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Maturation of the Biphasic Behavioral and Heart Rate Response in the Formalin Test

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BARR, G. A. *Maturation of the biphasic behavioral and heart rate response in the formalin test.* PHARMACOL BIOCHEM BEHAV **60**(2) 329–335, 1998.—The biological processes that mediate and modulate the perception of pain in the infant animal are not well studied and thus nociception during early development is poorly understood. In the adult animal, injection of formalin into the hind paw produces distinct phases of behavioral and autonomic responses: an early nociceptive response followed by a period of quiescence and a later second phase that matches or exceeds the initial response. The delayed reaction of the second phase has been suggested to be a model of inflammation-induced changes in neuronal sensitivity. Studies in the infant rat have demonstrated that the first phase is present in the fetus and neonate but the onset of the second phase is later maturing. We report here that the first phase occurs in 7- to 35-day-old pups in the formalin test when measured behaviorally and in 14- to 35-day-old pups when assessed by increased heart rate. However, the behavioral response in second phase is greatly attenuated or absent in 7- or 14-day-old pups, a finding consistent with that of others, appearing first at 21 days of age. The biphasic tachycardic response was not noted until even later, at 35 days of age. These data confirm that the neural mechanisms that mediate the secondary behavioral phase in the formalin test are late maturing, that the biphasic cardiovascular response does not occur until substantially later, after weaning, and that the behavioral and cardiovascular responses are dissociated developmentally. © 1998 Elsevier Science Inc.

Formalin Pain Biphasic response Heart rate Ontogeny Central facilitation

THE premature or seriously ill full-term infant is subjected to various and multiple physical insults, and thus pain is a serious clinical problem for human neonates (4). These painful procedures can and do cause localized inflammation, in part because of the immaturity of the infants' immune response (7). But how painful stimuli are processed similarly or differently in the adult or infant is not well understood.

In the rat, maturation of the physical and functional components of nociception begins prenatally and continues into the postnatal period. Aδ and C fibers enter the dorsal horn L4/L5 segments late on fetal day 19, reach lamina II at fetal day 19.5 (13) and demonstrate an adult-like distribution through the dorsal horn at birth (17). Substance P (SP) and fluoride-resistant acid phosphate (FRAP) are markers for C-fiber afferents (41); SP is found in the small primary afferents before birth (19,43) and FRAP is present in the spinal cord within 12 h of birth (19,37). Dorsal horn cells respond to pinching of the distal hindlimb at E19 (15) and express the Fos protein by E20 (56), and the flexor-withdrawal reflex in response to pinching or heating the hind paw is present on the day of birth (18). However, there are clear postnatal changes in these processes. Adult concentrations of SP and FRAP are not reached until the second week of postnatal life and the electrophysiological properties of fine-diameter nociceptors undergo significant maturation throughout that first postnatal week (14). Aδ fibers, which respond to high threshold mechanical stimulation, are functional earlier than are C-fibers, and the capsaicin-sensitive burst of spikes elicited by the electrical stimulation does not appear until postnatal day 9 (18). Thus, although the rat fetus does experience and react to noxious stimulation, the full elaboration of nociceptive processes continues well into the postnatal period.

There are differences in the development of responsivity to different noxious signals, presumably because of the late development of functional C-fiber input, and some aspects of spinal cord plasticity are clearly not present in the infant rat.

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For example, the onset of neurogenic edema, in response to application of mustard oil, histamine, or bradykinin, is delayed until 11 days of age (18), and does not stimulate c-*fos* expression in lamina II of the lumbar spinal cord until late into the second postnatal week (50), likely due to the immaturity of C-fibers and/or the late development of sympathetic postganglionic neurons (21).

Despite the clinical importance of changes in sensitivity to pain in the human infant, there is little known of the plastic changes in pain sensitivity at different stages of development. There is evidence for hyperinnervation and sprouting of primary afferents into denervated spinal cord or injured peripheral tissue in infant rats (46), and sensitization to repeated cutaneous mechanical stimulation (heel lance) in premature human infants (5). Deep tissue insults, such as the subcutaneous injection of turpentine or carrageenan, also produce severe inflammation in the fetal lamb (32).

