Neural correlates of competing fear behaviors evoked by an innately aversive stimulus

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Summary

Environment and experience influence defensive behaviors, but the neural circuits mediating such effects are not well understood. We describe a new experimental model in which either flight or freezing reactions can be elicited from mice by an innately aversive ultrasound. Flight and freezing are negatively correlated, suggesting a competition between fear motor systems. An unfamiliar environment or a prior aversive event, moreover, can alter the balance between these behaviors. To identify potential circuits controlling this competition, global activity patterns in the whole brain were surveyed in an unbiased manner by c-fos in situ hybridization, using novel experimental and analytical methods. Mice predominantly displaying freezing behavior had preferential neural activity in the lateral septum ventral and in several medial and periventricular hypothalamic nuclei, while mice predominantly displaying flight had more activity in cortical, amygdalar and striatal motor areas, the dorsolateral posterior zone of the hypothalamus and the vertical limb of the diagonal band. These complementary patterns of c-fos induction, taken together with known connections between these structures, suggest ways in which the brain may mediate the balance between these opponent defensive behaviors.
Introduction

Studies of defensive behaviors in rodents provide useful paradigms to understand how the environment influences the type of motor response evoked by aversive or fearful stimuli. In rodents, flight behaviors are predominantly observed in situations where a threat is proximal or when escape routes are available, while freezing most often occurs where threat is distal or inescapable (Blanchard et al., 1989; Blanchard et al., 2001). Most prior work on the neural circuitry of fear has focused on learned (conditioned) fear (Maren and Fanselow, 1996; Le Doux, 1995). Such work has the advantage that fear reactions can be elicited by a well-defined unimodal stimulus, such as an auditory cue. Therefore comparisons of brain activity evoked by the same stimulus before and after conditioning are possible. However, the predominant behavioral response to such conditioned stimuli (CS) is freezing. Both flight and freezing behaviors can be elicited from rodents exposed to a natural predator. However, these models usually involve complex, polymodal stimuli that are difficult to standardize or to quantify, variable experimental conditions and animal handling (Canteras et al., 1997; Dielenberg et al., 2001). Therefore, there have been relatively few brain imaging studies with innate fear stimuli.

We present here a new experimental model in which freezing and/or flight behaviors can be consistently evoked from inbred mice on first presentation by an innately aversive, well-defined auditory stimulus. The balance between these two behaviors can, moreover, be altered in a predictable manner by simple environmental manipulations. Naïve mice exposed to an ultrasonic stimulus in their home cage predominantly display flight, and freeze very little. By contrast, mice placed in an unfamiliar environment, or treated with foot-shocks the previous day, primarily display freezing and less flight. We find that occurrences of flight and freezing, which can in principle both be exhibited by an individual animal during different intervals of the same testing period, are negatively correlated. This observation suggests the existence of competing motor systems underlying these alternative defensive motor responses.
To elucidate neural correlates of this behavioral switch, we have used \textit{c-fos} mRNA expression to provide a global map of neural activity, with single-cell resolution, in the brains of naïve and shock-sensitized animals responding to the aversive ultrasonic stimulus. Two important features have been incorporated to facilitate the interpretation of the \textit{c-fos} mapping data. First, the aversive stimulus was delivered in animals’ home-cages, to avoid the influence of animal handling on \textit{c-fos} expression patterns. Second, the stimulus was of a single sensory modality, to permit precise control over stimulus parameters. In this way, the observed neural activity patterns predominantly reflect stimulus/response relationships, rather than stress or novelty imposed by the testing environment.

We have also developed a novel analytic approach to measure the density of \textit{c-fos}$^+$ cells in relevant regions across the entire brain. Cells expressing \textit{c-fos} mRNA are revealed by non-isotopic \textit{in situ} hybridization on thick (120 µm) floating sections, permitting analysis of virtually the entire brain with a manageable number of sections. Two methods have been developed to analyze these data. A computerized macroanalysis technique is first used to scan the whole brain to detect potential areas of differential \textit{c-fos} activity between animals under the two conditions. Subsequently, the densities of \textit{c-fos}$^+$ cells in these regions are rigorously quantified using design-based stereology, a method that avoids common histological biases (Geuna, 2000; Howard and Rose, 1998; Mayhew and Gundersen, 1996). This approach has allowed the analysis of over 70 different brain structures, and identified among them different regions that show preferential \textit{c-fos} mRNA expression under conditions of either flight or freezing. These results have been used, in conjunction with known connectional and functional data relevant to these regions, to construct a heuristic circuit that may control the switch between competing motile and immobile defensive behaviors.
**Experimental procedures**

Male C57Bl6/N mice from Harlan Sprague-Dawley (San Diego), aged between 6-12 weeks, were individually housed and maintained on a 12-hr light-dark cycle with free access to food and water. All mice were single-housed 2-4 days before any experimental procedure. On the first day, two groups of mice were sensitized using 30 foot-shocks (0.5 mA, 6s, with an average of 1 min intertrial intervals) and were subsequently placed back into their home cages. The behavioral apparatus (Coulbourn.com) used for the sensitization session consisted of four identical chambers (175 cm³) situated in a cabinet located in a dimly lit and isolated room. Foot-shocks were delivered through rods wired to a shock generator and a scrambler. The second day, all groups were tested for their innate fear reactions to a train of ultrasound stimuli (100 ms frequency sweeps between 17-20 kHz, 85 dB, alternately ON 2s then OFF 2s for 1 min following a 3-min baseline period). Flight behavior triggered during the ON periods is defined as an event of running from one side of the cage to the other followed by behavioral arrest, while the freezing behavior sampled every 4 s during the OFF period is defined as complete immobility except for respiration. Cages (165 cm wide, 275 cm long, 155 cm high) were placed into a plexiglass container, with a speaker (Bullet Horn Tweeter, Optimus, Tandy) attached to a lid, providing additional sound insulation, inside an isolated room different from the one in the foot-shocks were delivered. The ultrasound stimulus was produced using a function generator (Telulex, Model SG-100/A, korins.com). A portable sound pressure meter was calibrated using a microphone sensitive to 20 kHz ultrasounds and a computer-based spectrograph.

For the feeding-suppression test of anxiety, different groups of mice were single-housed and foot-shock sensitization was done as described above. All mice were deprived of their regular food 24 hours before the test and then brought to the standard testing environment, except that they were not exposed to any acoustic stimulus. The latency to feed was measured with a video camera from
the time the pellets were placed in the center of the cage until the animal began to feed.

