Published in final edited form as:
Brain Res. 2008 March 20; 1200: 58–65.

DEVELOPMENTAL EMERGENCE OF FEAR LEARNING CORRESPONDS WITH CHANGES IN AMYGDALA SYNAPTIC PLASTICITY

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Abstract

Mother-infant attachment is facilitated in altricial rodents through unique neural mechanisms that include impaired neonatal fear conditioning until the time that pups first begin to leave the nest (sensitive period). Here, we confirmed the developmental emergence of odor fear conditioning in neonatal rat pups, and examined synaptic plasticity of inputs to the basolateral amygdala *in vitro*. Coronal slices through the amygdala were obtained from sensitive (< 10 days) and post-sensitive (> 10, < 19 days) period pups. Field potentials were recorded in the basolateral amygdala in response to stimulation of either the external capsule (neocortical inputs) or fibers from the cortical nucleus of the amygdala (olfactory inputs). The effects of tetanic stimulation were examined in each pathway. In both pathways, tetanic stimulation induce significant long-term synaptic plasticity in post-sensitive period pups, but no significant plasticity in sensitive period pups incapable of learning odor aversions. $GABA_A$ receptor blockade in post-sensitive period slices reverts synaptic plasticity to sensitive period characteristics. The results suggest that sensitive period deficits in fear conditioning may be related to impaired amygdala synaptic plasticity and the immature state of GABAergic inhibition and/or it modulation in the neonatal amygdala.

Keywords

emotion; olfaction; fear memory; infant attachment; GABA; long-term potentiation

1. INTRODUCTION

Forming social attachments is fundamentally important for survival in many altricial species. This is highlighted by the presence of specialized learning circuits during ‘sensitive periods’ of social attachment formation where some forms of learning are facilitated, while others are attenuated. For example, altricial rat pups are dependent on maternal care for survival and exhibit facilitated sensitive period odor preference learning to the maternal odor, which is then used for approach to the caregiver and nipple attachment. Sensitive period pups also show attenuated aversion learning, presumably to prevent pups from learning to avoid the maternal odor. For rat pups, the temporal association of maternal odor with a variety of other maternally

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generated stimuli, such as grooming, warmth, or milk results in learned approach, nipple attachment and behavioral activation responses by the neonate on subsequent presentation of that odor (Galef and Sherry, 1973, Johanson and Hall, 1979, Johanson and Teicher, 1980, Brake, 1981, Pedersen et al., 1982, Alberts and May, 1984, Sullivan et al., 1986a, Sullivan et al., 1986b, Wilson and Sullivan, 1994). Importantly, the range of interactions with the mother includes painful stimuli, such as biting and being stepped upon, yet neonates fail to learn an aversion to odors paired with such painful stimulation and instead learn to prefer the odor (Haroutunian and Campbell, 1979, Sullivan et al., 1986a, Sullivan et al., 1986b, Camp and Rudy, 1988, Sullivan et al., 2000, Moriceau and Sullivan, 2004a, Roth and Sullivan, 2005). As pups mature and begin to explore the extra-nest environment around postnatal day (PN) 10 (Bolles and Woods, 1964), more 'adult-like' fear and inhibitory learning emerges (Haroutunian and Campbell, 1979, Blozovski and Dumery, 1987, Camp and Rudy, 1988, Sullivan et al., 2000, Moriceau and Sullivan, 2004a, Roth and Sullivan, 2005).

Here we explore the neural correlates of attenuated aversion learning and the emergence of fear conditioning in sensitive period and post-sensitive period pups. In adult rats, the amygdala plays a critical role in fear conditioning (Sananes and Campbell, 1989, Rosenkranz and Grace, 2002, Davis et al., 2003, Fanselow and Gale, 2003, LeDoux, 2003, Debiec and Ledoux, 2004, Sevelinges et al., 2004, Schroeder and Shinnick-Gallagher, 2005). Association of a conditioned stimulus and, for example, footshock in juvenile or adult rats causes activation of the amygdala, and induces a modification of conditioned stimulus-evoked responses of amygdala neurons (Rosenkranz and Grace, 2002). Lesions of the amygdala prevent or retard fear learning and memory (LaBar and LeDoux, 1996, Setlow et al., 2000, Gale et al., 2004). Furthermore, synaptic plasticity of cortical and thalamic inputs to the basolateral nucleus of the amygdala appears necessary for normal fear conditioning (Blair et al., 2001, Maren, 2005), such that manipulations that impair or enhance such plasticity also impair or enhance acquisition of behaviorally expressed learned fear (e.g., (Campeau et al., 1992, Davis et al., 1994, Szinyei et al., 2007).

