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Unique Characteristics of Neonatal Classical Conditioning: The Role of the Amygdala and Locus Coeruleus

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Abstract

The central nervous system of altricial infants is specialized for optimizing attachments to their caregiver. During the first postnatal days, infant rats show a sensitive period for learning and are particularly susceptible to learning an attraction to their mother's odor. Classical conditioning appears to underlie this learning that is expressed behaviorally as an *increased ability to acquire odor preferences and a decreased ability to acquire odor aversions*. Specifically, in neonatal rats, pairing an odor with moderately painful shock (0.5mA) or milk produces a subsequent relative *preference for that odor*. The neural circuitry supporting the *increased ability to acquire odor preferences* appears to be the heightened functioning of the noradrenergic pontine nucleus locus coeruleus. Indeed, norepinephrine from the locus coeruleus appears to be both necessary and sufficient for learning during the sensitive period. On the other hand, the *decreased ability to acquire odor aversions* seems to be due to the lack of participation of the amygdala in at least some aversive learning situations. The site of plasticity in the pup's brain appears to be limited to the olfactory bulb. This neonatal sensitive period for learning ends around postnatal day 9-10, at which time pups make the transition from crawling to walking and classical conditioning becomes "adultlike." The neonatal behavioral and neural induced changes are retained into adulthood where it modifies sexual behavior.

Keywords

classical conditioning; olfactory learning; imprinting; sensitive period; neonatal learning; odor conditioning; attachment; abuse; amygdala; fear conditioning; locus coeruleus; norepinephrine

IN ALTRICIAL SPECIES, such as humans and rats, rapid formation of attachment is critical for survival. While some prenatal experiences contribute to attachment formation, most species dependent on parental care exhibit some form of rapid, specialized postnatal learning, such as imprinting in avian species (Salzen, 1970). We have recently developed a mammalian model of imprinting in the rat which has enabled us to explore the neurobiology of attachment using a classical conditioning paradigm (for a review of other maternalinfant attachment models see Brennen & Keverne, 1997; Hudson, 1993).

According to Bowlby (1965), attachment is characterized by the infant seeking proximity to the caregiver and the infant's endurance of considerable abuse to maintain contact with the caregiver. These attachment characteristics are not unique to mammals and appears to occur throughout the animal kingdom. Specifically, attachment to an abusive caregiver has been clinically documented in both human infants (review, Helfer, Kempe & Krugman, 1997), and nonhuman infant primates (Harlow & Harlow, 1965). These results are consistent with experimental manipulations; chicks shocked during imprinting show enhanced following (Hess, 1962) and abused infant dogs form attachments to an abusive handler (Fisher, 1955

cited in Rajecki, Lamb & Obmascher, 1978). Bowlby (1965) & others (Hofer, 1981) have speculated that unique infant learning abilities may have evolved to ensure that altricial animals easily learn the proximity seeking behaviors necessary for survival, regardless of the quality of the care they receive from their caregiver.

Observations of naturalistic interactions between rat pups and their mother shows that it is not uncommon for the mother rat to hurt her pups. Indeed, pain elicited vocalizations from pups can be heard when the rat mother steps on pups while entering and leaving the nest or when the mother retrieves pups. The benefits of preventing neonatal pups from learning an aversion to their mother's odor ensures pups will learn only approach responses to the mother and are therefore critical for pups' survival. Indeed, without pups' approach responses to the mother, pups can not obtain the mother's milk, warmth and protection. Again, from an evolutionary perspective, it may be better for an altricial infant to have a bad caretaker than no caretaker. Although a learning based attachment system may seem precarious, the ecological niche of the neonatal pup, along with the motoric limitation of the pup, ensures neonatal rat pups will not venture from the nest. Therefore, the range of possible attachment figures is greatly limited. Furthermore, characteristics of neonatal rat learning greatly enhance the probability of pups learning the odor preference which underlies their attachment to the mother. This learning is temporally limited to a sensitive period during the first week and a half of the neonatal rat's life.

