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Associative Fear Conditioning of Enkephalin mRNA Levels in Central Amygdalar Neurons

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The central nucleus of the amygdala (CEA) is required for the expression of learned fear responses. This study used in situ hybridization to show that mRNA levels of the neuropeptide enkephalin are increased in CEA neurons after rats are placed in an environment that they associate with an unpleasant experience. In contrast, mRNA levels of another neuropeptide, corticotropin releasing hormone, do not change under the same conditions in the CEA of the same rats. Conditioned neuropeptide levels in amygdalar circuits may act as a reversible "gain control" for long-term modulation of subsequent fear responses.

It has long been known that the medial temporal lobe of the cerebral hemispheres, and more specifically the amygdala, plays an important role in the expression of emotional behavior (Brown & Schäfer, 1888; Kaada, 1972; Klüver & Bucy, 1939; Penfield, 1958; Weiskrantz, 1956); and recent evidence suggests that the amygdalar basolateral and central nuclei are important for the learning and expression, respectively, of conditioned fear responses (for review see Fanselow & LeDoux, 1999).

The central nucleus of the amygdala (CEA) has three structurally distinct parts: medial (CEAm), lateral (CEAl), and capsular (CEAc). The expression of fear responses is commonly attributed to the CEAm because of its descending axonal projections to regions that generate appropriate autonomic and behavioral responses (Hopkins & Holstege, 1978; Rizvi, Ennis, Behbehani, & Shipley, 1991; Schwaber, Kapp, Higgins, & Rapp, 1982), and because stimulation of neurons in this region produces autonomic and behavioral responses that mimic conditioned emotional responses (Applegate, Kapp, Underwood, & McNall, 1983; Kapp, Gallagher, Underwood, McNall, & Whitehorn, 1982)—which are abolished by large lesions of the CEA that include the CEAm (for reviews, see Davis, 1992; LeDoux, 1995). Axonal projections from the CEAl have been characterized recently (Petrovich & Swanson, 1997) and are very restricted, with dense inputs to the adjacent CEAm, to the bed nuclei of the stria terminalis (BST; via the stria terminalis) of the basal ganglia, and to the hindbrain parabrachial nucleus (via the ansa peduncularis and then medial forebrain bundle). On the basis of this and other evidence, it was hypothesized that changing levels of certain neuropeptides synthesized by CEAl neurons may act as a "gain control" for reversible, long-term modulation (LTM) of conditioned fear responses (Petrovich & Swanson).

As an initial test of this hypothesis, we used in situ hybridization to examine neuronal mRNA levels for two neuropeptides, corticotropin releasing hormone (CRH) and enkephalin (ENK), in the CEA of rats trained in a contextual fear conditioning paradigm, in which mild footshock acts as the unconditioned stimulus and a particular environment acts as the conditioned stimulus. Considerable evidence implicates CRH in behavioral aspects of stress, anxiety, and fear (for reviews, see Dunn & Berridge, 1990; Koob et al., 1993; but see also Weninger et al., 1999), whereas opioids, including ENK, may alter learning and memory (e.g., Aloyo, Romano, & Harvey, 1993; Gallagher, Kapp, & Pascoe, 1982; Rigter et al., 1980) and are also important in pain perception and analgesia, which are altered during conditioned fear responses (Helmstetter & Fanselow, 1987; Olson, Olson, Vaccarino, & Kastin, 1998).

Method

Subjects

Twenty-seven adult male Sprague–Dawley rats (250–275 g) were individually housed and maintained on a 12-hr light–dark cycle (lights on at 6 a.m.) with unlimited access to food and water. After arrival, rats were allowed 7 days to acclimate to the colony and were then handled daily (2 min per rat) for 7 days to familiarize with the experimenter and to acclimate to transportation from the colony to the experimental room. All experiments, including training and testing, were performed in the early morning hours (between 6 and 10 a.m.).