The failure of odor-pain association to induce learned fear in neonates may in part be due, therefore, to the immature state of amygdala circuitry during the early postnatal period. In the adult, neocortical and thalamic inputs to basolateral nucleus neurons demonstrate long-term synaptic plasticity following tetanic stimulation, and this plasticity may either be expressed as potentiation or depression depending on the conditions and presence or absence of $GABA_A$ receptor antagonists (Rogan et al., 1997, Heinbockel and Pape, 2000, Rammes et al., 2001, Kaschel et al., 2004). Furthermore, plasticity is expressed at both excitatory and inhibitory synapses (Rogan et al., 1997, Bauer and LeDoux, 2004, Szinyei et al., 2007). Both amygdala synaptic plasticity and learned fear are modulated by a number of factors, including neuromodulators (Rosenkranz and Grace, 2002, Azad et al., 2004), steroid hormones (Setlow et al., 2000) and level of GABAergic inhibition (Watanabe et al., 1995, Rammes et al., 2000). Importantly however, while GABA synthetic enzymes (e.g., GAD (Stork et al., 2000)) and receptor subunits (Zhang et al., 1991) are present at birth in the amygdala, they do not attain adult levels there until several weeks later, suggesting a potential late emergence for the mature expression of amygdala synaptic plasticity (Gilbert and Cain, 1981). In fact, odor-foot shock association that induces amygdala activation (e.g., c-fos labeling) and learned fear in PN12 rat pups, induces neither amygdala activation nor fear in PN10 pups (Sullivan et al., 2000, Moriceau and Sullivan, 2004b, Roth and Sullivan, 2005). It should be noted that pain threshold to footshock is very similar across this age range of pups (Emerich et al., 1985, Barr, 1995, Sullivan et al., 2000, Fitzgerald and Beggs, 2001).

The present report was an examination of synaptic plasticity in two afferent pathways to the basolateral nucleus of the amygdala *in vitro*, before and after the age at which fear conditioning emerges in the rat. Given that neonatal maternal recognition is primarily olfactory mediated,

we examined the putative input from the cortical nucleus of the amygdala to the basolateral nucleus. The cortical nucleus of the amygdala receives direct input from the olfactory bulb (Shipley and Ennis, 1996), and olfactory evoked responses within the amygdala are known to be modified by fear conditioning (Rosenkranz and Grace, 2002). To allow our results to be compared to the extant literature on amygdala synaptic plasticity, we also examined the neocortical input to basolateral nucleus. We hypothesized that the during the sensitive period for learned odor-guided attachment to the mother, plasticity within circuits mediating fear conditioning would be impaired or abnormal. The results suggest a GABAergic-dependent change in synaptic plasticity of both pathways coinciding with developmental emergence of learned fear.

2. RESULTS

Behavior

As shown in Figure 1, behavioural Y maze testing revealed that sensitive period (PN8) pups that received the paired odor-shock conditioning made a larger proportion of choices towards the CS odor, indicating a learned odor preference. Older, post-sensitive period (PN12) pups that received the same conditioning made a smaller proportion of choices towards the CS odor demonstrating a learned odor aversion (ANOVA, condition X age interaction, $F(2, 28) = 41.47$, $p < 0.001$; post hoc Fisher test revealed that the paired pups were significantly different from each of the age-matched control groups). This replicates previous results (Sullivan et al., 2000).

Electrophysiology

As shown in Fig. 2, both stimulation of the dorsal edge of lateral nucleus near the external capsule and stimulation of putative fibers from the cortical nucleus of the amygdala evoked short-latency, negative evoked responses recorded in the basolateral nucleus. The lateral nucleus input pathway has been well described by multiple groups, and is a glutamatergic excitatory response. Similarly, responses to the cortical nucleus input could be reversibly blocked by the glutamatergic receptor antagonist kynurenic acid (10 mM; n =3, data not shown).