I. During the sensitive period for learning, neonatal rat pup classical conditioning has unique characteristics

Pup classical conditioning is very similar to adult learning and has been demonstrated within the natural nest, as well as in more controlled situations outside the nest (Brunjes & Alberts, 1979; Campbell, 1984; Cornwell-Jones & Sobrian, 1977; Galef & Kaner, 1980; Leon, 1975; Miller, Jagielo & Spear, 1989; Pedersen, Williams & Blass, 1982; Rudy & Cheattle, 1977; Sullivan, Hofer, Brake & Williams, 1986a, Sullivan, Hofer & Brake, 1986b; Sullivan, Wilson, Wong, Correa & Leon, 1990; Terry & Johanson, 1996). However, there are unique characteristics of neonatal learning which greatly enhance the likelihood of pups developing odor preferences necessary for forming maternal attachment. First, inhibitory conditioning and passive avoidance do not appear to emerge until after postnatal days (PN) 10-11 (Blowovski & Cudennec, 1980; Collier, Mast, Meyer & Jacobs, 1979; Goldman & Tobach, 1967; Martin & Alberts, 1979; Stehower & Campbell, 1978; review Myslivecek, 1997). Second, pre-exposure to a conditioned stimulus (CS) facilitates conditioning in infant rats, whereas it retards conditioning in weanling and adult rats (Hoffmann & Spear, 1989). Thus, exposure to maternal odors without a reward would appear to enhance pups learning a preference to maternal odors. Third, when pups do learn, they appear to be *less selective* learners as compared to adult rats (Spear et al., 1989). That is, whereas an adult will focus on only a few environmental stimuli during learning, pups will acquire information about a broad range of stimuli, thus, facilitating acquisition of a myriad of maternal features. Fourth, many stimuli, even those with aversive qualities, can function as a positive reward and when paired with an odor, produce a subsequent *preference to that odor* (Figure 1; Alberts & May, 1984, Brake, 1981; Camp & Rudy, 1988; Dominguez, Lopez & Molina, 1999; Johanson & Teicher, 1980; Johanson & Hall, 1982; McLean, Darby-King, Sullivan & King, 1993; Pedersen et al., 1982; Sullivan, Hofer & Brake, 1986b; Sullivan, Brake, Hofer & Williams, 1986a; Sullivan & Leon, 1986; Sullivan & Hall, 1988; Sullivan, Landers, Yeaman & Wilson, 2000a; Sullivan, Stackenwalt, Nasr, Lemon & Wilson, 2000b; Weldon, Travis & Kennedy, 1991). The ability of stimuli such as shock (0.5mA) and tailpinch to support odor preference conditioning is not due to pups' inability to detect pain, and pain threshold does not change between PN4 and PN12 (Camp & Rudy, 1988; Haroutunian & Campbell, 1979; Emerich, Scalzo, Enters, Spear & Spear, 1985).

It should be noted that pups can learn an odor aversion if that odor is paired with malaise. Specially, pairing an odor with either LiCl or very strong shock (1.2mA-1.5mA), both of which produce illness in pups, results in a subsequent aversion for that odor. It should be noted that this high shock level is above that typically used in adult fear conditioning experiments (Ader & Peck, 1977; Gemberling & Domjan, 1982; Rudy & Cheattle 1977, 1978; Sullivan & Wilson, 1995). Moreover, according to work from Jerry Rudy's lab and Byron Campbell's lab, until about PN9-10, pups easily learn aversions about interoceptive cues (malaise or internal shock) but not exteroceptive cues (moderate 0.5mA tail or paw shock (Camp & Rudy, 1988; Haroutunian & Campbell, 1979). Indeed, Camp and Rudy have suggested that changes in categorization of appetitive and aversive events occur in pups sometime between PN8 and PN12. However, it should be noted that even Odor-LiCl learning has additional constraints during the neonatal period: pups presented with odor-LiCl pairings *while nursing* have a difficult time learning to avoid that odor (Martin & Alberts, 1982).

At PN9 to PN10, pups mature and begin to venture outside the nest (Bolles & Woods, 1965), the sensitive period ends (Sullivan et al., 2000a) and learning begins to more closely resemble learning in adults. Thus, the broad odor preference learning which underlies early attachment appears to end when pups are no longer confined to the nest. Indeed, pup learning appears to become more selective at a time in development when pups begin leaving the nest and encounter odors not associated with the safety of the nest.

The odor preferences acquired during the sensitive period are retained into adulthood

In rats, early attachment-related odors are retained into adulthood, where they take on a role in modulating reproductive behaviors (Fillion & Blass, 1986), albeit a limited role (Moore, Jordan & Wong, 1996). Thus, as has been documented in imprinting in other species, stimuli associated with early experience and attachment influence adult mate preference and enhance sexual performance.

Good memories of bad events

Recent work from our laboratory has further characterized the paradoxical odor-shock preference conditioning of neonatal rats. First, we found that the odor-shock induced odor-preference was very resilient and persisted even after 4 days of continued odor-shock conditioning after the sensitive period (Sullivan, Roth, Moriceau, Dikes & Holman, submitted, Figure 2). This memory seems persistent since it is still expressed as an odor preference at weanling. This suggests that once a neonatal memory is formed, the original memory trace continues to be strengthened by subsequent experience. In the present case, it is possible that the neonatal learning circuitry continues to be used, even after a more complex neural circuitry of adult learning emerges.