In the adult rat, formalin injection into the paw produces a biphasic pattern of nociceptive behavior, a first phase that lasts no longer than 10 min, followed by a period of quiescence (interphase), and then a resumption of nociceptive behaviors about 20–30 min after the initial formalin injection [second phase; (1,48)]. The behavioral response is paralleled by changes in heart rate and arterial blood pressure (51). Formalin injected into the hind paw of the late fetal or infant rat produces edema, an adult-like flexor response, generalized alterations in behavioral state, and expression of c-*fos* in the superficial and deeper lamina of the dorsal horn of the spinal cord (24,39,56,57). The second phase of the biphasic behavioral response, which may represent sensitization of primary afferents and spinal cord dorsal horn neurons, however, is not seen until the start of the third postnatal week in the infant rat (24). Although there is a biphasic pattern of cardioacceleration in the adult, both phasic and tonic control of heart rate by the autonomic nervous system matures during the weaning period (23,45), and it is not known if the infant rat shows the same biphasic cardiovascular response. The goal of this study was to assess the response of the infant rat to formalin injection into the hind paw, replicating the studies on the age of onset of the biphasic pattern of behavioral responding, and to determine if similar changes in the cardiovascular system occur in the infant as they do in the adult.

METHOD

Subjects

The subjects were the pups of Long–Evans Hooded rats mated in our animal facility. All rats were housed in plastic tubs measuring 40 × 20 × 24 cm and the environmental temperature was maintained at a constant 22 ± 1°C. Parent animals were fed Purina Lab Chow and water ad lib. Cages were checked daily at approximately 1000 and 1800 h. Pups found on that day at either time were termed 0 days of age. Following parturition, litters were culled to 10 pups, without regard for the ratio of males to females. Only one pup per condition per litter was tested. All experimental protocols were approved by the Institutional Animal Care and Use Committee.

Implantation of Cardiorespiratory Leads

Pups were anesthetized with methoxyflurane on the morning of the test day and implanted with subcutaneous silver wire electrodes (47). The procedure required less than 5 min. Following surgery, pups recovered as a litter in an incubator maintained at 32°C.

Behavioral Tests

Rats, aged 7, 14, 21, or 35 days of age, were placed in a 10-gallon aquarium with wood shavings on the floor. An anesthetized littermate was placed into the cage with the test pup. Pups were connected to the polygraph, and behavior and heart rate were recorded for a 5-min baseline period. Pups were then injected with formalin (10 μl, 10%) or saline (10 μl) into the plantar pad of the right hind paw using a 30-gauge needle, and observed each minute for 60 min. Initially these injections were conducted in a blind manner, but it was always immediately obvious as to the content of the injection, and thus most of the data were collected in a nonblind method. Specific pain-related behavioral responses were recorded at the sampling time according to the following rating scale: 0 = the injected paw is not favored at all; 1 = the injected paw is slightly favored with less than full weight support but is still in contact with the floor; 2 = the injected paw is elevated and is not touching the floor ("paw lift"); 3 = animals lift the injected leg and shake it rapidly and vigorously ("paw shake"); 4 = animals lick the injected paw ("paw lift"). These behavioral ratings for the formalin test have been used and validated in the adult (1,12) and the infant (24,39,57) with the exception of the "paw shake" (score 3). This behavior was prominent, seemed intermediate to the paw "lift" and "lick," and was thus added; however, the results of the data analysis with or without that behavior (e.g., when "paw lick" was assigned the value of 3) were virtually identical. Saline-treated pups scored "0" on almost all observations.

Heart Rate Data Acquisition

Heart rate was scored by computer using methods previously described (45). Briefly, data sampled every 100 ms during the 65-min session were analyzed in 30-s epochs. Mean heart rate was defined as the inverse of the mean of the RR intervals (×60,000) during each epoch. To control for artifacts of movement or electrical noise, RR intervals were excluded if they exceeded 300 ms (<200 bpm) or were less than 80 ms (>750 bpm). Any RR interval change from one beat to the next greater than 15 ms was considered artifactual, and any epoch with fewer than 10 acceptable RR intervals changes was also excluded.

Statistics

For the behavioral analysis there were no behavioral responses to the saline injection or during the baseline period, and thus only responses following formalin injection were analyzed. The behavioral data were averaged into 3-min bins to decrease minute by minute variability, and a factorial analysis of variance (ANOVA) was performed on the behavioral scores. Age was a between-subject variable and the 20 3-min bins a within-subject variable. The omnibus ANOVA was significant and subsequent individual ANOVAs were performed at each age. These were followed by post hoc analysis using the Sheffé statistic with $\alpha = 0.10$ as recommended by Sheffé. Although the rating scale can be considered an ordinal scale, parametric statistics were used because the distribution of the scores was reasonably normal. A discussion of the type of analysis appropriate to types of statistical variables is found in Marascuilo and McSweeney (35)

Heart rate was recorded in 30-s bins. As for the behavioral data, to reduce variability and to account for bins in which the data were not usable due to artifact, we averaged the bins into 3-min epochs. An analysis of variance was performed on both

formalin- and saline-treated animals to ascertain if the formalin treatment altered heart rate compared to the saline control pups.