Design and Procedure

Rats were randomly assigned to one experimental group and two control groups ($n = 9$ for each group). Experimental rats (conditioned fear group) were trained in an experimental chamber for 2 days. During the morning of each training day, rats were placed in the chamber and allowed to explore freely for 3 min before three, 1-s-long, 1-mA footshocks were delivered, 1 min apart, through the grid floor. Immediately after the last shock, rats

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were quickly returned to their home cages and taken back to their colony. They were left undisturbed on Day 3 to allow for possible training-induced changes in neuropeptide mRNA levels to return to baseline. On Day 4, each rat was brought back to the experimental chamber (at the same time of the morning that training had taken place) for 30 min to measure a learned fear response associated with this particular environment (context). No footshocks were delivered during testing. After testing, rats were perfused and the brains were collected and pretreated for anatomical procedures.

Freezing, a characteristic species-specific defensive fear response (Blanchard & Blanchard, 1969; Fanselow, 1994), was used as a behavioral measure of conditioned fear expression. Freezing behavior was assessed independently by two observers unaware of rats' group assignments, who scored the behavior of each rat every fifth minute during the 30-min testing period, which was videotaped. In addition, each rat's movement (or immobility) was measured continuously during the testing period with an infrared activity sensor system. Both measurements are presented as a percentage of total observations made during the testing period.

Rats in one control group (no training) followed the same protocol as the experimental group except that they received no footshocks; this group controlled for rat's exposure to handling, transportation, and the training environment alone. The other control group (training only) provided information about possible training-induced changes in peptide mRNA levels. These rats received the same training as the experimental group, including footshocks, but were not tested for the conditioned fear response; instead, they were perfused at the time testing would have begun on Day 4.

Behavioral Apparatus

A well-lit metal box (30 cm wide, 26 cm long, and 32 cm high) with a glass front wall and a stainless steel rod floor (Coulbourn Instruments, Allentown, PA) through which footshocks were delivered was used as an experimental chamber. The entire box was carefully wiped with 5% ammonium hydroxide solution before each rat was placed inside on training and testing days. The box was exposed to 80 dB of background noise.

A 24-cell infrared activity sensor was mounted on the top of the experimental chamber to monitor rats' movement (or immobility), by measuring the emitted infrared (1300 nm) body heat image from the rat in the *x*, *y*, and *z* axes. During the testing period, rats' movement (or immobility) was measured continuously with an L2T2 LabLinc System (Coulbourn Instruments, Allentown, PA). The procedure has been described in detail previously by Lee and Kim (1998).

Anatomical Procedures

Exactly 75 min after the testing period ended, rats were quickly and deeply anesthetized with pentobarbital and then perfused transcardially with 4% paraformaldehyde according to the protocol described elsewhere (Swanson & Simmons, 1989). Five rats were chosen randomly from each experimental group ($n = 9$) for anatomical procedures.

For histochemical analysis, frozen brains were cut on a sliding microtome into five adjacent series of 24-μm-thick transverse sections. Two series were processed for in situ hybridization with cRNA probes for CRH or ENK mRNA, and a third was stained with thionin for cytoarchitecture.

Sections were hybridized with ^{35}S-UTP-labeled cRNA probes transcribed from a 700 bp cDNA sequence that codes for part of Exon 1 and all of Exon 2 of preproCRH (Frim, Robinson, Pasieka, & Majzoub, 1990), and a 935 bp cDNA sequence containing the entire coding sequence of preproENK. The ^{35}S-UTP-labeled probes were synthesized and in situ hybridization performed according to the protocol described previously (Swanson & Simmons, 1989). Briefly, sections were prehybridized and then hybridized for 21 hr at 60 °C with a probe concentration of 5×10^6 cpm/ml. After posthybridization treatment (RNAse treatment and washes in descending concentrations of sodium saline citrate (SSC), followed by alcohols), sections were exposed to Microvision-C X-ray film (Sterling Diagnostic Imaging, Newark, DE) for different periods of time to find the optimal exposure length for each probe (15 hr for ENK and 48 hr for CRH), then dipped in nuclear track emulsion (Kodak NTB-2) and exposed (ENK for 36 hr and CRH for 3 days), developed, and counterstained with thionin.