For \textit{c-fos} analysis naïve and shock-sensitized mice were sacrificed 30 min after delivery of the ultrasound stimulus in their home cages. For baseline \textit{c-fos} expression analysis, we sacrificed naïve and sensitized mice taken directly from their home cages, without ultrasound exposure. Their brains were collected, cut in 3-4 mm coronal slabs using a block and fixed overnight in 4 % paraformaldehyde. In brief, a free-floating-section \textit{in situ} hybridization was performed as follows: First, 120 \textmu m-thick coronal sections were made from the tissue slabs using a vibratome. Then, the sections were gently digested for 30 min using proteinase K, fixed with 4 % paraformaldehyde and hybridized at 60 °C overnight with a cRNA digoxygenin-labeled probe specifically binding \textit{c-fos} mRNA. The non-hybridized probe was washed off at 60 °C and digested with RNAse A at 37 °C for 30 min. Immunohistochemistry was performed using anti-digoxygenin conjugated with alkaline phosphatase (Roche, IN). Development was performed with an alkaline phosphatase substrate generating a blue product and the sections were counterstained with Nuclear Fast Red (Vector, CA). Preliminary tests confirmed that all the reagents adequately penetrated inside the 120 \textmu m-thick sections.

For the macroanalysis process, whole-section mosaics of high magnification photomicrographs were assembled using a computerized stage and a CCD camera using the Neurolucida software (microbrightfield.com). Cell profiles from the most densely stained cells were then thresholded (on the blue channel) and transformed into vectors (yellow markers) to provide a preliminary population estimate for stereological measurements, and to identify regions with possible changes between groups. The images from 3 sections were overlaid to make 360 \textmu m virtual sections and better view the cell distribution in regions of low cell density. These virtual sections were not necessary in cases when the cell distribution was already obvious from a microphotograph.
For stereology, the outlines of local brain areas to be counted, derived from a standard mouse brain atlas (Paxinos and Franklin, 2001), were digitally fitted at low magnification (4x) on the original specimens and manually corrected for shrinkage and section distortion. Cell counts were performed at 40x magnification using the Optical Fractionator (50 µm x 50 µm x 60 µm counting bricks randomly sampled) automatically operated by the StereoInvestigator software (microbrightfield.com). The identity of each brain area was confirmed using strict distance measurements from anatomical cues in all directions that were clearly visible by virtue of the Nuclear Fast Red counterstaining on thick sections. Importantly, brain areas were never defined by the c-fos staining. Volumes were measured by Planimetry and coefficients of variation of the sampling distribution for cell density estimates were always smaller or equal to 5% (Schaeffer test). Three different animals were analyzed from each group (naïve and sensitized) and the Student’s t-test was used to evaluate the statistical differences in between groups. Detailed information about the in situ hybridization procedure, the stereological quantification and the macroanalysis processes can be found in the Appendix A.
Results

Identification of innately aversive unimodal stimuli in mice

We initially undertook to identify unimodal sensory stimuli that could reliably elicit robust defensive behaviors in inbred strains of laboratory mice upon first presentation. Predator odors produced risk-assessment behaviors such as stretch-attend and inhibition of grooming, but intense fear reactions such as flight or freezing were not observed in C57Bl6/N mice. Moreover, the intensity and duration of these olfactory stimuli were difficult to control and to normalize to neutral control stimuli. Subsequently, we experimented with ultrasonic tones in the ~20 kHz range because rats are known to emit alarm vocalizations in this range and to respond to such auditory stimuli with defensive behaviors (Blanchard et al., 1992; Cuomo et al., 1992; Beckett et al., 1996).

Preliminary studies indicated that explosive defensive responses could be reliably elicited in C57Bl6/N mice using a continuous 20 kHz square-pulse signal, and that this stimulus was more effective than a sine-wave signal. Nevertheless, we chose to use sine-wave signals because ordinary sound pressure meters cannot detect the contribution to overall dB levels of the harmonics generated by square-pulse signals. We found that patterning the ultrasound stimulus, by introducing fast frequency sweeps between 17-20 kHz, was more effective in producing fear reactions than were continuous tones at any given frequency within this range. Our optimized stimulus consisted of a train of 100 msec 17-20 kHz frequency sweeps delivered at 85 dB, alternately ON for 2 sec then OFF for 2 sec, for 1 minute.

The most frequent reactions elicited by this ultrasound from naïve mice in their home cages were flight (which is defined as an event of running from one side of the cage to the other followed by arrest), rapid breathing and circa-strike behaviors. Occasionally we also observed tail rattling and jumping. Importantly, none of these behaviors occurred during baseline observations. In contrast, behaviors such as grooming and rearing, which frequently occurred during baseline, were markedly decreased during presentation of the stimulus. In
addition to these changes in motor behavior, the ultrasonic stimulus elicited a rise in serum corticosterone and heart rate (measured by radio-immuno assay and telemetry; data not shown). These behavioral, endocrine and autonomic responses support the inference that the ultrasonic stimulus elicits strong and reliable fear reactions in laboratory mice.

Anxiogenic manipulations cause changes in ultrasound-evoked defense behaviors

When mice in their home cage were exposed to the ultrasonic stimulus, frequent flight responses (6-8 events / min) were observed during the ON periods (Fig. 1A, home cage, blue bar) but there were very few bouts of freezing (measured during the OFF periods as complete immobility except for respiration; Fig. 1B). By contrast, when animals were exposed to the ultrasound in an unfamiliar cage, the frequency of flight events was reduced to about half that of home cage mice (Fig. 1A, new cage, blue bar) and significantly more episodes of freezing were observed (Fig. 1B, new cage, blue bar; cf. home cage). A similar reduction of flight and an increase in freezing were observed in animals tested in their home cage after being exposed 24 hrs earlier to a series of foot-shocks (30 x 6 sec shocks of 0.5 mA; Fig. 1A, B; home cage, red bar, cf. blue bars). When the foot-shocked animals were tested in a new cage, flight responses were almost completely suppressed and freezing was maximal (Fig. 1A, B; new cage; red bars). Freezing events were seldom observed (6.7 +/- 3.4 % of time) in shock-sensitized mice during exposure to a neutral auditory tone (2 kHz sine-wave; 1 min; 85 dB) compared to a group of mice exposed to the aversive ultrasound at the same sound pressure (54.6 +/- 10.6 % of time).

Flight was triggered primarily during the stimulus ON period, while freezing occurred during the OFF period. Therefore we measured flight responses during the ON period and freezing responses during the OFF period. Hence, we could theoretically measure maximal flight and freezing responses from an individual animal during a given experiment. Nevertheless, when the
frequency of flight and freezing to the stimulus for individual mice (n=38) was plotted for all animals tested (collapsing both sensitization and new cage factors), a significant negative correlation between the two behaviors was observed (Fig. 1C; P<0.001, r²=0.6). This strong negative correlation is not simply a trivial consequence of the fact that flight and freezing are physically mutually exclusive. Bouts of freezing occupied limited intervals during the testing session (they rarely occurred during the ON period). Therefore there was ample of time for animals that freeze to also display flight behavior. Conversely, episodes of flight are typically interrupted by pauses (during the stimulus OFF period) during which the animal can freeze. Indeed, many individual animals displayed episodes of both flight and freezing during the same testing period (Fig. 1C). The fact that flight occurrences nevertheless became much less frequent as the frequency of freezing increased therefore suggests that these behaviors are in competition with one another for reasons other than simple motor incompatibility.