II. Sensitive period learning is characterized by learning-dependent olfactory bulb plasticity

The development of a specific olfactory-based attachment system in the neonatal rat pup is associated with the acquisition of odor-specific olfactory bulb neural changes. Similarly to the acquisition of the attachment based learning, these induced olfactory bulb changes are only acquired during the sensitive period (Figure 3). We found that rat pups express this modified olfactory bulb response to both natural maternal and artificial odors experienced in the nest, as well as to odors in controlled learning experiments (Sullivan & Leon, 1986; Johnson, Woo, Duong, Nguyen & Leon, 1995; Wilson & Sullivan, 1991; Wilson, Sullivan & Leon, 1987). The modified olfactory bulb response is characterized by enhanced immediate-early gene activity (c-fos), and enhanced 2-deoxyglucose (2-DG) uptake in focal, odor-specific glomerular regions in response to the conditioned odor. In addition, modified single-unit response patterns

of mitral/tufted cells near the enhanced glomerular foci were found (Wilson et al., 1987; Wilson & Leon, 1988a,b; Wilson & Sullivan, 1990), olfactory bulb anatomical changes reflected in enlarged glomeruli were found within these foci (Woo, Coopersmith & Leon, 1987), as well as an increase in pCREB (McLean, Harley, Darby-King & Yuan, 1999). As with the behavioral changes in attachment, these neural changes are retained into adulthood, but their acquisition is dependent upon experiences during infancy (Woo & Leon, 1988; Pager, 1974).

III. Without the neural structures involved in adult learning circuitry, the neonate relies on a unique neural circuitry for learning

A pre and early postnatal rat can be classically conditioned prior to maturation of brain areas important in adult learning, such as the hippocampus and amygdala. Specifically, major nuclei subdivision of the amygdala does not occur until around PN7 with major neurogenesis occurring from PN10-20 (Bayer, 1980; Berdel, Morys & Maciejewska, 1997; Mizukawa, Tseng & Otsuka, 1989). Maturation of the hippocampus occurs even later, since primary cortical input to the hippocampus from the entorhinal cortex is only beginning to develop during the first postnatal week (Crain, Cotman, Taylor & Lynch, 1973). Indeed, based on behavioral studies, the hippocampus does not appear to be functioning in learning until weaning or later (Rudy & Morledge, 1994; Rudy, Stadler-Morris & Alberts, 1987; Stanton, 2000). Furthermore, the frontal cortex is still undeveloped during this early neonatal period (Vermer, VanVulpen & VanUum, 1996) and there is evidence that the cortex may not be involved in neonatal sensitive period learning (Landers & Sullivan, 1999). The failure of brain areas such as the amygdala, hippocampus and frontal cortex to function during learning may be due to a lack of the functional connectivity not emerging until after the first week and a half of life (Nair & Gonzalez-Lima, 1999). Thus, neural immaturity, along with lack of functional connectivity between brain areas may underlie the infant rat's reliance on another learning neural circuitry.

Without the adult learning circuitry, the neonate appears to depend upon a unique, simplistic neural circuit for learning. Specifically, based upon the results of a series of experiments from both our lab and others, it appears as though the heightened release of norepinephrine (NE) from the locus coeruleus (LC) into the olfactory bulb underlies the olfactory bulb plasticity responsible for neonatal odor learning.

The importance of Norepinephrine (NE) in neonatal learning

Many neurotransmitters have a role in early olfactory learning in neonatal rats (5-HT—McLean et al., 1993; DA—Weldon et al., 1991; Barr & Wang, 1992; glutamate—Mickley et al., 1998; Lincoln et al., 1988; GABA - Okutani, Yagi & Kaba, 1999; and opiates—Barr & Rossi, 1992; Kehoe & Blass, 1988; Roth & Sullivan, in press), although the action of NE appears particularly important in neural plasticity during early development and learning induced-plasticity used in early attachment. We have shown this through manipulating the site of NE action in the olfactory bulb, as well as through the manipulation of the source of NE, the LC.

NE within the olfactory bulb—The notion that NE plasticity underlies the neural and behavioral changes of the rapid, olfactory based learning underlying rat pups' attachment, has received strong support from a variety of labs. First, work in Michael Leon's lab using microdialysis showed that olfactory bulb NE increases during infant odor learning but only during the sensitive period (Rangel & Leon, 1995). Second, recordings from olfactory bulb mitral cells during learning indicates that mitral cells maintain their responsiveness to odors in the experimental learning groups, but not control groups (Wilson & Sullivan, 1992). Third, infusion of NE into the olfactory bulb during an odor presentation is sufficient for pups to acquire an odor preference (Langdon, Harley & McLean, 1997; Sullivan, Stackenwalt, Nasr,

Lemon & Wilson, 2000b). Fourth, blocking olfactory bulb NE during learning, prevents neonatal odor learning (Sullivan, Zyzak, Skierkowski & Wilson, 1992). Thus, it is the contingent events of increasing olfactory bulb NE and odor stimulation that underlies the neural plasticity responsible for olfactory-based attachment formation.