Data Analysis

Researchers who were unaware of the subjects group assignments measured levels of mRNA (mean gray levels) within the three parts of the CEA and the ventrolateral part of the ventromedial nucleus of the hypothalamus. The exposed Microvision-C X-ray films were photographed with an SC501 CCD camera (VSP Laboratories, Ann Arbor, MI) connected through a Perceptics Pixel Buffer frame grabber and *IPLab* Spectrum software (v2.51; Signal Analytics Corp., Vienna, VA), as described previously (Watts & Sanchez-Watts, 1995). The anatomical region chosen for analysis was determined on the film with careful reference to local cytoarchitectonics on the adjacent thionin-stained sections and the corresponding dipped autoradiographs. For each rat, the entire area of interest was measured on both sides of the brain. Six consecutive rostrocaudal sections, which contained the entire CEAl, were measured (in a one-in-five series of sections) on each side of the brain, to obtain neuropeptide mRNA levels in the CEAl; whereas seven and five consecutive sections were measured on each side of the brain for the CEAc and CEAm, respectively. Because no systematic differences were found between the left and right sides of the brain (or along the rostrocaudal axis), all measurements, from each side of the brain, were pooled together to obtain the mean value for each rat. As shown in Figure 3, the many CEA neurons that express the ENK gene are packed so closely together that reliable measures of mRNA content/cell (i.e., silver grain counts) could not be obtained (see Watts & Sanchez-Watts, 1995). All nomenclature was adopted from Swanson (1999).

Significance of differences between groups was determined by single-factor ANOVA tests followed by Fisher's post hoc tests for comparison with control values.

Results

Behavioral analysis shows that, on average, the conditioned fear group displayed freezing behavior more than 60% of the time during the testing period, whereas the no training group froze less than 10% of the time (see Figure 1). These results demonstrate that the experimental rats, but not control rats, associated the contextual environment with an unpleasant experience (footshock). Subjects from the training only group were not tested behaviorally because they were perfused at the time testing would have begun to provide information about possible training-induced changes in neuropeptide mRNA levels.

Anatomical analysis revealed no detectable differences in CRH mRNA levels between the three groups in parts of the CEA where this neuropeptide is expressed in measurable amounts: the CEAl, $F(2, 12) = 0.14$, $p = .86$; and CEAm, $F(2, 12) = 0.42$, $p = .66$ (see Figure 2). In contrast (Figures 3 and 4), ENK mRNA levels were increased specifically in two CEA regions of the conditioned fear group, as compared with either control group. An ANOVA revealed significant differences among the three groups for ENK mRNA levels in the CEAl, $F(2, 12) = 7.10$, $p < .01$; and CEAc, $F(2, 12) = 7.45$, $p < .01$. Post hoc tests (Fisher) indicated that in both the CEAl and the CEAc, the conditioned fear group differed from the no training ($p < .05$) and training only ($p < .01$) groups, whereas no differences ($p > .05$) were found between the two control groups (the no training and training only groups).