RESULTS

The most common behavior in reaction to the formalin injection was vocalization during injection, which occurred at all ages. For saline-injected animals, vocalization occurred occasionally, but was rarer. Edema occurred in all formalin-treated pups, whereas it was seen only in a few animals treated with saline.

Behavioral Scores

Data were complete for 8 to 11 subjects at each age. Analysis of the behavior was the same whether the four- or five-point scale was used and, thus, we present only the data from the latter measure. The analyses showed that there was a difference between the four ages, $F(2, 627) = 15.18$, $p < 0.001$, an effect over time, $F(19, 627) = 29.51$, $p < 0.001$, and an interaction between the age of the animal and the behavior over the hour observation period, $F(57, 627) = 4.25$, $p < 0.01$. Figure 1 shows those data. (Plots of the medians yielded virtually identical curves, data not shown). There was a significant increase in the overall pain score, collapsed over bins, from 7 to 14 days of age and again from 14 to 21 days of age. When the pattern of responding over 60 min was analyzed, the 7- and 14-day-old pups did not differ from each other; both younger ages differed from the 21- and 35-day-old pups. The two older groups did not differ from each other. The Sheffé tests showed that the first bin (3 min) after the formalin injection was different from the last 5–15 min at each age. The first bin differed from bins of the interphase (minutes 6–12) only at 21 and 35 days of age. Furthermore, the interphase differed from the second phase (minutes 18–22) again only at the two older ages. Thus, there is little statistical evidence for a biphasic pattern of responding at 7 or 14 days of age.

Heart Rate

Because of artifactual readings and other experimental error, data was lost on some subjects for whom there were available behavioral data. Data were available for six to seven formalin-treated and saline-treated animals at each age. Baseline heart rates for the last 3 min of the baseline period were 335 ± 21, 454 ± 12, 405 ± 12, and 484 ± 9 bpm for the 7, 14, 21, and 35-day-old animals, respectively. For the analysis of the formalin data only, there was an effect of age, $F(3, 21) = 22.05$, $p < 0.001$, and of 3-min bins, $F(19, 399) = 4.91$, $p < 0.001$, and an interaction of the two main effects, $F(57, 399) = 4.91$, $p < 0.001$. When saline data were added to the analysis, the results were similar. There were significant effects of age, $F(3, 41) = 28.17$, $p < 0.001$, and treatment [saline vs. formalin; $F(1, 41) = 5.20$, $p < 0.03$, but no significant interaction between these two variables. There was a significant interaction of bins, treatment, and age, $F(57, 779) = 1.34$, $p < 0.05$. Post hoc comparisons showed that the overall heart rate, collapsed over bins, was significantly highest for the 35-day-old pups, equal between the 14- and 21-day-old animals, and significantly lowest for the 7-day-old pups. The heart rate was increased in the formalin treated 14-, 21-, and 35-day-old pups, but not 7-day-old pups, compared to saline-treated animals. The biphasic pattern of heart rate did not appear until 35 days of age. Inspection of the data shown in Fig. 2, shows an elevated and reasonably steady or slightly declining heart rate over time at