Tetanic stimulation of the lateral nucleus/external capsule pathway in post-sensitive period pups (> PN11; Fig. 3A) induced a long-term depression of responses (repeated measures ANOVA main effect of time, $F(30,300) = 3.49$, $p < 0.001$), as has been reported previously (Heinbockel and Pape, 2000,Rammes et al., 2001,Kaschel et al., 2004). In contrast, in slices from sensitive period pups, the same stimulation protocol induced no significant change (repeated measures $F(30,120) = 1.12$, N.S.), though there was a clear trend toward potentiation. There was a significant difference between sensitive period and post-sensitive period responses post-trains (ANOVA, $F(1,46) = 11.77$, $p < 0.01$).

In the cortical nucleus – basolateral nucleus pathway (Fig. 3B), tetanic stimulation in post-sensitive period pups induced a long-term depression, similar to that seen in the lateral nucleus pathway (repeated measures ANOVA, $F(30,330) = 2.66$, $p < 0.001$). The magnitude of this change was not as great as that in the external capsule pathway, reflecting either a real difference in plasticity in these two pathways, or perhaps more likely a difference in density of fibers being stimulated at the two sites. Nonetheless, no significant long-term change in synaptic strength was observed after tetanic stimulation of the cortical nucleus pathway in sensitive period pups (repeated measures $F(30,180) = 0.87$, N.S.). There was a significant difference between sensitive period and post-sensitive period responses post-trains (ANOVA, $F(1,55) = 4.74$, $p < 0.05$).

To determine whether the late maturation of GABAergic circuits could contribute to the changes in synaptic plasticity observed here around the time of sensitive period termination, we examined the effect of the $GABA_A$ receptor antagonist picrotoxin (100 μM) on plasticity in these two pathways in slices from post-sensitive period pups. Blockade of $GABA_A$ receptors in post-sensitive period slices induced changes in slice physiology comparable to that observed in sensitive period pups. In the lateral nucleus to basolateral nucleus pathway of slices from post-sensitive period pups (Fig. 4A), tetanic stimulation in the presence of picrotoxin induced a significant long-term potentiation (repeated measures ANOVA $F(30,240) = 4.32$, $p < 0.01$), in contrast to the long-term depression observed under the same conditions in the presence of aCSF above. There was a significant difference between aCSF and picrotoxin treated slices post-trains (ANOVA, $F(1, 58) = 23.03$, $p < 0.01$).

Similarly, in the cortical nucleus to basolateral pathway of slices from post-sensitive period pups (Fig. 4B), picrotoxin reduced the magnitude of long-term depression, though a short-term depression remained. Analysis of train effects greater than 8 min post trains revealed a significant depression in aCSF-treated slices (repeated measures ANOVA, $F(9,99) = 3.05$, $p < 0.01$), but no lasting depression in picrotoxin treated slices (repeated measures ANOVA, $F(9,81) = 0.68$, N.S.). The difference between aCSF and picrotoxin treated post-train effects in this pathway did not quite reach statistical significance (ANOVA, $F(1,64) = 3.58$, $p = 0.062$).

3. DISCUSSION

An association of odor and 0.5mA footshock in sensitive period rat pups (< PN10) induces a relative preference for that odor, while similar conditioning in post-sensitive period pups induces a relative aversion, replicating previous results (Camp and Rudy, 1988, Sullivan et al., 2000). While it may seem paradoxical for infant rats to learn to prefer an odor paired with pain, this attenuated learning can prevent avoidance of the mother and increase caregiver proximity-seeking behaviors, regardless of the quality of caretaker treatment. Similar paradoxical attachment occurs in other species, including chicks, infant dogs, nonhuman primates and children (e.g., (Harlow and Harlow, 1965, Salzen, 1967)).

In adults, conditioned fear of stimuli in a variety of sensory modalities is associated with synaptic plasticity of inputs to the basolateral amygdala (Rogan et al., 1997, Rosenkranz and Grace, 2002). While adults display long-term potentiation of afferent input to the amygdala associated with learning *in vivo* (Rogan et al., 1997), in adult *in vitro* slices this plasticity is expressed as either long-term potentiation or long-term depression, depending on the stimulation characteristics and presence or absence of $GABA_A$ antagonists in the bath, similar to that shown in the post-sensitive period slices here. The present results suggest that at an age where pups do not acquire learned odor aversions, neither neocortical nor cortical nucleus of the amygdala inputs to the basolateral nucleus display the mature form of long-term synaptic plasticity. In fact, in the cortical to the basolateral nucleus pathway, which conveys odor input from the olfactory bulb, no stable synaptic plasticity could be evoked in sensitive period pups that fail to learn aversions. Several days later, when normal learned odor fear can be acquired, normal long-term synaptic plasticity is expressed. Age-dependent changes were observed in both afferent pathways, though the magnitude of plasticity was less in the cortical nucleus of the amygdala pathway compared to the external capsule pathway. These results suggest that modified or impaired synaptic plasticity of inputs to the basolateral amygdala during early development may contribute to the reduced ability of animals at this age to learn odor aversions, and in turn contribute to infant-maternal attachment.