This olfactory bulb NE is not intrinsic to the olfactory bulb and arrives via LC fibers terminating in the granule cell layer (McLean & Shipley, 1991). The inhibitory interneurons of the granule cell layer modulate the output of the olfactory bulb through the adjacent mitral cells (Lauder & Bloom, 1974; Trombley & Shepherd, 1992; Wilson & Leon, 1988b). Wilson (Wilson & Sullivan, 1992) has shown that activation of the NE input to the infant rat's olfactory bulb during an odor presentation maintains mitral cell responsiveness to that odor, preventing the habituation these cells normally exhibit to repeated odor presentations. Subsequent work has shown that NE increases mitral cell responses to olfactory nerve input, suggesting that NE functions to increase the signal to noise ratio (Jiang, Griff, Ennis, Zimmer & Shipley, 1996).

Manipulating the source of olfactory bulb NE, the LC—Direct manipulation of the LC is also able to induce or block neonatal odor learning. First, destroying the LC is sufficient to prevent pups from learning (Sullivan, Wilson, Lemon & Gerhardt, 1994; Figure 5). Second, stimulating release of NE by the LC with either idoxoxane or ACh (Sullivan, Stackenwalt, Nasr, Lemon & Wilson, 2000b; Figure 4) during an odor presentation is sufficient to induce an odor preference. Thus, NE appears both necessary and sufficient for learning during the neonatal sensitive period.

Developmental changes in the LC may account for the end of the sensitive period—Developmental changes within the LC appear to underlie the termination of the infant rat's unique learning abilities during the first week and a half of postnatal life. In neonatal pups, the LC is not completely mature during the first week of postnatal life, nor is it an immature version of the adult LC. Indeed, it has unique characteristics that result in an enhanced response of neonatal LC neurons to sensory stimulation. The infant LC is more responsive to sensory stimuli than the adult LC. Although the adult LC is activated by sensory stimuli (Aston-Jones, Rajkowski & Cohen, 1999; Aston-Jones, Rajkowski, Kubiak & Alexinsky 1994; Foote, Aston-Jones & Bloom, 1980; Harley & Sara, 1992; Sara, Dyon-Laurent & Herve, 1995; Vankov, Herve-Minvielle & Sara, 1995), in comparison to the infant, the adult LC is less likely to respond to non-noxious stimuli (Kimura & Nakamura, 1985; Nakamura, Kimura & Sakaguchi, 1987; Nakamura & Sakaguchi, 1990). Furthermore, the adult LC habituates after repeated presentation of the stimuli (Vankov, Herve-Minvielle & Sara, 1995), whereas the infant LC fails to exhibit habituation (Kimura & Nakamura, 1985; Nakamura & Sakaguchi, 1990). Even more striking is the duration of the response of the neonatal LC as compared to the preweanling/adult LC. Indeed, a 1 sec presentation of tactile stimulation is likely to cause a few ms response in the adult LC, but a 20-30 sec response in the infant LC (Kimura & Nakamura, 1985; Nakamura & Sakaguchi, 1990). Finally, the early infant LC shows far more extensive electrotonic coupling, a process that appears to potentiate the neonatal LC's response (Christie & Jelinek, 1993; Marshall, Christie, Finlayson & Williams, 1991). The infant LC appears to take on adult characteristics at around PN9-10 (Nakamura et al., 1987; Nakamura & Sakaguchi, 1990; Marshall et al., 1991). Together, these results suggest that the infant rat's olfactory bulb receives a uniquely large input of NE from the LC during the first 9 days of life. When the LC matures, it brings to a close the early sensitive period for the imprinting like learning underlying attachment in this species.

As illustrated in Figure 5, the change in LC functioning around PN10 appears to be based on the functional development of the $\alpha 2$ inhibitory autoreceptors on LC dendrites and cell bodies. Specifically, the LC contains recurrent collaterals which feedback onto the LC, potentiating the neonatal response, but shutting down the older LC response to stimulation via the $\alpha 2$

inhibitory autoreceptors (Kimura & Nakamura, 1985; Nakamura et al., 1987; Nakamura & Sakaguchi, 1990). Reduction in LC electrotonic coupling (Marshall, Christi, Finlayson & Williams, 1991), as well as changes in tyrosine hydroxylase levels (Bezin, Marcel, Rousset, Pujolm & Weissmann, 1994a; 1994b) at PN10 may also contribute to LC maturational changes underlying the massive reduction in LC NE release after the sensitive period (Rangel & Leon, 1995).

After the sensitive period, NE is no longer necessary for learning but remains sufficient—This effect of LC/NE in the neonate is in sharp contrast to the modulatory or attentional role in learning of LC/NE function in older pups and adults (Harris & Fitzgerald, 1991; Ferry, Roozendaal & McGaugh, 1999; Liang, 1998; McGaugh, Cahill & Roozendaal, 1996; Moffat, Suh & Fleming, 1993; Sara et al., 1995). Additionally, electrophysiological studies on the *adult LC* during learning clearly support the notion that NE is involved in adult learning but appears to *modulate* behavior and attention (Sara et al., 1995).

