle loro specifiche caratteristiche eco-etologiche, che sono profondamente diverse da quelle degli adulti della stessa specie. Attraverso stimoli olfattivi, tattili e termici la madre modula le risposte fisiologiche e comportamentali del neonato. Perciò, ogni interferenza con la relazione madre-prole nelle prime due settimane di vita può indurre effetti permanenti sullo sviluppo del neonato, in particolare sul sistema nervoso e sul comportamento. La considerazione delle specifiche caratteristiche età-dipendenti dei soggetti sperimentali, oltre a rappresentare un indispensabile requisito per il miglioramento del loro benessere, previene errori sistematici nella interpretazione dei risultati, migliorando la qualità del dato sperimentale ottenuto.

Parole chiave: roditori neonati, sviluppo neurocomportamentale, interazione madre-prole, apprendimento olfattivo, ipotermia, benessere animale.

Introduction

In certain branches of biomedical research, newborns or immature animals are used as experimental subjects. For example, in preclinical studies on the developmental toxicity of drugs, adverse effects are evaluated by assessing morphologic, somatic, and neurobehavioural parameters in immature rodents [1]. Moreover, the analysis of physiological parameters in immature rodents, such as metabolite levels or the age of expression of a behavioural response, can greatly contribute to understanding both genetic and acquired human infant diseases [2].

Nonetheless, the use of immature animals in research poses certain problems, in that the specific ecological niche of immature animals, which differs from that of adults, must be taken into account. In general, the development of an organism is gradual and continuous and results from a dynamic interaction between endogenous factors and environmental stimuli. The changes that occur during development are often accompanied by a complete reorganisation of the organism and its behaviour, which Bateson has fittingly compared to the metamorphosis of a caterpillar into a butterfly [3]. For this reason, research experiments must be adapted to the specific characteristics of the immature animal, and their extreme susceptibility to environmental factors must be considered, so as to minimise distress and improve both the animal’s welfare and the quality of data. The present work provides some ethological and methodological considerations in the maintenance, handling, and testing of newborn rats and mice in biomedical research.

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The overall development of newborn rats and mice

Rats and mice are altricial: at birth their nervous system is immature and the animals are blind, and they are unable to regulate their body temperature, to move efficiently, or to nourish themselves autonomously, requiring maternal care until weaning. The behavioural repertoire of the newborn revolves around solving the problems of an immature organism, such as maintaining contact with the mother’s body (i.e., its source of food and heat) and locating the mother’s nipple by means of thermal, tactile, and olfactory cues. Despite its foetal appearance, as early as the first hours of life the newborn acquires and stores information that is essential for its survival (mainly information of an olfactory nature). In the first two weeks of life, the newborn’s activities are mainly related to feeding, and the animal appears to have rather limited locomotor abilities, spending all of its time huddled with its siblings under the mother. As vision and hearing mature and the thermoregulatory mechanisms develop, the behavioural repertoire increases greatly, eventually resembling that of adults, which occurs at around the time of weaning (at about three weeks of life). Late in the second week of life, the animals begin to explore the surrounding environment, though for short periods. At this stage, they are generally hyperactive, and only around the time of weaning do they display a habituation profile similar to that of adults when placed in an unfamiliar experimental environment, showing intense exploratory activity at the beginning of the test, with subsequent decreases in the activity level (see [4] for a comprehensive review of the behavioural development of altricial rodents).

An olfactory world

Several ontogenetic studies have demonstrated the importance of early olfactory experiences in the neurobehavioural development of rodents. In rodents, the memory of early olfactory experiences is retained through adulthood and plays an important role in establishing social, sexual, and parental behaviour. In the first week of life, newborns develop a strong preference for odours associated with the nest or with tactile stimulation similar to that received by their mother [5], and this preference, which lasts through adulthood, is associated with permanent physiological and neuroanatomical changes in the glomerular layer of the olfactory bulb [6]. Thus any interference with the nest’s natural odours can have significant repercussions on the behaviour of not only the newborn but also the adult, affecting the ability to respond to environmental stimuli. Nest odour has also been shown to have a quieting effect in rat pups, reducing their behavioural arousal when placed in a new environment [7]. Moreover, home-cage environmental stimuli facilitate learning in preweaning rats, confirming that a testing environment that closely resembles the ecological niche favours the more precocious expression of associative capabilities [8].

An important olfactory stimuli for the newborn is the odour of the amniotic fluid that the mother spreads onto her nipples while licking herself during labour and delivery and which leads the newborn to the nipples; the newborn recognises this as a familiar scent from its intrauterine life [9]. Olfaction also appears to be the principal sensory route through which newborns exert their effect on prolactin release [10].