Furthermore, the abnormal characteristics of sensitive period synaptic plasticity can be mimicked in post-sensitive period pups by blockade of $GABA_A$ receptors with picrotoxin in both pathways. This finding, combined with the known slow ontogeny of GABA receptors in

the amygdala (Zhang et al., 1991, Stork et al., 2000) and limbic system, leads to the hypothesis that developmental emergence of amygdala plasticity and amygdala-dependent behaviors may reflect, in part, maturation of GABAergic function. In support of this hypothesis, manipulations known to modulate GABAergic function also modulate the age of sensitive period termination. For example, corticosterone (CORT) modulates GABAergic inhibition and principal cell excitability in the adult amygdala (Duvarci and Pare, 2007) and other limbic regions (Verkuyl et al., 2005). Elevating CORT levels within the amygdala of sensitive period pups allows precocial emergence of fear conditioning (Moriceau and Sullivan, 2004a), while reducing CORT levels in post-sensitive period pups either by adrenalectomy or maternal presence (Wiedenmayer et al., 2003) reinstates the sensitive period blockade on fear conditioning (Moriceau and Sullivan, 2004b, Moriceau and Sullivan, 2006).

The potential link between the developmental emergence of fear learning with GABAergic function and modulation of amygdala synaptic plasticity also has ramifications for the known long-term effects of early stress and fear learning. For example, early neonatal handling and maternal separation are correlated with changes in limbic system GABA receptor sub-unit expression and receptor function in adults (Hsu et al., 2003), as well as several behavioral effects such as reduced fear responses (Ladd et al., 2000). Similarly, the postnatal sensitive period odor-shock conditioning paradigm used in the present study reduces odor fear conditioning when those animals are later trained as adults (Sevelinges et al., in press). Integrating the effects of early experience on GABAergic maturation, endocrine function and amygdala synaptic plasticity may provide important clues toward understanding the behavioral consequences of infant stress and trauma.

4. EXPERIMENTAL PROCEDURES

The subjects were male and female preweanling Long Evans hooded rat pups bred and born in the University of Oklahoma animal care facilities. Rats were housed in rectangular polypropylene cages (34×29×17 cm) lined with wood chips and kept in a temperature (20o C) and light (0800–2000 hr) controlled room with a*d lib* food and water. The birth date was considered PN0, litters were culled to 12 pups on PN1 and only 1 male and/or female from each litter was used in any training/test condition. Animal care and experimental procedures conformed to US Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Behavior

Pups were randomly assigned to one of three groups for odor-shock conditioning. The *paired group* (n=6 PN8, n = 6 PN12)received 10 pairings of the unconditioned stimulus (US -moderate tail shock; 0.5 mA, 1 sec.) paired with the last second of a 30 sec conditioned stimulus (CS, 2L/min, 1:10 peppermint vapor:air) delivered through an olfactometer controlled by a Chrontrol at a 4 min inter-trial interval. The *unpaired group* (n=5 PN8, n = 6 PN12)received the shock 1.5–2 min after the CS odor presentation, while the *odor-only group* (n=5 PN8, n = 6 PN12) received only the odor CS. Pups were removed from the nest immediately prior to conditioning and given 10 min to habituate to the training apparatus (600 ml Pyrex jars) prior to the training session, and returned immediately to the nest following training. Pups' behaviors were monitored during training and all pups in the paired odor-shock group showed learning curves (documented by a behavioral activity scale related to the number of limbs moving), while control pups showed no indication of an acquisition curve. All pups also showed strong responsiveness to shock regardless of the conditioning groups.