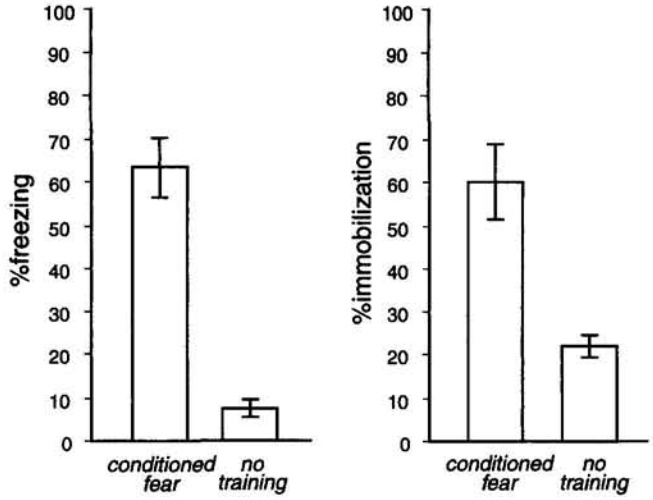

Figure 1. On Day 4 of the experimental protocol, rats' behavior during the 30-min test period was measured as percentage of time freezing (left) and percentage of time immobile (right). Rats in the conditioned fear group displayed robust freezing behavior (> 60% of the time), whereas those in the control (no training) group spent less than 10% of the time displaying this behavior. Lack of movement (immobilization) is a less sensitive measure of conditioned fear because rats in the control group did not move, especially in the second half of the testing period, for reasons other than expression of freezing behavior (e.g., they may have been resting or sleeping). Freezing is expressed as a mean (± *SEM*) percentage of total observations during the 30-min test period; immobilization is expressed as a mean (± *SEM*) percentage of total behavior during the 30-min test period. (n = 9 for all groups.)

The increase in ENK mRNA levels observed in the conditioned fear group is region-specific because significant changes were not found in the CEAm, $F(2, 12) = 3.13$, $p = .08$, or in the ventrolateral ventromedial hypothalamic nucleus (not shown), $F(2, 12) = 1.24$, $p = .32$, a cell group that has been implicated in reproductive behavior (see Risold, Thompson, & Swanson, 1997). The importance of measuring separate CEA parts is underscored

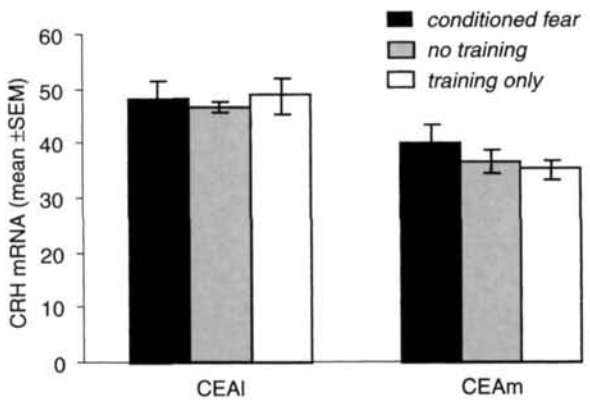

Figure 2. No significant differences were found in corticotropin releasing hormone (CRH) mRNA levels between the three groups of rats in the central nucleus of the amygdala, lateral (CEAl; left) or medial CEA (CEAm; right) part. Neurons in the capsular CEA do not express detectable amounts of CRH mRNA. (n = 5 for all groups.)

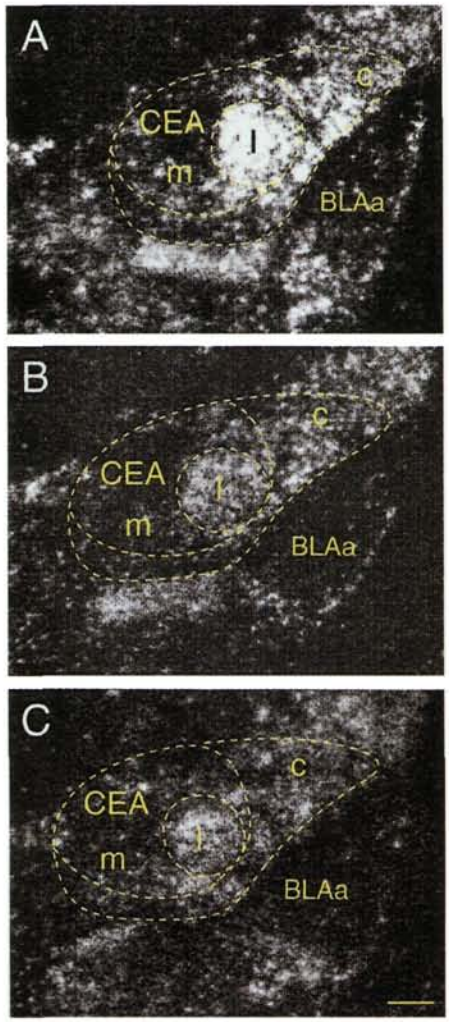

Figure 3. Darkfield photomicrographs of enkephalin mRNA hybridization in and around the central nucleus of the amygdala (CEA) of the conditioned fear (A), no training (B), and training only (C) groups. c = capsular part, l = lateral part, m = medial part; BLAa = anterior basolateral amygdalar nucleus. Right side of transverse sections (medial to the left, dorsal to the top); scale bar = 250 μm.

by the observation that when data from the three parts of the CEA were pooled, no significant differences between control and experimental groups were observed for ENK mRNA.