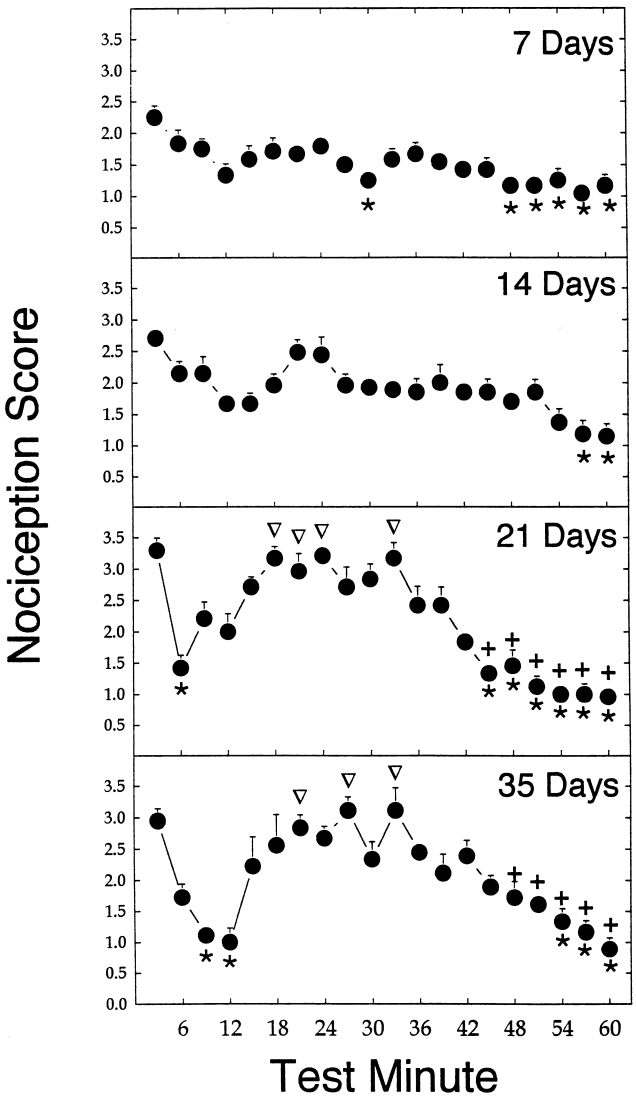

FIG. 1 The change in behavior following injection of formalin (10 μl; 10%) into the hind paw. There are three symbols that denote statistically significant differences between the test bins (minutes), thus defining the first phase, the interphase, the second phase, and the decline seen at the end of the test session. The asterisks (*) denote differences from the first bin (minutes 1–3). This defines both the interphase and the decline at the end of the session. Thus, for all animals there was a significant decline in responding during the last 5–15 min of the session. The interphase (a significant decline from minutes 1–3 to minutes 6–15) only occurred at 21 and 35 days of age. The inverted triangles (▽) denote difference from the lowest score of minutes 6–15 (the interphase) from all other bins at each age is defined as an increase in responding during a second phase. There is no evidence of a statistically significant second phase at 7 or 14 days of age, and the second phase is seen only at 21 and 35 days of age. The crosses (+) denote significant differences from the peak score of minutes 15–36 to subsequent minutes. For 21- and 35-day-old pups, but not the younger pups, the last six bins differed from the second phase and, thus, the second phase is limited in time from about minute 18 to minute 33. Saline data are not presented because these scores were almost exclusively zeroes.

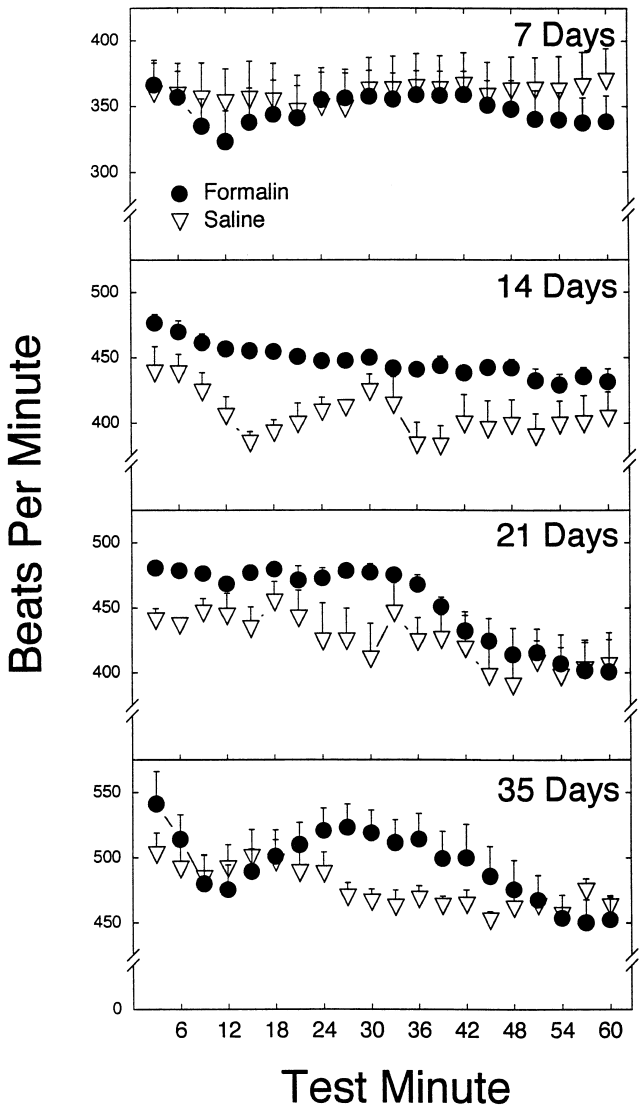

FIG. 2. Heart rate in beats per minute is shown for saline and formalin treatment conditions. There was no significant tachycardia induced by formalin at 7 days of age, and no biphasic pattern of responding until 35 days of age. Note that the scale for the maximum rate (Y axis) changes with age, although the range remains constant.