The next day, pups were tested in a Y-maze to assess their expression of an odor preference or aversion using a video-tracking system (Columbus Instruments). The Y-maze required pups to choose between the CS odor and a familiar odor (the same wood shavings used as nest

bedding but clean) placed at the end of the two arms of a Plexiglas Y-maze (choice arms: 8.5x24x8 cm). Pups were placed in the start box for 5 sec, the alley doors opened, and the pups were given 60 sec to choose an arm (pup's entire body was past the alley entrance). Each pup completed 5 trials. Between trials, the pup was placed in a holding cage for 5 sec and he floor wiped clean with water and dried.

Electrophysiology

Amygdala slices were obtained from pups in one of two age groups, PN7-PN10 (<20g, sensitive period pups) and PN11-PN19 (>20g post-sensitive period pups). Brains were rapidly dissected from isoflurane anesthetized pups and placed in ice-cold oxygenated artificial cerebrospinal fluid (aCSF) composed of 124mM NaCl, 5 mM KCl, 1.24 mM KH_2PO_4, 2.4 mM CaCl, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$ and 10 mM glucose. Coronal, 400μ thick slices including the amygdala were cut with a vibratome. Slices were incubated at room temperature for 1 hour in aCSF. The slices were then moved to a superfusion chamber at room temperature for recording. In all procedures described below, electrical stimulation was provided with a concentric bipolar stainless steel stimulating electrode and field potentials were recorded using tungsten microelectrodes (A-M Systems). Amplified and bandpass (5Hz-1kHz) filtered signals were acquired at 5 kHz and analyzed using Spike2 software (CED, Inc).

Evoked responses and plasticity were examined in two different pathways afferent to the basolateral amygdala in different slices. Amygdalar nuclei were located using neuroanatomical landmarks. Activation of neocortical inputs was mimicked by electrical stimulation of the dorsal lateral amygdala near the external capsule. Activation of inputs from the olfactory system was mimicked by stimulation near the dorsal edge of the cortical nucleus of the amygdala, which receives direct input from mitral/tufted cells of the ipsilateral olfactory bulb (Shipley and Ennis, 1996). Following recording of baseline responses to 0.1 ms duration, 10–100 μA test pulses as 0.1 Hz, high frequency tetanic stimulation was delivered (100 Hz, 1 sec trains with 3 min between trains, 5 trains total, same pulse duration and intensity as the test stimuli), followed by a return to 0.1 Hz test pulse stimulation for up to 60 min post-trains. Only one pathway was tested in each slice.

In a subset of slices, the effect of the $GABA_A$ receptor antagonist picrotoxin (100 μM) on response to tetanic stimulation was tested. Following recording of baseline responses in aCSF, slices were superfused with picrotoxin for at least 10 min, followed by the stimulation protocol described above.

Evoked responses were quantified by measuring the slope of the initial phase of evoked waveforms averaged from three stimuli. Effects of the tetanic stimulation were statistically examined with repeated measures ANOVA over data from 3 min pre-trains and the first 10 min post-trains. Mean effect of tetanic stimulation was compared across age groups or drug condition with ANOVA of the data at least 8 min post-trains.

Acknowledgements

This work was funded by grants NIDCD-DC03906 to DAW and NICHD-HD33402, NSF IOB-0544406 & the Oklahoma Center for the Advancement of Science and Technology to RMS.

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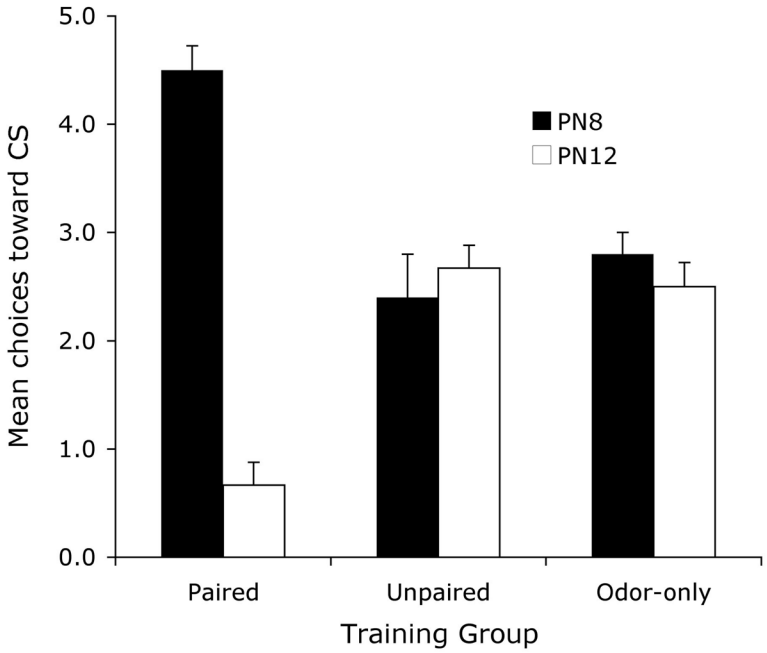