However, direct infusions of NE into the site of plasticity within the brain still remains sufficient for learning. Recent work in our lab has shown that the infusion of NE directly into the olfactory bulb, even after the sensitive period has ended, still results in an odor preference (Sullivan, Stackenwalt, Nasr, Lemon & Wilson, 2000b; Moriceau & Sullivan, 2000). This is consistent with work from a number of other laboratories where direct infusion of NE into the brain was able to support learning in a variety of sensory systems (Dahl & Li, 1994; Harley, 1998; Lacaille & Harley, 1985).

IV. The failure of the amygdala to participate in odor-shock conditioning may underlie pups' difficulties in learning odor aversions

The amygdala is a brain area strongly implicated in adult and preweanling rat conditioned fear (i.e. odor-shock conditioning; Cahill, Weinberger, Roozendaal & McGaugh, 1999; Fanselow & LeDoux, 1999; Fanselow & Rudy, 1998; Hunt & Campbell, 1999; Fendt & Fanselow, 1999; Sananes & Campbell, 1989; Schoenbaum, Chiba & Gallagher, 1999). Evidence suggests that the lack of a functional amygdala during the neonatal period may underlie pups' difficulty in learning and expressing fear. *First*, behaviors associated with amygdala function emerge around PN10 (inhibitory conditioning and passive avoidance: Blozovski & Cudennec, 1980; Blozovski & Hennocq, 1982; Collier, Mast & Meyer, 1979; review Myslivecek, 1997). *Second*, amygdala lesions during the neonatal sensitive period (PN1-9) do not prevent the acquisition of an odor preference, although it can slightly retard odor preference conditioning with no effect on specific conditioned responses (Sullivan & Wilson, 1993). *Third*, the amygdala does not appear to participate in the odor-shock induced odor preference during the sensitive period (Figure 6; Sullivan et al., 2000a). However, following the termination of the sensitive period, when odor-shock conditioning produces an odor aversion, the amygdala is involved in learning. Our data indicates that during the neonatal rat's sensitive period, when aversions are difficult to learn, the amygdala may not be active.

Other unlearned behaviors associated with the amygdala, such as immobility to a novel male odor (males eat pups) also emerges at PN10 (Takahashi, 1994a; Wiedenmayer & Barr, 1998). However, certain amygdala related olfactory conditioned behaviors emerge later in development, such as heart rate changes (PN15, Sananes, Gaddy & Campbell, 1988). Moreover, amygdala related behaviors in other sensory systems emerge even later in development, with immobility to auditory and visual stimuli emerging at PN16 and 18 respectively (Hunt & Campbell, 1999; Hunt, Hess & Campbell, 1997). Thus, microcircuitry within the amygdala or connections within each sensory specific system exhibits different developmental progression making global statements concerning amygdala function difficult.

IV. Summary and Conclusion

Based on the content of this review, the immature brain is designed to maximize attachment to the mother. Specifically, during the sensitive period, ontogenetically unique characteristics of the neonatal LC appear to underlie the neonates propensity for odor preference learning. On the other hand, the failure of the amygdala to participate in odor-shock conditioning may underlie pups' difficulties in learning odor aversions, and underlie the odor preference expressed by older pups.

Human infant attachment is also characterized by the infant seeking proximity to the caregiver and maintaining contact with the caregiver regardless of the quality of the care they receive (Bowlby, 1965). As has been reviewed above, this is true for a wide range of species including humans and rats. Based on our work on rats, the neural basis of attachment may be due to reduced amygdala function and heightened LC function. Although human attachment may not use the same neural circuitry as the rat, or use the circuitry in a different manner, these studies provide us with a new conceptual framework in which to explore human attachments.