One explanation for the effect of a novel environment and/or prior foot-shock sensitization on the balance between flight and freezing responses is that these two manipulations increase anxiety. To test this, we used an animal model of anxiety called the feeding suppression task (Gross et al., 2000; Bodnoff et al., 1988). In this paradigm, mice are presented with food in a given environment 24 hours after being food-deprived. In both rats and mice, the latency to feed in this environment is greater with increasing anxiety-like states. Consistent with the idea that the novel environment increased anxiety, food-deprived mice transferred to a new cage had a significantly greater latency to feed compared to home cage controls (home-cage = 110±19 sec, new-cage = 189± 18 sec, n=8, P = 0.01 using the Student’s t-test). Combining the new-cage and sensitization factors had an even larger effect, as foot-shocked mice placed in a new cage 24 hrs later had a greater latency to feed compared to naive animals in the new cage (naive = 173± 20 sec, sensitized = 230± 14 sec, n=6-7, P < 0.05). These data indicate that there is a correlation between manipulations that decrease
flight and increase freezing, and those that increase anxiety as determined by an independent behavioral test. Consistent with this interpretation, earlier studies have shown that shock sensitization increases anxiety in other behavioral tests, such as the elevated plus-maze (Steenbergen et al., 1990).

We have also assessed ultrasound induced-defense following a long-term treatment with the anxiolytic drug alprazolam (1 mg/kg/day i.p. for 10 days; dissolved in 0.9 % saline with few drops of Tween 80; tested in home-cage 1 hour after the last injection). After a control intraperitoneal saline injection, (which is a stressful manipulation like shock sensitization or placement in a new cage), mice typically display some freezing and little flight in reaction to the aversive ultrasound. However, freezing was significantly reduced in mice injected with alprazolam compared to controls (saline = 25.7±6.8 % of time spent freezing vs. alprazolam = 6.7±4.2 % of time; P < 0.05; n = 6). In contrast, the frequency of flight tended to change in the opposite direction (saline = 2.8±1.1 flight events vs. alprazolam = 6.8±2 events; P = 0.11; n = 6). These data indicate that reducing anxiety increases USS-induced flight while decreasing freezing, supporting the idea that anxiety conversely increases freezing and decreases flight.

**Functional imaging using quantitative analysis of c-fos mRNA-expressing cells**

We next sought to map global patterns of neuronal activity in the brains of naïve (N) and sensitized (S) mice following their first exposure to the ultrasonic stimulus. To do this we used a non-isotopic in situ hybridization method with single-cell resolution to examine expression of c-fos mRNA, the best characterized marker of neuronal metabolic activity (Herdegen and Leah, 1998). We chose to examine c-fos mRNA rather than protein because it is a more proximate indicator of c-fos expression. Induction of c-fos transcription in neurons occurs within 2-5 minutes in response to depolarization-induced calcium entry (Finkbeiner and Greenberg, 1998), and peaks after ~30 minutes
(Greenberg and Ziff, 1984), the time at which ultrasound-exposed animals were sacrificed for analysis. C-fos expression can also be induced by factors that elevate intracellular cAMP expression, such as monoamines or neuropeptides, as well as by stress hormones (glucocorticoids; reviewed in Herdegen and Leah, 1998). However, glucocorticoids are not the principal influence on c-fos expression in tasks involving stress (Helmreich et al., 1996; Anokhin et al., 1991).

Exposure of mice to the ultrasonic stimulus in their home cages was a key feature of our paradigm because the handling of animals necessary to transfer them to a new testing cage induces a substantial number of c-fos$^+$ cells. By testing animals in their home cage, background levels of c-fos mRNA expression in control animals were kept very low and the ultrasound was therefore the main external stimulus affecting c-fos expression (Fig. 2A). A reflection of the specificity of c-fos mRNA induction under these conditions is seen in the inferior colliculus (IC), which contains a tonotopic map of frequency-responsive neurons (Ryan et al., 1988; Ehret and Fischer, 1991). Exposure to the ultrasonic stimulus induced massive c-fos mRNA expression in a zone of the IC corresponding to the region sensitive to frequencies in the 17-20 kHz range (Fig. 2B, arrow). That the expression of c-fos reflects motor output as well as sensory input is illustrated by its expression in the forelimb region of the motor cortex (Li and Waters, 1991), where there was a higher level of expression in N mice (which exhibit primarily flight) than in S mice (which exhibit primarily freezing; Fig. 2C; see below for quantification). As expected (Castro-Alamancos et al., 1992), there was relatively little c-fos expression in regions of motor cortex not involved in the escape response (see nose region in Supplemental Figure S7A; Supplemental Figures S1-S8 can be found in Appendix B).

To accurately quantify the induction of c-fos mRNA, we measured the density of c-fos$^+$ cells in various brain regions using unbiased stereology with the Optical Fractionator. This method is considered the most accurate way to
estimate cell densities in a given volume of brain tissue using a random sampling method. Surprisingly, however, it had not previously been used to map *c-fos* expression for functional imaging studies. By performing non-isotopic *in situ* hybridization on free-floating thick (120 µm) sections, we could obtain a large “counting brick” (60 µm depth) with sufficient cellular resolution to estimate cell densities by optical dissection (Fig. 2G). To control for any differential shrinkage between sections, the volumes of brain regions sampled were calculated from section thickness and area measurements using well-defined anatomical landmarks. For example, in the forelimb motor cortex we digitally fitted the anatomical boundaries (M1 and M2 located between AP = - 0.5 to - 1.9 mm) from a digital brain atlas (Paxinos and Franklin, 2001) using landmarks such as the interhemispheric fissure and the corpus callosum. The measured volume for this entire region did not differ between groups (N = 0.94±0.06 mm³; S = 0.95±0.03 mm³; in no case did we observe significant differences in volumes between groups for the regions sampled, and therefore the volumes are presented as a single value derived from both groups), but the density of *c-fos*⁺ cells was found to be higher in N than in S mice (Fig. 2E), consistent with the higher level of motor activity in the former group.

Because it was impractical to perform stereological cell counts through all brain regions in each of the 3 animals analyzed for each condition, we developed a method, called Macroanalysis, to initially survey large regions of the brain to identify potential areas of differential *c-fos* expression and to obtain preliminary estimates of cell density. Briefly, this method uses the Neurolucida software to assemble low-magnification views of entire coronal sections with single-cell resolution, by assembling a mosaic or “virtual slice” from a series of contiguous high-magnification fields (Fig. 2D, top panels). The positions of the most strongly stained *c-fos*⁺ cells in each section are then extracted using a thresholding program that identifies cell profiles based on their color, dimension and shape (Fig. 2D, bottom panel). Data from 3 consecutive virtual slices (360 µm) can then be overlaid to clearly reveal the cell profiles distribution within a
given region (such as the functional columns within the periaqueductal gray; PAG; Fig. 2F). Automated counting of cell profiles could be performed within coarsely bounded regions for an animal of each group. Regions showing potential differences were then further analyzed by stereological counting in more tightly bounded regions.