Figure 1.
Mean (± sem) number of choices toward the conditioned stimulus (CS) odor during the Y-maze test (total of 5 trials) for PN8 and PN12 pups.

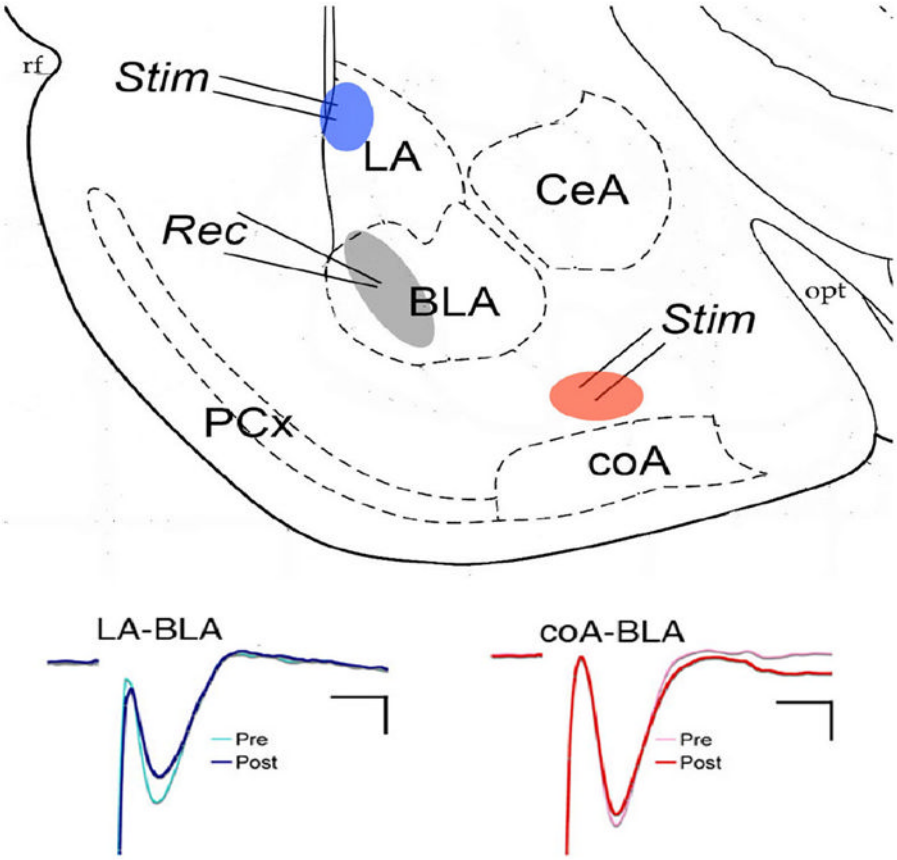

Figure 2.

(**TOP**) Schematic representation of coronal amygdala slice showing approximate stimulation (Stim) and recording (Rec) electrode placements. Only one pathway was tested in each slice. (**BOTTOM**) Examples of evoked potentials before and after tetanic stimulation of the two pathways. LA = lateral nucleus, BLA = basolateral nucleus, ceA = central nucleus, coA = cortical nucleus, PCx = piriform cortex. Calibration is 2 ms and 2 mV for LA-BLA pathway and 2 ms and 5 mV for coA-BLA pathway.