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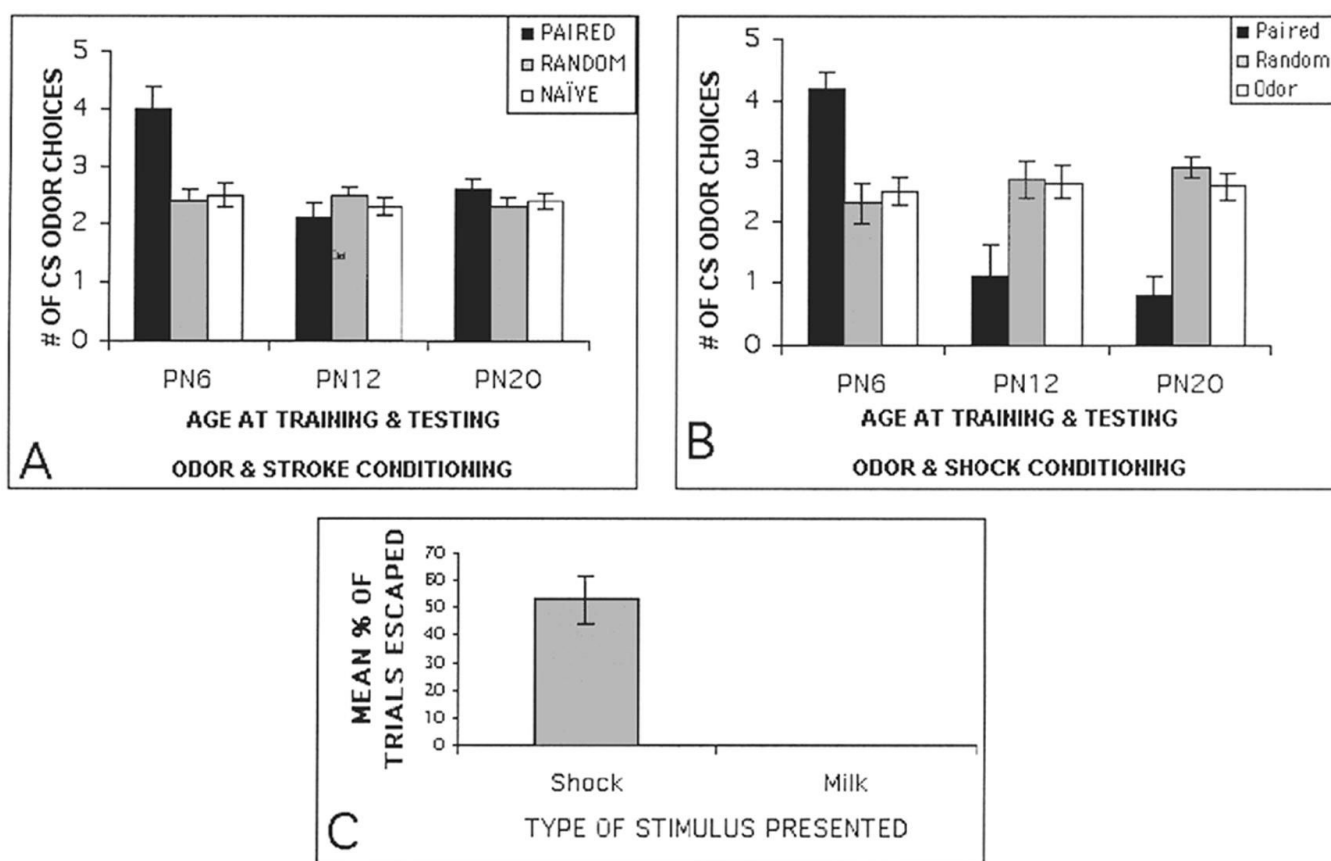
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**FIG. 1.**

Odor conditioning in PN6, PN12 and PN21 pups. **(A)** Odor-stroking (tactile stimulation) produces an odor preference in PN6 pups, but fails to support learning in older pups **(B)** Odor-shock conditioning produces a subsequent relative odor preference in PN6 pups, while older pups expressed an aversion to the conditioned odor. **(C)** Comparison of PN9 and PN12 pain responses to 0.5mA tail shock illustrates that shock is painful to pups during the time it supports odor preference conditioning.

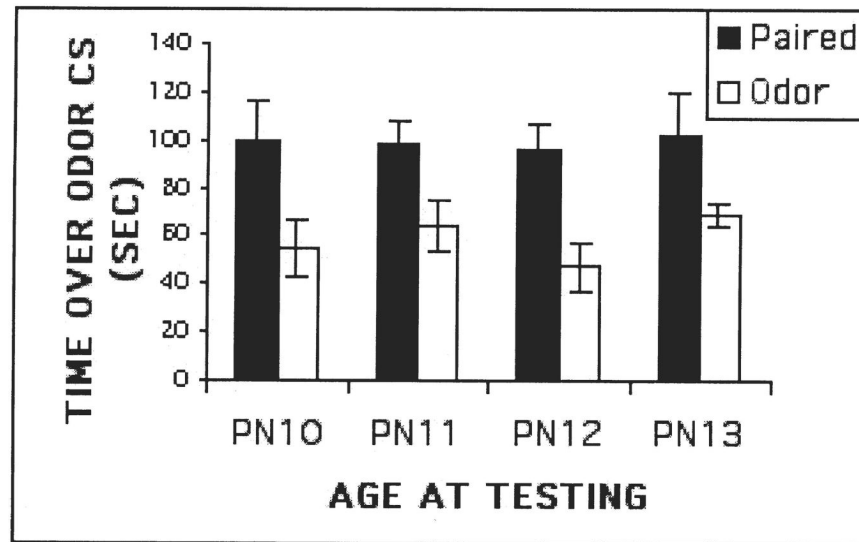


FIG. 2.