**Neural correlates of the switch between flight and freezing behaviors**

For *c-fos* analysis, three naïve (N) animals and three animals sensitized by foot-shock 24 hrs previously (S) were exposed to the ultrasonic stimulus in their home cages. The behavioral data for these animals confirmed our previous findings: home-cage N mice; flight = 7.0±1.2 events, freezing = 4.3±4.3 % of time, home-cage S mice; flight = 1.7±0.9 events, freezing = 60±14 % of time. The frequency of both flight and freezing were significantly different between the two groups (P < 0.05, using the Student's t-test). The brain of each of these animals was cut into three large slabs (rostral, intermediate, caudal) and each of these slabs was in turn sectioned at 120 µm using a vibratome for *in situ* hybridization.

Approximately 70 different areas or nuclei were examined. (Table 1 in the appendix B summarizes the intensity of staining observed in various regions of the brain in N and S mice exposed to the ultrasound stimulus.) Exposure to the aversive ultrasound produced massive increases of *c-fos*+ cells in cortical, amygdalar, septo-hippocampal and diencephalic areas, but less so in the basal ganglia and the brainstem. About 80% of the regions examined did not show signs of differential activity. Approximately 14 regions were identified that showed higher levels of *c-fos* expression in N mice than in S mice, while only half as many showed higher activity in S mice (Fig. 7). Strikingly, all but one of the latter regions are clustered in the hypothalamus. About 8 areas showed strong activity in both N and S mice. Below we systematically compare the detailed patterns of activity between N and S mice, beginning with the mesencephalon and ending with the cortex.
**Mesencephalon**

The midbrain periaqueductal gray (PAG) is thought to serve as a final common pathway for the initiation of flight and freezing behaviors elicited by fearful stimuli (Bandler et al., 2000). Consistent with this, the PAG was strongly activated in mice exposed to the ultrasonic stimulus (Fig. 2F). However, there were no significant group differences in any region of this mesencephalic structure as assessed by stereological counting (Figs. S1, S6A). There were also no apparent differences at the level of the inferior and superior colliculi (Fig. 2B) (Fig. S3), in the median and dorsal raphe nuclei (containing serotonin cell bodies; (Fig. S2A, S6A) and in the locus coeruleus (containing noradrenaline cell bodies; (Fig. S2C) although staining was strong in these areas. There were very few c-fos+ cells in the ventral tegmental area or the substantia nigra (Fig. S2B). The only area of the midbrain where we did observe a difference between N and S mice was at the lateral edges of the ventral PAG, at the junction between the pedunculopontine tegmentum (PPTg) and the cuneiform nucleus (Cnf) (Fig. 2A, arrow). Stereological cell counting in this area was performed within an arbitrarily defined octagonal region (Fig. S6B), because this cluster of staining did not fit any known boundaries in the atlas. These measurements confirmed a significantly higher (55%) density of c-fos cells within this defined boundary, in N vs. S mice. (N = 2594±312 cells/mm³; S = 1795±23 cells/mm³; P < 0.05; between AP = - 4.4 to – 5.1 mm; vol. = 0.182±0.003 mm³).

**Diencephalon**

In contrast to the mesencephalon, the hypothalamus displayed numerous areas of differential activity that correlated with differences in either flight or freezing responses. In general, the lateral and posterior hypothalamus showed preferential activity in N mice, while many periventricular and medial hypothalamic nuclei showed preferential activity in S mice (Fig. 3). The hypothalamus has been subdivided into a series of four zones from rostral to caudal: the anterior, preoptic, tuberal and mamillary zones. In the mamillary
zone, the lateral portion of the posterior hypothalamus (PH) was strongly activated in both groups, but was somewhat higher (47%) in N mice (Fig. 3A, arrow; 3B). In the tuberal zone, there was strong labeling in both groups in the dorsomedial hypothalamus (DMH) and in the dorsomedial portion of the ventromedial hypothalamic nucleus (VMHdm; Fig. 3C), two areas implicated in defensive behaviors (Graeff, 1990; Canteras, 2002). By contrast, the ventrolateral portion of this nucleus (VMHvl), which has been implicated in reproductive behaviors, was weakly labeled (Fig. 3C). The lateral hypothalamus (LH), also in the tuberal zone, had a significantly higher level of activity in N mice (80%; p<0.05; Fig. 3D), mostly in the dorsal aspect that mediates aversion (Fig. S4A) (Cazala and Schmitt, 1987).

A number of other hypothalamic areas showed preferential activity, conversely, in S mice (Fig. 3G-L). In the anterior zone, the paraventricular nucleus (Pa) showed an overall increase of 48% in S mice, but the difference was more apparent in the dorsal portion of the nucleus (Fig. 3E, arrow; F). There was no difference between N and S mice in the anterior hypothalamic nuclei, although it was activated in both cases (Fig. 3E). In the preoptic hypothalamic zone, there were two areas that showed strongly preferential c-fos expression in S mice: the medial preoptic nucleus and the anterodorsal preoptic nucleus (MPO, ADP; Fig. 3G-J). In the ADP, there was a nearly 3-fold activity increase in S relative to N mice (Fig. 3I-J), while in the MPO the level of activity was doubled (Fig. 3G-H). This latter result was somewhat surprising, as the MPO is usually considered to be part of the medial hypothalamic behavioral control column for reproductive behaviors (Pfaus et al., 1993). The c-fos+ cells were particularly dense around the midline and also at the ventral junction with the medial preoptic area (MPA; Fig. 3G), but there was a relative gap in c-fos labeling in the more lateral domain of the MPO (Fig. 3G). In other studies, this domain has been shown to strongly express c-fos in animals performing reproductive behaviors (Pfaus et al., 1993). These observations suggest that the MPO may be subdivided into regions involved in defense and reproduction.
In the premammillary zone, there was strong but equivalent \textit{c-fos} expression in both groups in the dorsal premammillary nucleus (PMd; Fig. 3K), a structure required for both freezing and flight responses to a predator (Canteras et al., 1997). In this same region, S mice displayed higher activity (61\%) in a domain at the junction of the premammillary ventral and the arcuate nuclei (PMv, Arc; Fig. 3K, arrow; 3L). The central region of the PMv, previously implicated in sexual behaviors (Yokosuka et al., 1999), had little staining. Finally, although we found a substantial amount of \textit{c-fos} activity in other regions of the diencephalon, there were no apparent differences between N and S mice in any portion of the epithalamus, the subthalamus or the thalamus (Fig. S4B).