Discussion

The major conclusion to be drawn from our results is that ENK mRNA levels increase selectively in CEAl and CEAc neurons when rats are placed in an environment they have learned to associate with an unpleasant experience; that is, when they express a conditioned fear response.

At least two different mechanisms could be responsible for the conditioned changes in central nucleus ENK mRNA levels observed in the present study. First, it is possible that ENK in the CEA is involved in some aspects of the learning and/or memory of conditioned fear. However, the role of the amygdala in learning

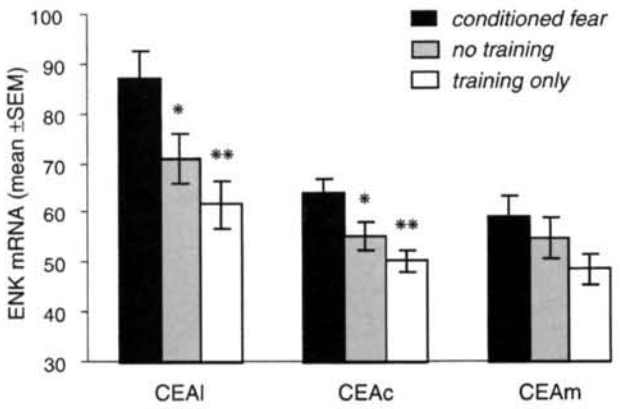

Figure 4. Conditioned stimulus significantly increases enkephalin (ENK) mRNA levels in the experimental group (conditioned fear) compared with the control groups (no training; training only) in the central nucleus of the amygdala, lateral (CEAl), and the capsular CEA (CEAc) but not in the medial CEA (CEAm). No difference was found between the two control groups. ($n = 5$ for all groups.) * $p < .05$. ** $p < .01$.

and memory is controversial. One interpretation is that the learning and memory of conditioned emotional responses occurs within the amygdala itself (Fanselow & LeDoux, 1999), whereas others argue that the amygdala influences (or is influenced by) other brain regions where these processes actually take place (Cahill, Weinberger, Roozendaal, & McGaugh, 1999). In either case, the basolateral amygdala is somehow involved in learning and memory mechanisms, which may require mRNA synthesis (Bailey, Kim, Sun, Thompson, & Helmstetter, 1999), whereas the CEA, which receives a direct input from the basolateral amygdala, is involved in at least the expression of learned fear responses.

Second, it is also possible that conditioned changes in central nucleus ENK mRNA levels are associated specifically with the expression, and not the learning and memory, of conditioned fear. This would imply that ENK responses are not critical for learned fear and presumably could be produced by the expression of fear induced by any source. However, it is not clear at the present time whether the CEA is part of the circuitry necessary for the expression of innate fear.

Within this context, it is important to mention that in our study, learned fear was inferred from measurements of its behavioral expression (freezing); and it is impossible to separate the effects of the two on ENK mRNA levels in the CEA. Thus, one could even speculate that it was not the "state of fear" but a difference in motor activity between conditioned rats (exhibiting freezing) and control rats (not exhibiting freezing) that was associated with changes in ENK mRNA levels. One approach to resolving this issue would be to examine ENK mRNA levels in conditioned subjects with ventral periaqueductal gray lesions, which would specifically prevent the freezing response (LeDoux, Iwata, Cicchetti, & Reis, 1988).

Clearly, future experiments are needed to clarify the mechanisms responsible for the conditioned changes in the central nucleus ENK mRNA levels observed in the present study, and to determine whether mechanisms underlying conditioned mRNA levels reside in CEA neurons or in neurons that project to the CEA and induce changes in mRNA.