14 and 21 days of age, and a biphasic pattern of heart rate change only at 35 days of age.

DISCUSSION

The biphasic behavioral response and the biphasic heart rate response following formalin injection in the hind paw were both late maturing, and dissociated developmentally. The biphasic pattern of behavioral response in the formalin test appeared only at around 2–3 weeks of age, substantially prior to the onset of the biphasic heart rate response, which appeared between 21 and 35 days of age.

The behavioral response of the adult animal to the injection of formalin into the hind paw consists of three distinct phases (12). Young rat pups and rat fetuses show the initial response to the formalin injection, similar to adult animals, measured behaviorally (24,39), and there is an adult-like pattern of c-*fos* expression in the dorsal horn of the spinal cord beginning on gestation day 20 (53,56,57). In the present experiment, all pups responded behaviorally to the formalin injection, and over the first 15 min the pattern of the behavioral response was similar for all ages. Furthermore, the eventual decline in responding seen in the last 10 min of the 60-min test was also similar across ages. The developmental difference was limited to the time roughly from 15 to 40 min after the injection, when the 21- and 35-day-old animals show a tremendous increase in their behavioral response, matching the response seen in the first 3 min. This second phase of nociceptive behavior was absent or attenuated in the 7- and 14-day-old pups. This result is consistent with the findings of Guy and Abbott (24), who reported the onset of the biphasic pattern of responding at 15 days of age, but not with those of McLaughlin and co-workers, who interpreted an increase in pain responding in 3-day-old pups 20 min after formalin injection as perhaps the start of the second phase (39). Thus, the mechanisms that mediate the biphasic behavioral response in the formalin test mature sometime between 14 and 21 days of age. That a similar developmental course occurs for neuropathic pain, where mechanical allodynia (normally innocuous stimuli inducing pain) following peripheral nerve injury first occurred between 14 and 21 days of age (34), suggests the fundamental neuroplastic processes that mediate increased sensitization to noxious stimuli are relatively late maturing.

In the adult rat, formalin injection induces a hypertensive and tachycardic response that mimics the behavioral response (51). The initial tachycardia, in the first phase, to the formalin injection was absent in the 7-day-old, occurring only in 14-day and older animals. The biphasic pattern of heart rate change was not seen until 35 days of age, although the biphasic behavioral pattern was clearly present at 21 days. There are a number of possible explanations for this. First, it is possible that the cardiovascular system of the young pup cannot maintain a tachycardic response for the full hour. Although the autonomic system is still developing, even as weaning approaches (3,23,45,49), we know of no data that address this hypothesis directly. Second, it may be that the specific autonomic mechanisms that mediate the heart rate response in the first and second phases change from 21 to 35 days of age. There are data, using other stimuli, that are consistent with this hypothesis. In particular, Campbell and colleagues have reported that the parasympathetic system modulates phasic heart rate change in the preweaning rat. The cardiac deceleration induced by white noise or a nonnoxious auditory sound is mediated by parasympathetic activation, whereas the cardiac acceleration that is evoked by brief electric shock is due to parasympathetic withdrawal. This latter parasympathetic response lasts through 30 days of age and contrasts with the sympathetically mediated tachycardia induced by similar stimuli in the adult (26,33). Thus, in the present study the initial response to formalin may be mediated by parasympathetic withdrawal through 21 days of age, but the tachycardic response of the second phase requires the late maturing sympathetic activation. Direct proof of this hypothesis will require pharmacological studies.

It is also possible that the first and second phases of the formalin test induce distinct subjective states that are responded to in a similar behavioral manner but evoke different cardiovascular responses in the immature animal. The cardiac response of a young animal to a proximal threat such as pain is cardiac acceleration, as seen in the initial response to the formalin challenge or when subjected to electric shock (26).

In contrast, distal threats, such as a loud but distant noise presented to an infant rat (33), or the sound of threatening but distant stimuli to a deer fawn results in cardiac deceleration (28,29). Similar stimuli presented to adults of these species produce tachycardia. It is possible that the second phase is a different experience of "pain" and one that is less comparable to the immediate experience of pain, and more similar to that of a distal threat.