Pain-induced odor preference is maintained despite odor-shock training following the termination of the sensitive period. Pups received odor-shock conditioning during (PN8-PN9) and after (PN10, 11, 12, 13) the sensitive period until testing. Pups were tested on a 2-odor choice test at either PN10, PN11, PN12 or PN13. All experimental pups continued to exhibit a conditioned relative odor preference to the conditioned odor.

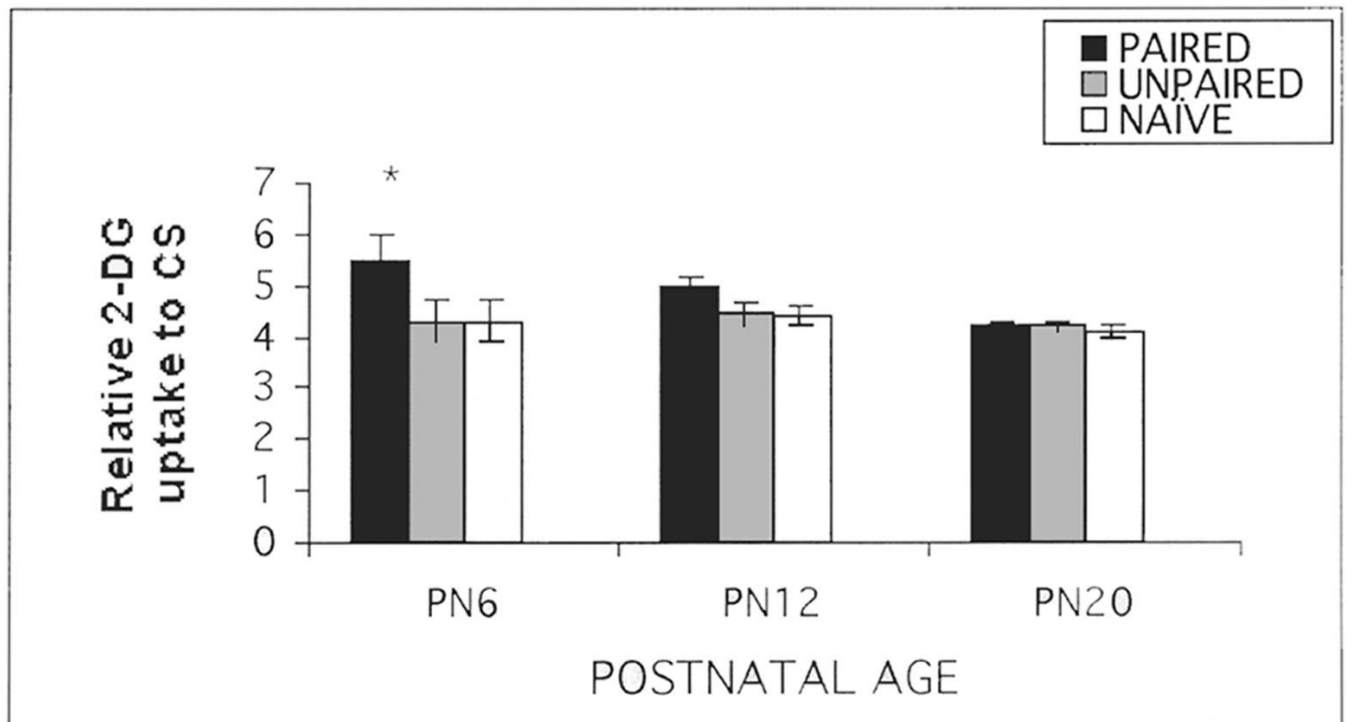
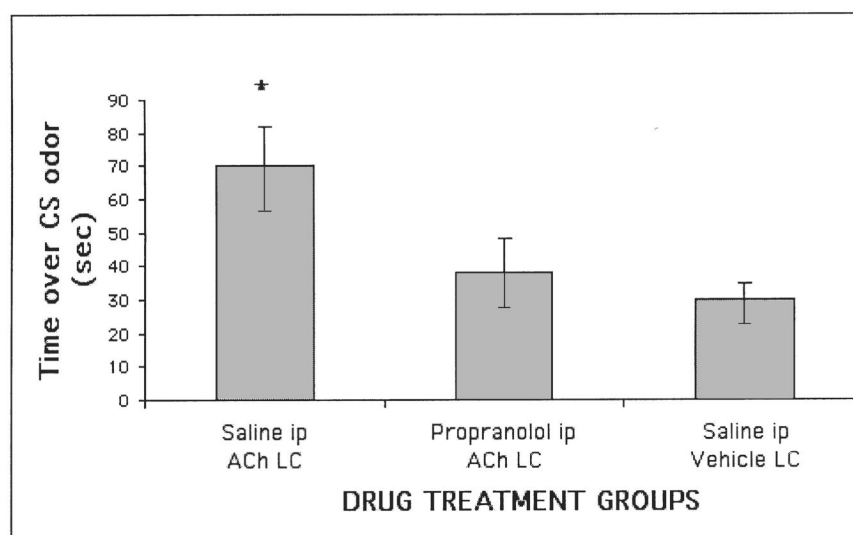
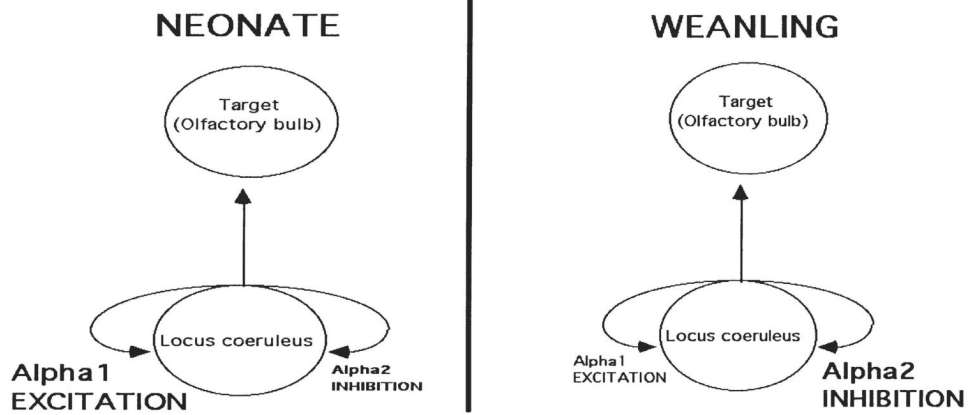