The fact that we did not observe changes in CRH mRNA under the conditioning paradigm used here might appear surprising given a large body of evidence implicating this neuropeptide in behavioral aspects of stress, anxiety, and fear (see introduction section). However, we observed no obvious changes in mRNA after training with three weak footshocks on 2 successive days; training with more footshocks, or with more intense footshocks, might result in altered levels of CRH mRNA when the rats are exposed to the conditioning stimulus. In any event, changes in neurotransmitter/neuromodulator levels certainly are not requisite for involvement in the function of a neural circuit.

What are the axonal terminal fields of neurons in the CEAc and CEAl, where conditioned changes in ENK mRNA levels were observed? Although the distribution of CEAc outputs remains to be determined systematically, a recent *Phaseolus vulgaris leukoagglutinin* (PHAL) analysis (Petrovich & Swanson, 1997) showed that major projections of the CEAl are quite restricted to the CEAm, the BST (oval and fusiform nuclei and anterolateral area), and the parabrachial nucleus. Furthermore, combined retrograde tracer/histochemical studies indicate that enkephalinergic neurons in the CEA do not project to the parabrachial nucleus (Moga & Gray, 1985; Veening, Swanson, & Sawchenko, 1984), whereas there are enkephalinergic terminal fields in the CEAm (Veening, Swanson, & Sawchenko) and BST (Woodhams, Roberts, Polak, & Crow, 1983); and opiate receptors are expressed in both (Mansour, Fox, Akil, & Watson, 1995). Thus, conditioned changes in CEAl ENK levels could influence neuronal responses in the CEAm and/or BST.

In light of recent suggestions that the CEA is a visceromotor region of the caudal striatum (Swanson & Petrovich, 1998), a possible enkephalinergic input to the CEAm from the CEAl is intriguing because local axon collaterals of dorsal striatal enkephalinergic projection neurons apparently dampen activation of other dorsal striatal projection neurons (Steiner & Gerfen, 1998). Such peptidergic modulation would effectively result in disinhibition because enkephalinergic neurons in the dorsal striatum, as in the CEA, also contain GABA as the "classical" inhibitory neurotransmitter (Petrovich & Swanson, 1997; Steiner & Gerfen, 1998). Such a mechanism could act as a "gain control" on the expression of conditioned emotional responses (Petrovich & Swanson, 1997). The functional significance of CEAl projections to the BST is less clear. However, amygdalar modulatory effects on memory involve GABAergic and opioid peptidergic mechanisms and are exerted via projections through the stria terminalis (Liang, McGaugh, & Yao, 1990; McGaugh, Cahill, & Roozendaal, 1996), many of which end in the BST. Recent PHAL studies of the oval and fusiform parts of the BST, which receive a dense input from the CEAl and contain abundant CRH neurons (Ju, Swanson, & Simerly, 1989; Petrovich & Swanson, 1997), indicate that they preferentially and densely innervate visceromotor-related cell groups in the hypothalamus and lower brainstem (Dong, Petrovich, & Swanson, 1999).

Although our results demonstrate that levels of mRNA for a neurotransmitter/neuromodulator can be associatively conditioned in neurons of a circuit that controls the expression of mammalian learned fear responses, it remains to be determined whether increased ENK mRNA levels are translated into increased ENK peptide levels and increased synaptic release in the CEAm and/or BST. In addition, at the systems level, our results also point to the

need for more neuroanatomical work to characterize the differential projections of GABAergic neurons in the CEAl (and CEAc) that also express either ENK or CRH. It is known that separate neuron populations in the CEA express these two peptides (Veinante, Stoeckel, & Freund-Mercier, 1997); and it has been shown, for example, that CRH-expressing neurons project to the parabrachial nucleus, whereas ENK-expressing neurons do not (Moga & Gray, 1985; Veening, Swanson, & Sawchenko, 1984). However, both CRH and ENK-expressing neurons project to the BST, although it is not clear whether they innervate the same parts (Arluison et al., 1994; Sakanaka, Shibasaki, & Lederis, 1986). It is now important to determine exactly which projections from the CEA are involved in modulating various components of conditioned fear responses including freezing, changes in heart and respiration rates, and analgesia (for reviews, see Davis, 1992; Fanselow, 1994; LeDoux, 1995), and how neuropeptides modulate those responses.

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