\textbf{Septal and hippocampal areas}

The most striking difference between S and N mice was in the lateral septum ventral (LSV). As shown in Fig 4, there was a major increase (+ 173\%) in the density of positive cells in the LSV of S mice (Fig. 4A, 4D). In fact, the LSV was one of the few regions where a difference in \textit{c-fos} expression between N and S mice was evident by visual inspection of the sections, without the need for Macroanalysis. In the most caudal portion of the septum, the stained cells were highly clustered within the boundary of the LSV (Fig. 4A, arrows). At more rostral levels, \textit{c-fos} cells in S mice clustered in the LSV along the edge of the lateral ventricle and there was also a substantial amount of staining in the adjacent lateral septum intermediate (LSI). However, the density of positive cells in the LSI of S mice was not significantly different from that of N mice (Fig. 4E). There was relatively little activity, and no apparent difference between N and S mice, in the lateral septum dorsal (LSD). Cell density was generally low in the medial septum (MS) and the horizontal limb of the diagonal band (HDB). In sharp contrast to the LSV, the vertical limb of the diagonal band (VDB) showed a much higher (136\%) density of \textit{c-fos} cells in N than in S mice (Fig. 4B, 4C). The positive cells appeared as a well-defined cluster at the base of the septum (Fig. 4B).
The bed nucleus of the stria terminalis (BNST) is composed of multiple subnuclei, whose classification varies according to different authors (Alheid et al., 1995). The contours that were used for stereological measurements in this analysis did not follow those defined by Paxinos and Franklin (2001). We refer here to the BNSTa (anterior) as all the subdivisions of the BNST within the region bounded caudally by the posterior part of the anterior commissure and rostrally by the shell of the nucleus accumbens. There was an increased density of c-fos+ cells (73%) in the BNSTa of N compared to S mice (Fig. 4F, arrows; 4I). In contrast, the bed nucleus of the commissural component of the stria terminalis (CST, Alheid et al., 1995) did not reveal any group differences (Fig. 4G, 4J). In other areas of the BNST c-fos+ cell density was generally low and there was no evidence of differential activity. In the dorsal hippocampus, there were high densities of c-fos+ cells in CA1, moderate densities in CA2 and in CA3 and a low density in the dentate gyrus (Fig. 4H), but no apparent group differences in any of these regions.

**Amygdalar and striatal areas**

In both N and S mice, positive cells in the lateral and basolateral amygdala (La, BLA) tended to cluster in the most medial portion of these nuclei. There were no apparent group differences in these predominantly sensory regions of the amygdala (La, BLA; Fig. 5C). By contrast, in the basomedial amygdala there was a greater than 2-fold increase in the density of positive cells in N mice, and the cells tended to cluster toward the corticomedial amygdala (Fig. 5A, 5B). A similar change in N mice (+ 123%) was found in the medial amygdala anterior (MeA; Fig. 5E,5F). In the most caudal sections there was an apparent cluster of c-fos+ cells encompassing both the medial amygdala posterior ventral (MePV) and the anterior cortical nucleus (Aco) (Fig. 5D,5K), whose density was ~2-fold higher in N mice (Fig. 5I). Although the central nucleus of the amygdala (Ce) is known to be involved in the expression of conditioned freezing, there were no significant differences between N and S mice.
throughout the rostro-caudal extent of this structure (Ce; Fig. 5A, 5L). Strikingly, in no case did we identify any amygdalar regions that showed more activity in S than in N mice.

Few areas of the basal ganglia had any staining. There were hardly any c-fos+ cells in any of the pallidal areas, while activity in the caudate putamen was restricted to areas innervated by the auditory cortex (McGeorge and Faull, 1989). These included the dorsomedial portion of the rostral caudate putamen (CPu) and the posterior CPu (Fig. 5G, 5H). There are no boundaries in the mouse atlas corresponding to the dorsomedial CPu. Therefore this region was arbitrarily defined by measuring a triangular area delimited by nodes 500 µm perpendiculars to the lateral ventricle and connected to the most ventral edge of the lateral ventricle. Mice displaying predominantly flight responses had twice the density of c-fos+ cells in that area (Fig. 5M). In the posterior striatum, including the amygdalo-striatal transition area (Astr), cell density was not as high (Fig. 5A, H), but N mice had again significantly more cells than S mice (51%; Fig. 5N). A particularly striking difference was observed in the shell of the nucleus accumbens (Acb), where N mice displayed a 147% higher density of c-fos+ cells than S mice (Fig. 5J, 5O).

**Cortical areas**

The only region of differential activity among posterior cortical areas was in the retrosplenial cortex (RS; also known as the posterior cingulate cortex; Fig. 6A). This change was particularly evident in the agranular layer of RS (RSA) where cells were denser. There was a 43 % increase in activity in RSA (Fig. 6D) and there was also an apparent change in RSG (Fig. 6A). In contrast, there was no evidence of differential activity in the parietal association cortex, which is contiguous with RS in the posterior cortex, or in the temporal association cortex (Fig. S7). In the motor cortex, which is adjacent to the RS at rostral levels, there was 58% more activity in N mice (as mentioned earlier; Fig. 2C,E). More ventrally, in the limb component of the somatosensory cortex there were no
apparent changes (Fig. S7F). As expected, staining was particularly strong in the auditory cortex, but again there was no indication of differential activity (Fig. S7G). Cell density also appeared equal between groups in regions of the temporal lobe including the pyriform cortex, which had dense staining (Fig. S7D).

In area 2 of the anterior cingulate cortex (Cg2) quantification revealed an increased activity in N animals of even greater amplitude than that in the posterior cingulate cortex (Fig. 6B, 6E). The enhanced activity observed in the anterior cingulate cortex of N mice was contiguous with a similar change in the dorsal prefrontal cortex; there was a clear cluster of staining encompassing both area 1 of the anterior cingulate and the prelimbic cortices (Cg1, PrL; Fig. 6C). Both regions had more c-fos+ cells in N than S mice, but a dorsoventral trend in cell distribution was often observed such that N mice displayed more activity in Cg1 (51%) than in prelimbic (30%) cortex (Fig. 6F, 6G). Other areas of the prefrontal cortex, such as the infralimbic, the insular and the orbital cortex (IL, AI, O) did not show any signs of differential activity (Fig. S5).
**Discussion**

We have defined a novel behavioral paradigm in which defensive behaviors are reliably elicited from mice by an aversive unimodal stimulus upon first presentation. Naïve animals in their home cage predominantly display flight responses to a patterned ultrasonic stimulus, while a novel environment or prior foot-shock sensitization enhances freezing and suppresses flight. Flight and freezing behaviors are negatively correlated, suggesting the existence of opponent neural circuits mediating these motor responses. Our behavioral data suggest, moreover, that the balance between these behaviors is shifted from flight to freezing by increased stress or anxiety. As a first step towards elucidating the circuitry mediating this competition, we have compared the global patterns of *c-fos* activation in mice displaying predominantly flight (naïve; N) or freezing (sensitized; S). An analysis of several brain areas has identified subsets of regions preferentially activated in N or S animals (Fig. 7).