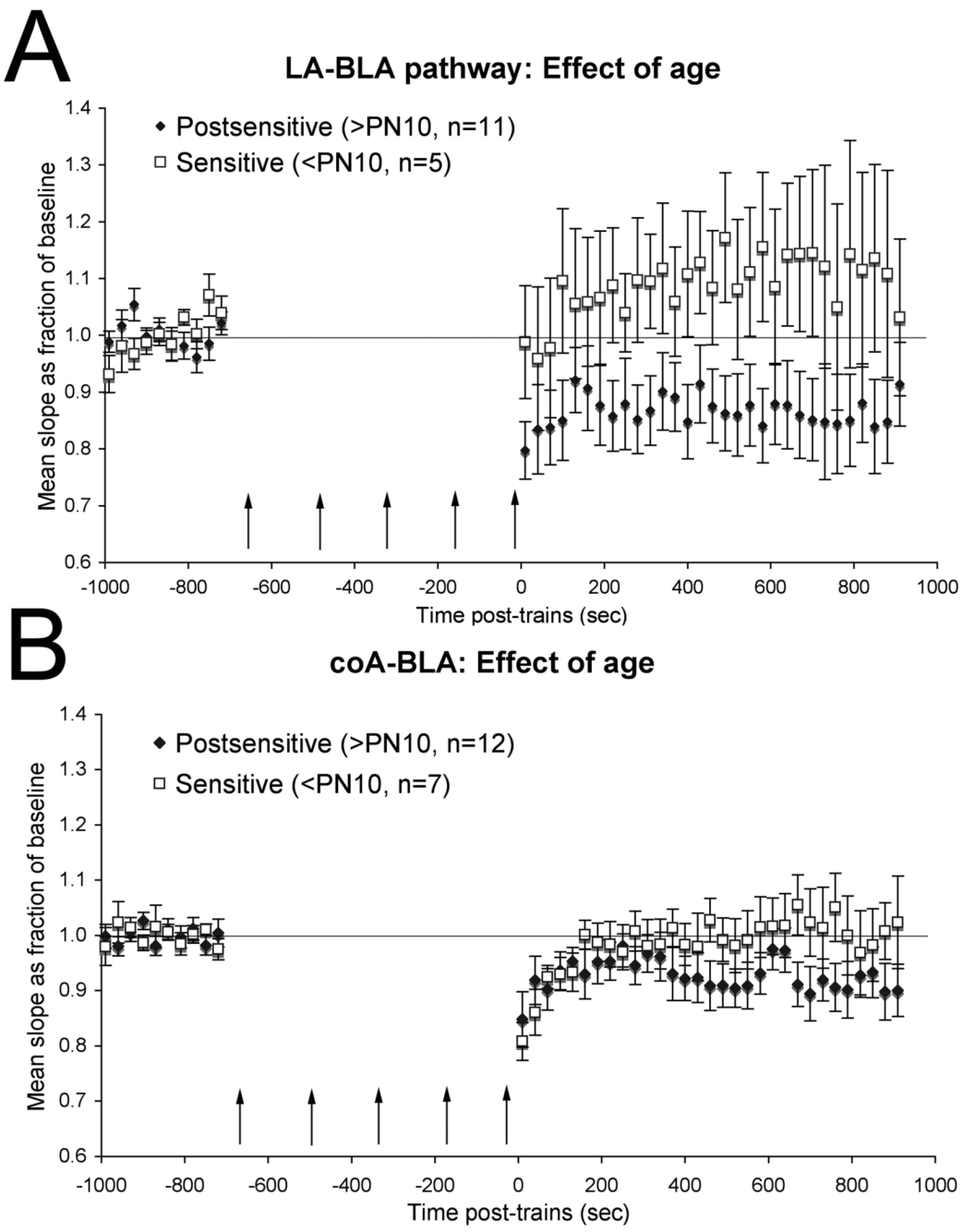

Figure 3.

Effect of age on synaptic plasticity in the basolateral amygdala. (**A**). Mean slope of evoked field potential as a proportion of baseline recorded in the BLA before and after tetanic stimulation of LA inputs for slices from postsensitive and sensitive period pups. Tetanic stimulation of the lateral amygdala/external capsule induced a long-term depression of synaptic potentials recorded in the basolateral amygdala of post-sensitive period pups. Similar stimulation in slices from sensitive period pups produced no statistically significant change, thought there was a mild potentiation. (**B**). Same as A, for stimulation of coA inputs. Tetanic stimulation of the putative cortical nucleus input to the basolateral amygdala also produced a

significant long-term depression in slices from post-sensitive period pups, but no long-term change in slices from sensitive period pups.