Olfactory stimuli are also important in eliciting and directing maternal behaviour [11]. Several studies have indicated that female mice rendered anosmic have serious difficulties in establishing the mother-offspring bond, neglecting the pups and manifesting abnormal anxiety/arousal, which can result in cannibalism [12]. To reduce the risk of this behaviour in the lactating mother, the researcher should avoid cleaning the home cage just before birth and for two to three days after birth, and should in general leave the litter as undisturbed as possible. If manipulation of the newborns is necessary, gloves should be worn, so as not to leave any unnatural scent on the animal’s body. If newborns are removed from the nest in the first two to three days after birth, shavings from the home cage should be spread on their body when returning them to the mother, which minimises the risk of rejection.

The mother-offspring bond

In mammal species, the mother is the main source of environmental stimuli for newborns for a considerable period after delivery, and separation can induce long-term, often irreversible effects on physiology and behaviour [13]. An extremely important aspect of mother-offspring contact is the maintenance of optimal body temperature in pups, which is assured by the behaviour of the lactating mother. Specifically, mother-offspring contact serves as a source of heat and prevents heat loss. It also prevents hyperthermia by increasing the pup’s respiration and consequently decreasing its body temperature (newborns are extremely vulnerable to elevated temperatures) [14]. Furthermore, in rats, in the first two weeks after delivery, the mother progressively decreases the amount of contact with the pups as soon as their thermoregulatory mechanisms begin to develop [15].

In performing research, the mother-offspring bond must be considered as a unified system whose components exchange physiological resources and a
variety of stimuli. For example, in rats, odours and sounds have profound behavioural and physiological effects on each member of the dyad: olfactory and acoustic cues, such as ultrasonic vocalisations, can regulate behavioural arousal and even increase or decrease hormone titres in both the mother and offspring [16, 17]. It is clear that any disturbance to this system in the first two weeks after delivery can greatly change the physiological and psychological state of the offspring. For instance, it is well known that the brief isolation of a rat pup from its mother and siblings on a daily basis, although apparently innocuous, actually has long-lasting effects on behaviour [18]. Handling during development has also been shown to reduce anxiety levels when animals, as adults, are subjected to open-field tests [19]. There exists evidence that is consistent with the hypothesis that the mother mediates the effects of handling: infant rodents are extremely distressed by separation from the mother in the neonatal period and respond to separation with increased ultrasonic vocalisations. These vocalisations are likely to continue when the pups are returned to the mother, which readily responds by paying increased attention to them [11]. Caldji et al. [20] have shown that offspring that are frequently licked, groomed, and nursed by their mothers show substantially less fear in response to novelty, compared to offspring that receive these forms of attention less frequently. Furthermore, separation from the mother early in life can result in long-term changes in basal glucocorticoid levels and in the morphology of hippocampal neurons [21, 22]. Early handling also has long-term effects on immune responses [23] and on the behavioural sensitivity to psychotrophic drugs [24]. In a review of the role of pre-weaning experience as a modifier of prenatal drug effects, Clausing et al. [25] provides several examples of the striking effects on neuroendocrine and behavioural functions of both the holding of pups for 3-5 min in the researcher’s hand and the separation of the pup from the mother for periods varying from 3 to 20 min. Experimental evidence indicates that the HPA axis, the benzodiazepine receptor system, and the dopaminergic system are affected [26]. Furthermore, in studies in which rodents were used as models of foetal alcohol syndrome, some of the adverse effects of prenatal ethanol exposure were apparently counteracted by preweaning handling/maternal deprivation.

Various efforts can be made to reduce the impact of maternal separation on response variables. To prevent hypothermia, an incubator set to the temperature of the nest (30 ± 1 °C), which can also maintain the level of humidity (around 60%), should be used. The duration of separation from the mother should be as brief as possible yet allow enough time to perform the experiment. When possible, the litter should be kept together, since huddling with siblings reduces both heat loss and isolation-induced stress. Familiar olfactory cues (e.g., shavings from the home cage) should be used during separation. Finally, when using control animals, it should be ensured that the duration of maternal separation is the same as that for the study animals, given that separation could have a greater effect than the treatment itself on the response variable. Considering that maternal separation can affect behaviour even in adulthood, it should be ensured that the controls and the study animals have undergone the same degree of handling throughout their life, especially when conducting longitudinal studies and when repeatedly subjecting animals to experimental procedures from birth to adulthood.

**Age-specific experiments for measuring behaviour in developing rats**

Immature mammals obviously cannot be considered as miniature adults, and their specific physiology must be taken into account when performing research. In this respect, behavioural studies are paradigmatic. The studies carried out since the early 1970s, which have commonly used altricial rodents such as rats and mice, have revealed the richness and complexity of the behavioural repertoire of newborns, and once the species-specific ecology and the motor and sensory characteristics of immature animals began to be considered, very complex learning and retention capacities emerged. The ecological approach to the ontogeny of behaviour, which highlighted the adaptive value of the newborn’s behavioural repertoire, eventually led to a methodological revolution. Experiments were designed to be suited to the specific capabilities of immature animals, with olfactory and thermotactile stimuli replacing the auditory and visual stimuli used in classical conditioning experiments. Equal importance was placed on the choice of behavioural responses for measuring learning, such as sniffing or probing for the nipple, and the type of reinforcement for inducing instrumental conditioning [27].