**Ultrasound-induced defense and its modulation**

The design of this paradigm allowed both flight and freezing behaviors to be independently performed at maximal level during the testing session. In fact, some mice displayed flight during the ON period and then freezing immediately after during the OFF period, followed again by flight and so on (Fig. 1C). However, when assessed using a large number of animals a negative correlation between flight and freezing behaviors was observed. This negative correlation is consistent with ethological observations of flight and freezing in responses to predators (Blanchard et al., 1989; Blanchard et al., 2001). We also observed an acoustic startle response to the USS in some mice. However, in contrast to flight, the acoustic startle defense reflex, which was most often observed in S mice or mice placed in a new cage, is positively correlated with freezing behavior (Leaton and Borszcz, 1985) and facilitated by prior foot-shock sensitization (Gewirtz et al., 1998). The fact that freezing is, like startle, enhanced by foot-shock sensitization suggests that these two behaviors are modulated by aversive
events in a similar manner, contrary to freezing and flight which appear as opponent defense behaviors.

Why do foot-shock sensitization or a novel environment inhibit flight and promote freezing? Our feeding suppression data, which are consistent with previous studies (Bodnoff et al., 1988; Steenbergen et al., 1990), and our anxiolytic drug administration data suggest that anxiety is an important factor. One simple interpretation of our data, therefore, is that freezing requires a higher threshold level of anticipatory fear or anxiety to be elicited by the USS. This would be consistent with Gray’s view that ‘‘‘prepared’ [or] ‘innate stimuli for fear’...require some additional source of emotional disturbance before...they elicit...a full-blooded fear reaction’” (Gray, 1971). In this view, the USS is a ‘prepared’ (innately fearful) stimulus for the expression of freezing, and becomes a releaser of this behavior in the presence of elevated anticipatory fear or anxiety. The fact that flight is, conversely, suppressed under the same conditions also fits with the idea that anticipatory anxiety suppresses panic-like behaviors, such as flight reactions (Deakin et al., 1992). Nevertheless, it should be noted that flight in response to a threat can sometimes reflect a higher state of acute fear than does freezing, as it displaces freezing when danger becomes more imminent or proximal (Blanchard et al., 1989).

The fact that foot-shocks can promote a freezing response to the USS 24 hr later suggests that this phenomenon reflects sensitization, and not classical conditioning (Gray, 1971). An alternative interpretation is that the freezing that occurs in sensitized mice is a conditioned response, that reflects prior associative learning between the foot-shocks and the context in which it was delivered, and results from a generalization of this contextual fear conditioning the next day (Fanselow, 1980). However, this interpretation seems very unlikely, as there were no common features between the training and the testing contexts. Furthermore, it does not account for the fact that a similar shift from flight to freezing is also caused simply by placing the animal in an unfamiliar cage, where mnemonic effects are excluded.
It is also worth mentioning that the present sensitization model resembles the learned helplessness (LH) model in which mice exposed to inescapable foot-shocks typically show performance deficits when later given the opportunity to learn to escape the foot-shocks (i.e. in a shuttlebox). However, the foot-shock protocol used here is milder than that required to induce LH. More importantly, what is measured in LH is not flight per se, but an operant response – the ability of the animal to learn to escape a shock. By contrast, the undirected flight assessed here appears to be an innate behavior. Nevertheless, the suppression of flight and enhancement of freezing we observe in (S) mice may involve some processes in common with LH.

Overview of the neural correlates of USS-induced flight vs. freezing

Efforts to elucidate the neuroanatomy of fear-mediated motor responses have led to the concept of a hierarchy of neural systems mediating defensive behaviors such as flight and freezing (LeDoux, 1995; Gray and McNaughton, 2000). From the lowest to the highest levels of this hierarchy are the midbrain periaqueductal gray, the hypothalamus, the amygdala, the septo-hippocampal areas and the cingulate cortex. All of these systems receive sensory information about fearful stimuli through various routes. Components of this hierarchy are believed to regulate progressively more evolved forms of defense, such that phylogenetically older neural systems generate ‘quick and dirty’ strategies dealing with imminent danger, while more evolved brain areas produce slower but more sophisticated reactions (Graeff, 1994). These multiple systems are known to interact with each other, but the neural mechanisms that mediate switches between alternative motor defensive behaviors remain to be elucidated. The goal of the present study was to observe whether there was any change in brain activity correlating with different defense behaviors elicited by the same stimulus. Our results identify several areas of the forebrain that exhibit differences in c-fos expression during flight vs. freezing behaviors. While there are no pre-existing theoretical models to provide a framework for the
interpretation of these data, an examination of the literature on the function and connectivity of these areas suggests a heuristic circuit controlling the switch between these behaviors, which makes testable predictions.

Two main features stand out in our analysis of c-fos activation patterns in the forebrains of N and S mice (summarized in Fig. 7). The first is the preferential activation in N mice of a cortico-amygdalo-striatal processing stream mediating active motor defenses (Fig. 7, blue shading). It is not surprising that these areas are less active in S mice, since these animals are less motile. The second and less expected feature is a pattern of reciprocal activation in septal and hypothalamic areas of N and S mice. In N mice, there is preferential activity in the vertical limb of the diagonal band (VDB) of the septum, and in the dorsolateral posterior zone of the hypothalamus (which projects to the VDB; Vertes et al., 1995; Fig. 8A). The VDB sends ascending excitatory projections to the retrosplenial cortex (Gonzalo-Ruiz and Morte, 2000) associated with motor programming areas, which are also more active in N mice and which project in turn back to the dorsolateral posterior hypothalamus (Floyd et al., 2001; Fig. 8A, blue arrows; Fig. S8). This apparent positive-feedback loop could potentially reinforce active motor behavior in N mice.

By contrast, in S mice there is enhanced activity in the lateral septum ventral (LSV), and in the medial periventricular zone of the hypothalamus (Fig. 8B, blunt arrow). These regions are heavily interconnected (Sakanaka et al., 1988; Jakab and Leranth, 1995; Risold and Swanson, 1997) and may also form a self-reinforcing circuit (Fig. 8C). Outputs from the lateral septum and/or medial periventricular hypothalamus could, in principle, inhibit the cortico-amygdalo-striatal motor processing stream at several levels, including the VDB and the shell of the nucleus accumbens (Acb) in the striatum (Staiger and Nurnberger, 1991; Simerly and Swanson, 1988; Fig. 8B). Importantly, the VDB in turn is thought to send inhibitory projections back to the LSV (Jakab and Leranth, 1995; Kiss et al., 1997; Staiger and Nurnberger, 1989). The reciprocal inhibition between the VDB and LSV could, therefore, comprise part of a bi-stable switch
circuit that controls flight vs. freezing behaviors. Additional evidence in support of this interpretation from prior connectional, lesioning and stimulation studies is discussed in more detail in the appendix C.