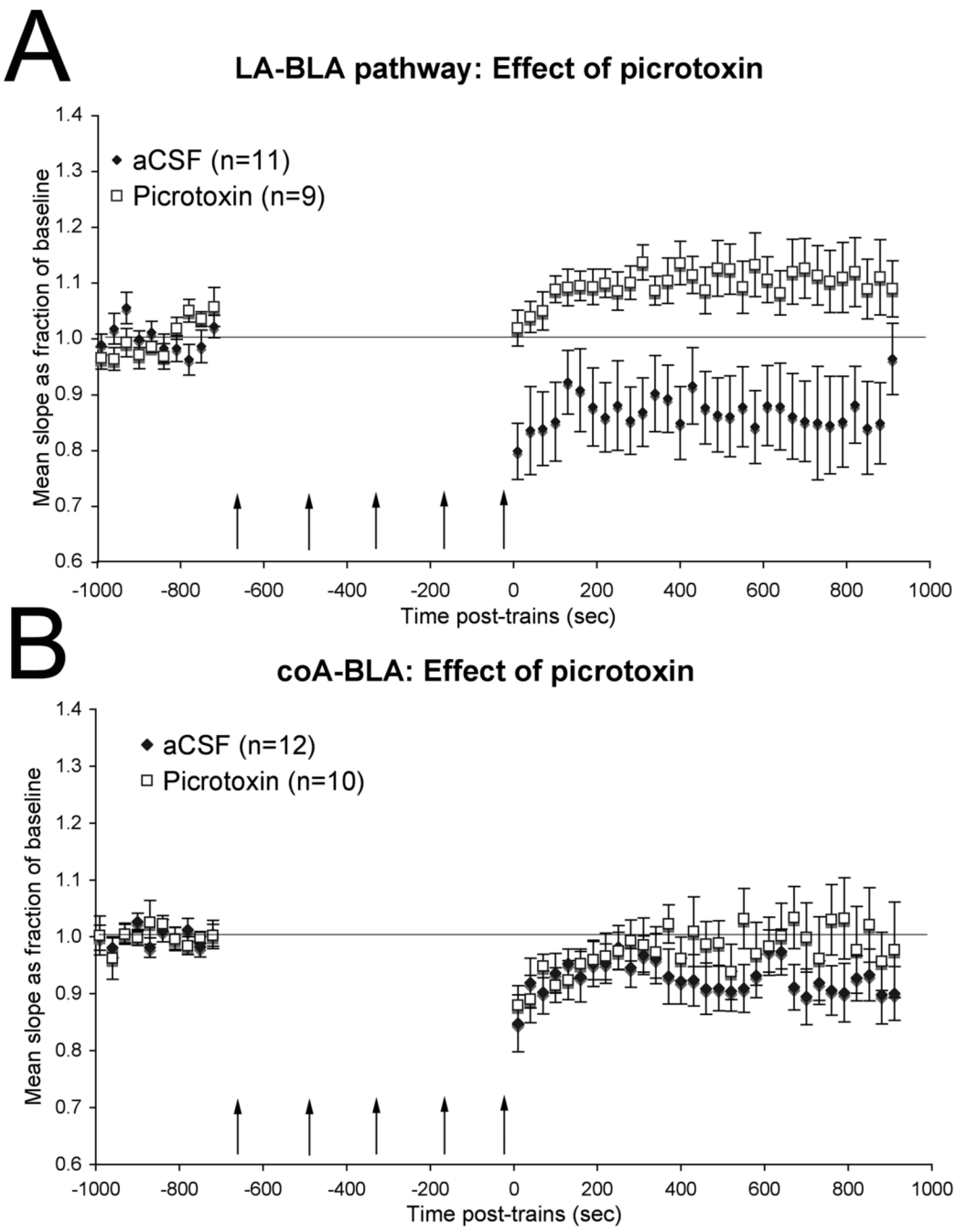

Figure 4.

$GABA_A$ receptor blockade in post-sensitive period slices reverts synaptic plasticity to sensitive period characteristics. Data for aCSF recordings is the same as shown in Fig. 3. (**A**) Mean slope of evoked field potential as a proportion of baseline recorded in the BLA before and after tetanic stimulation of LA inputs for slices from postsensitive period pups with and without added picrotoxin. Tetanic stimulation of the lateral amygdala input to basolateral amygdala induced long-term depression in post-sensitive period control slices but long-term potentiation in the presence of picrotoxin. (B) Same as A, for stimulation of coA inputs. Picrotoxin blocked the long-term depression of cortical nucleus input to basolateral amygdala seen in control post-sensitive period slices.