FIG. 3. Neonatal (PN6) odor conditioning produces learning associated changes in the olfactory bulb as measured by ^{14}C 2-DG autoradiography. These changes are only acquired during the sensitive period. PN6, 12 or 20 pups were conditioned with odor-shock. The next day, the olfactory bulbs were assessed for learning associated changes using ^{14}C 2-deoxyglucose autoradiography (Sullivan & Wilson, 1995).

**FIG. 4.**

Infusing ACh bilaterally into the LC during an odor presentation results in neonatal pups acquiring a subsequent preference for that odor. This results in the LC releasing NE. The effect of the LC infusion can be blocked by injecting pups systemically with propranolol prior to the LC infusion (Sullivan et al., 2000b).

**FIG. 5.**

The LC has recurrent collaterals that activate autoreceptors within the LC and modulate its behavior. In the neonate, the LC autoreceptors are primarily excitatory and result in prolonged LC activation (20-30 sec). At around PN10, the LC autoreceptors are functionally primarily inhibitory and result in a very brief excitation (few millisecond; Nakamura et al., 1987; Nakamura & Sakaguchi, 1990).

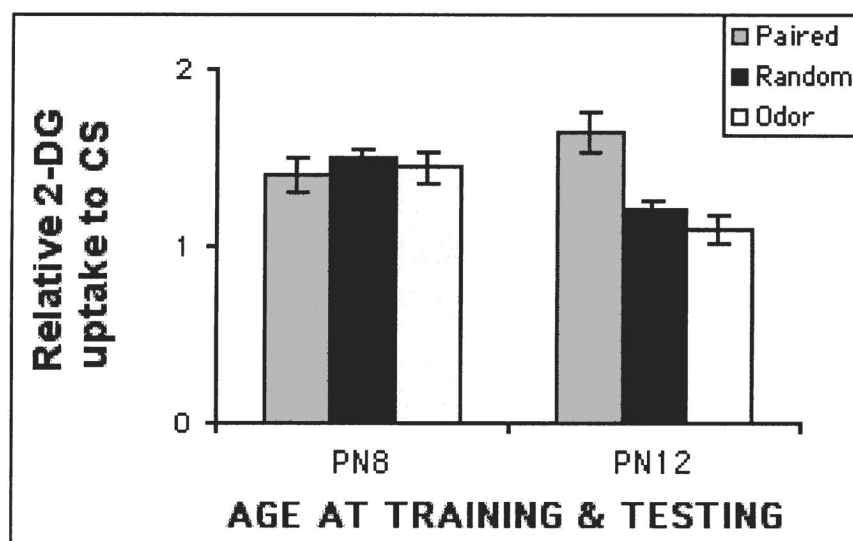


FIG. 6.

The amygdala does not appear to participate in the odor preference induced by odor-shock conditioning during the sensitive period. However, following the termination of the sensitive period, when pups easily learn an odor aversion, the amygdala is activated during odor-shock training (Sullivan et al,2000a)