As mentioned earlier, much more is known about the circuits for learned (conditioned) fear compared to those for innate fear, because of difficulties in controlling the quality and quantity of stimuli used to induce the latter. In principle, the circuits for innate fear could be identical, partially overlapping or completely independent from those for learned fear. Some evidence suggests that innately fearful or anxiogenic stimuli may be processed by different circuits than those for conditioned fearful stimuli (Walker and Davis, 1997; Wallace and Rosen, 2001; Fendt et al., 2003). With the present paradigm, the ability to reliably elicit defensive responses from laboratory mice by a parametrically well-defined auditory stimulus on first presentation now provides an opportunity to make more direct comparisons between the circuits underlying innate and conditioned fear reactions to auditory cues.

Finally, the present studies define a novel experimental paradigm for understanding the neural basis of contextual and experiential influences on defensive reactions to an innately aversive stimulus. Our results suggest that flight and freezing can compete for the expression of fear depending on levels of anxiety present before the presentation of this stimulus. These observations in turn raise the question of where and how anxiety modifies defensive behavioral outputs to an aversive stimulus. The *c-fos* mapping data provide a heuristic circuit for the regulation of these competing behaviors by anxiety or stress, which may now be tested by systematic functional perturbation experiments. This system may also provide a useful model for understanding the neural substrates of human fear disorders, such as panic and anxiety, as well as for drugs used to treat them.
Reference list


**Figure 1**

Modulation of defense reactions to an innately aversive ultrasound (USS). The frequency of flight (A) and freezing (B) is compared for naive (N, blue bars) and sensitized (S, red bars) mice. A) S mice showed significantly less flight than N mice (ANOVA, P<0.01), as did mice exposed to the USS in a new cage (P<0.05). B) S mice showed significantly more freezing than N mice in their home cages (P<0.01), as did mice in a new cage (P=0.01). Data represent mean +/- S.E.M, n=5-7 mice. C) Correlation analysis with all parameters combined revealed a significant (ANOVA P<0.001; r²=0.6) negative correlation between the frequency of flight and freezing. Each point represents a single animal (n=38). The coefficient of variation of the slope was 9%.

**Figure 2**

Functional imaging using quantitative analysis of c-fos+ cells. A) Background levels of c-fos mRNA in naive (N, blue box) and sensitized (S, red box). Control mice in their home cages had extremely low staining. The region illustrated is the periaqueductal gray (PAG) and is representative of other regions examined. The USS induced c-fos+ cells in the dorsomedial and the lateral periaqueductal gray (DMPAG and LPG) and in the dorsal raphe (DR), as well as at the boundary of the cuneiform and the pedunculopontine nuclei (CnF; PPTg; white arrow). (B, C) Photomicrographs illustrated in B) indicate c-fos+ cells in the region of the inferior colliculus (IC; arrows) topographically appropriate to the USS, and in C) show higher density of c-fos+ cells in the motor cortex of N mice exhibiting more flight than S mice (see E for quantification). D) Images illustrating the Macroanalysis procedure. High magnification photographs taken with a 6x objective are automatically assembled into a low-magnification mosaic of entire coronal sections using the Virtual Slice module of the Neuroulucida program. This mosaic is then automatically transformed to a vector image representing the distribution of strongly stained cells (yellow dots). E) Stereological data (cells/mm² +/- S.E.M.) showing the enhancement in motor cortical activity in N vs. S mice. Student’s t-tests indicated a significant (P<0.05) increase in the hindlimb area of the motor cortex (M1, M2). F) Portions of overlaid macroanalysis images from 3 sections spanning 360 µm were used to view the distribution of densely stained cell profiles in functional columns of the rostral portion of the DMPAG and LPG (arrows). G) Photomicrographs (40x) illustrating single-cell resolution of the c-fos mRNA in situ hybridization signals used for stereological measurements. See experimental procedure for details.
Data indicating changes in the density and the distribution of c-fos* cells in the hypothalamus. In N mice staining was more intense in A) the lateral portion of the posterior hypothalamus (PH; arrow), and in C) the dorsal portion of the lateral hypothalamus (LH), but not in the medial hypothalamus (dorsal and ventral nuclei; DM, VM). Stereological counting (cells / mm² +/- S.E.M.; in this and all subsequent figures, blue bars = naive (N) mice, red bars = sensitized (S) mice) indicated a greater density of c-fos* cells in N vs. S mice in B) the PH located between AP = - 1.8 to - 2.5 mm (vol. = 0.193 +/- 0.015 mm³, P = 0.059), and in D) the dorsal LH located between AP = - 1.3 to - 1.8 mm (vol. = 0.113 +/- 0.001 mm³, P < 0.05). Virtual sections and arrows show in S mice more intense staining in E) the dorsal and magnocellular portions of the paraventricular nucleus (PaD, PaM; no change in the central or lateral portion of the anterior hypothalamus, AH, LA) and in G) the medial and in the ventral portion of the medial preoptic nucleus (MPO). (I, K) Photomicrographs show in I) a cluster of cells in the anterodorsal preoptic nucleus (ADP) of S mice and its relative absence in N mice, and in K) cells more apparent at the boundary of the ventral premammillary nucleus (PMv) and the arcuate nucleus (Arc) in S mice. There was the same apparent number of cells in the dorsal premammillary nucleus (PMd). Stereological counting (cells / mm² +/- S.E.M.) indicated a greater density of c-fos* cells in S vs. N mice in F) the Pa located between AP = - 0.5 to - 1.1 mm (vol. = 0.059 +/- 0.001 mm³; P < 0.05), H) the MPO located between AP = 0.0 to - 0.6 mm (vol. = 0.235 +/- 0.002 mm³; P < 0.05), J) the ADP located between AP = + 0.3 to - 0.3 mm (vol. = 0.037 +/- 0.002 mm³; P < 0.01), and L) the PMv/Arc located between AP = - 2.1 to - 2.7 mm (vol. = 0.045 +/- 0.003 mm³, P < 0.01).
Pattern and density of staining in septo-hippocampal areas. Photomicrographs of the pattern of staining in A) the dorsal, intermediate and ventral portion of the lateral septum (LSD, LSI, LSV), and in B) the vertical limb of the diagonal band (VDB). Stereological counting (cells / mm³ +/- S.E.M.) in C) the VDB located between AP = +1.2 to +1.3 mm (vol. = 0.041 +/- 0.004 mm³), D) the LSV located between AP = +0.1 to −0.7 mm (vol. = 0.120 +/- 0.004 mm³), E) the LSI located between AP = +0.5 to +0.5 mm (vol. = 0.255 +/- 0.018 mm³). Student’s t-tests indicated significant group differences for the LSV (P < 0.01) and the VDB (P = 0.01), but not for the LSI. H) There were no differences between N and S mice at the level of the dorsal hippocampus. Note the high density of cells in the pyramidal layer of CA1, CA2 and CA3. There were contrasting effects in the anterior and commissural portion of the bed nucleus of the stria terminalis (BNSTa; CST). (I,J) Photomicrographs show the distribution of cells in I) the BNSTa (arrows indicate the regions particularly stained in N mice) and J) the CST (surrounded by the lateral septum ventral, LSV, and the anterodorsal preoptic nucleus of the hypothalamus, ADP). Stereological counting (cells / mm³ +/- S.E.M.) revealed a significant change in F) the BNSTa located between AP = +0.5 to +0.4 mm (vol. = 0.071 +/- 0.002 mm³; P < 0.05), but not in G) the CST located between AP = +0.1 to −0.2 mm (vol. = 0.131 +/- 0.001 mm³).
Figure 5