Although the mechanisms by which the first and second phases are mediated in the formalin test are not fully understood, there is increasing evidence that multiple mechanisms are involved. First, both the first and second phases of the formalin test are accompanied by increased activity in Aδ and C-fiber primary afferents (38,44) that might account in part for the biphasic response pattern. We are unaware of any developmental data that might clarify the role of the primary afferents in the maturation of the second phase response and, thus, will not discuss this further.

Both AMPA and NMDA glutamate receptors have been implicated in the behavioral and neural response to formalin. The AMPA glutamate receptor appears to mediate the initial electrophysiological response of convergent, lumbar dorsal horn cells and the behavioral reaction to injection of formalin into the hind paws [(8,27), but also see (10)]. In the neonate or fetus, formalin injection into the hind paw or forepaw induces an immediate behavioral response that is similar to the first phase in adults, and increased FOS expression in the dorsal horn (24,38,52,55,56). The AMPA receptor is overexpressed during early prenatal development, and is quite dense in the ventral horn of the spinal cord. The adult pattern of localization, restricted largely to the substantia gelatinosa only occurs between 14 and 21 days of age (30). If the AMPA receptor mediates the initial response in the formalin test, it does so prior to the onset of the adult like density and distribution.

Descending inhibitory pathways may play a role in the decline of responsiveness in the interphase (36). These pathways are immature in the infant rat, developing between 10 and 20 days of age (6,16,20). In the present experiments it is difficult to define an interphase in the absence of a well-developed second phase. Nonetheless, there is no evidence for dramatic developmental changes in this stage, with a slightly enhanced interphase seen only at 35 days of age. This is consistent with the later maturation of descending inhibition to the lumbar spinal cord.

Formalin likely induces central sensitization of spinal cord neurons, increasing their excitability to continued primary afferent input (9,11,54), and resulting in the resumption of nociceptive drive in the second phase. This sensitization of the nociceptive spinal cord neurons is likely mediated, at least in part, by the NMDA glutamate receptor. Administration of NMDA antagonists inhibit the second-phase behavioral nociceptive reaction and the electrophysiological response of the dorsal horn neurons following formalin injection (25,27,40, but see 42), although there is some controversy about the effectiveness of NMDA blockers administered after formalin (10, 52,55). In the present study, there is good agreement between the developmental appearance of the second phase and the maturation of the NMDA receptor in the dorsal horn of the spinal cord. NMDA receptors are transiently overexpressed in the spinal cord of infant rats, reaching an adult-like distribution towards the end of the third week of postnatal life (22, 31). The functional significance of that overexpression is not known, but it is possible that although the receptors are overexpressed, they are not fully functional, which would explain the late onset of the second phase. There are pharmacological data that are consistent with this hypothesis: the NMDA antagonist AP5 depressed spontaneous dorsal horn neural activity only after 21 days of age in the hamster, implying that the receptors become functional only at that age (2). Thus, it is possible that the large increase in the behavioral response that constitutes the second phase, and that appears after 14 days of age is dependent on the mature appearance and function of the NMDA receptor. Note that this hypothesis differs from that hypothesized for the AMPA receptor where the first phase is present even though the distribution and density of AMPA receptors in immature. Alternatively, maturation of specific circuits or other receptor systems (e.g., neurokinins) mediating the response to the formalin test might account for these data.

Regardless of the mechanisms, autonomic function and behavioral responses are not linked in immature animals as they are in the adult, and the question of when and how the link between the behavioral and cardiovascular responses occurs is an important one. These results also demonstrate that the cardiovascular changes in the formalin test do not reflect only changes in somatomotor behavior, because the biphasic behavioral response is present in the 21-day-old pup in the absence of a similar pattern of heart rate change, a result in agreement with that in the adult (51).

In summary, the second phase of nociceptive behavioral responding in the formalin test is late appearing, developing sometime late in the second week of life. This functional maturation parallels the appearance of the adult pattern of NMDA receptors in the superficial dorsal horn, lending support to the hypothesis that these processes are necessary for the behavioral and neural sensitization that occurs in the spinal cord after injection of irritants into the paw. The cardiovascular changes appear substantially later and likely reflect the late involvement of sympathetic activation of heart rate. The late appearance of the sensitization suggests that the full development of processes that are dependent of plastic changes in the spinal cord may also be late maturing.

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