As mentioned above, it has been shown that rodents already begin to acquire and retain olfactory signals in the uterus. In a study conducted on one-day-old rat pups that were administered milk through an intra-oral tube, the pups learned to move a paddle with their head to receive milk and to distinguish between two paddles marked with different odors [28]. At 7 days of age, using olfactory cues, rats can orient themselves in a small arena to reach a nest-scented area. They can also make their way through a Y-maze to reach the anaesthetised mother [29]. Although
immature rats, given their obvious motor limitations, have demonstrated a poorer learning capacity than adults in more complex conditioning tests based on the use of spatial information for orienting themselves in a maze, they have shown a good learning capacity in tests modified to take into account their sensory and motor characteristics. For example, before weaning, rats and mice find it difficult to learn an active or passive avoidance response, which is generally used to test associative capacities in the adult and calls for the animal to inhibit an escape response or to move among compartments to avoid punishment, generally a mild electric shock. The difficulties in learning the avoidance task are related to the expression of the conditioned response, hindered both by the high levels of activation provoked in the immature animal by the electric shock and by the poorer ability to find the escape route. Eliminating the directional component of the escape response and replacing the electric shock with less stressful aversive stimuli, such as a cold drought, has been shown to appreciably lower the age at which learning appears [30].

The context in which the learning and memory capacity is evaluated is also of fundamental importance. As mentioned, a test environment that closely resembles the ecological niche favours a more precocious expression of learning capabilities and the expression of spontaneous behaviours that are amenable to an adequate quantitative and qualitative analysis. The presence of siblings or of shavings from the home cage facilitates the acquisition of a passive avoidance response in young rats [7]. In fact, isolation from the nest environment and from the mother probably prevents the newborn from acquiring important information on task contingencies. The same consideration applies when analysing neonatal behaviour, because pups have a very low level of spontaneous behaviour when separated from the mother. However, the potential effects of a prenatal/neonatal treatment can be assessed by evaluating a specific neonatal pattern of great adaptive value, such as suckling, observing pups in a temperature-controlled environment in the presence of the anaesthetised mother [31-33].

Hypothermia-induced anaesthesia

In biomedical research aimed at obtaining animal models of infant neurological diseases, it is often necessary to perform surgery, which requires anaesthesia. The newborn of small altricial rodents is very amenable to hypothermic anaesthesia (as mentioned, newborn rats and mice are functionally poikilothermic until the third week of life) [34]. In fact, it has been shown that, during hypothermia, the core body temperature of rodent pups, determined by measuring rectal temperature, remains well below the level necessary for producing physiological analgesia [35].

There exist several methods for inducing hypothermia in newborn rodents, which, compared to lowering the body temperature of adults, is relatively simple, given the body mass of infants. Specifically, rapid core cooling can be achieved simply by surface cooling, which can be done by placing the pup directly onto crushed ice, packed in aluminium foil to avoid freeze damage to the skin. Profound torpor is achieved in about 10 min and generally lasts from 10 to 15 min. If longer-lasting anaesthesia is needed, the surgery can be performed on a chilled surface, for example, a metal plate placed on a cold pack during the operation. To avoid warming the pups, a fiber optic light should be used as light source.

Recovery from hypothermia-induced anaesthesia is rapid: when placed in an incubator maintained at nest temperature, the pups become active and responsive within 15 to 20 min. Pups should be returned to the mother after surgery only once they are completely awake; normally, they spontaneously crawl toward the mother to begin suckling (see [36] for a description of the handling procedures for mothers and newborns for avoiding cannibalism).

Conclusions

Failure to take into account the ethological and ecological characteristics of a species, including those specific to the animal’s age, could significantly bias the outcome of an experiment.

The ethological/ecological approach described herein could very likely contribute to improving the quality of data obtained from experiments on immature rodents, which is especially important when assessing phenomena such as the potential health risks associated with developmental exposure to pharmaceuticals or environmental contaminants. In fact, most behavioural teratology data have been provided by experiments on animals, and in many cases the observed effects have greatly contributed to focussing the attention of regulatory authorities on suspected teratogens, thereby influencing public-health and environmental policies.

Another fundamental aspect of this ethological/ecological approach is animal welfare. Within the framework of a functioning-based approach [37], one of the most readily observable indicators of welfare is behaviour, which provides information on the animals’ needs and internal states; however, the welfare of experimental animals is generally determined based on the physiologic and behavioural needs of adults. If the wellbeing of an animal is indeed
related to the extent to which its needs are met [38], then research on animal welfare should address that of newborns and developing animals as a separate field, with its own methodological and ethical implications.

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REFERENCES