Data showing the greater density of staining in amygdalo-striatal areas of N vs. S mice. Virtual sections show the distribution of cells in A) the basomedial and central amygdala (BMA, Ce), C) the lateral and the basolateral amygdala (La, BLA), E) the medial amygdala anterior (MeA), K) the ventral and dorsal portion of the medial amygdala posterior (MePV, MePD). The microphotograph in D) shows the dense cluster of cells at the boundary of the anterior cortical nucleus (ACo) and the MeA. Stereological counting (cells / mm³ +/- S.E.M.) revealed significant changes in B) the BMA located between AP = -0.7 to -1.5 mm (vol. = 0.273 +/- 0.003 mm³; P < 0.05), F) the MeA located between AP = -0.6 to -1.3 mm (vol. = 0.129 +/- 0.002 mm³; P < 0.01), I) the MePV / ACo located between AP = -0.9 to 2.1 mm (vol. = 0.424 +/- 0.009 mm³; P < 0.01). L) There was no significant change in Ce located between AP = -0.8 to -1.5 mm (vol. = 0.259 +/- 0.006 mm³). The other virtual sections show the distribution of cells in G) the dorsomedial portion of the caudate putamen (CPu), H) the posterior portion of the CPU, and J) the nucleus accumbens (Acb; notice in N mice the higher density in the shell compared to the core of the accumbens, AdbSh, AdbC). Stereological counting (cells / mm³ +/- S.E.M.) revealed significant changes in M) the CPU mediodorsal located between AP = +0.0 to -1.2 mm (vol. = 0.768 +/- 0.057 mm³; P = 0.01), N) the posterior CPU and the Astr located between AP = -1.0 to -2.0 mm (vol. = 0.693 +/- 0.039 mm³; P = 0.05), and O) the Acb located between AP = +1.7 to +1.3 mm (vol. = 0.645 +/- 0.019 mm³; P < 0.01).
Evidence for greater activity in cingulate and dorsal prefrontal cortices of N vs. S mice. Photomicrographs show the differential activity in A) the granular and agranular layers of the retrosplenial cortex (RSG, RSA), B) area 1 and 2 of the anterior cingulate cortex (Cg1, Cg2), C) the prelimbic cortex and the most rostral part of the anterior cingulate cortex (PrL, Cg1, no change in the adjacent nose component of the motor cortex, M2). Stereological counting (cells / mm$^3$ + - S.E.M.) shows an enhancement in cortical activity in N vs. S mice. Student's t-tests indicated significant effects in D) the granular retrosplenial cortex located between AP = - 1.8 to 2.7 mm (vol. = 0.280 +/- 0.011 mm$^3$; P < 0.05), E) Cg2 located between AP = + 0.1 to -0.7 mm (vol. = 0.168 +/- 0.005 mm$^3$; P < 0.05), F) Cg1 located between AP = + 2.1 to +1.6 mm (vol. = 0.260 +/- 0.008 mm$^3$; P < 0.01), and G) PrL located between AP = + 2.1 to +1.6 mm (vol. = 0.277 +/- 0.006 mm$^3$; P < 0.05).

Figure 7

Cross sectional diagram of the forebrain summarizing the results of the present study. Areas of preferential activity in N and S mice are indicated in blue and in red, respectively, with the color brightness representing the approximate intensity of the differences. Areas showing strong but equal c-fos expression in both N and S mice are omitted for clarity (see Table 1). See text for details. An animated 3D version of this summary diagram can be seen on-line.
Enhancement of cellular activity in N mice vs. S mice

Acb - accumbens nu.
Arc - arcuate nu.
Astr - amygdalostriatal transition area
BSTa - bed nu. stria terminalis anterior
Cg - anterior cingulate cortex
CPu - caudate putamen
LHD - lateral hypothalamus dorsal
LSV - lateral septum ventral
MeA - medial amygdala anterior
MePV - medial amygdala posterior ventral
M - motor cortex
MPO - medial preoptic nucleus
Pa - paraventricular nu.
PH - posterior hypothalamus
PMv - premammillary nu. ventral
RS - retrosplenial cortex
VDB - vertical limb diagonal band
Hypothetical circuit illustrating the interactions between brain areas leading to either A) motile defense and B) immobile defense. C) The septal and hypothalamic nuclei believed to be involved in behavioral inhibition in the context of other known functional columns. A) A stream of activity in motor programming cortical areas (i.e. Cg, RS, PrL, ...) and amygdalo-striatal motor regions would trigger motile defense via the mesencephalic motor pattern initiators (PAG, CnF, PPTg, ...). A putative positive feedback loop (blue arrows) from the motor programming cortical areas to the dorsolateral posterior hypothalamic zone (PH, LH) may maintain activity in the septal VDB, which in turn could limit behavioral inhibition through its inhibitory projections to the LSV (blunt arrows). The LSV could also be inhibited via projections from the medial amygdala. B) The LSV would inhibit flight by suppressing activity in the VDB. This inhibition could be reinforced by positive feedback interactions with hypothalamic nuclei of the medial periventricular zone (red arrows; ADP, MPO, Pa, ...). This hypothalamic zone could also independently inhibit areas subserving motile defense through direct and indirect projections (blunt arrows). The lateral septum, through descending GABAergic projections, could also decrease the activity of the dorsolateral posterior hypothalamic zone. C) Regions of the medial periventricular zone of the hypothalamus and the LSV are extensively interconnected (red represents pathways predominantly active in S mice). Black represents areas involved in defense and that have equal, but moderate to intense, c-fos activity in both groups. Light gray represents adjacent hypothalamic areas, involved in sexual behaviors, which displayed low activity to the aversive ultrasound. All these hypothalamic zones control behaviors through their projections to the PAG or the amygdala. The LSV, via the hypothalamus is also likely to modulate the release of stress factors such as CRH, which induce c-fos activity in the LSV.


**APPENDIX A**

**EXPERIMENTAL PROCEDURES**

**In situ hybridization procedure**

Mice are sacrificed by cervical dislocation 30 min following the end of the behavioral experiment. Brains are dissected out of the skull with great care not to damage any areas and precisely placed into a brain blocker. This blocker is required to correctly adjust the plane of brain sectioning to that of the reference atlas (Paxinos and Franklin, 2001). After aligning the left and right temporal cortex, slabs 2-4 mm thick are cut using razor blades. Three slabs are obtained for each brain corresponding to the rostral and the caudal forebrain (AP = +3 to –1 mm and AP = +1 to –3 mm) and the midbrain (AP = -3 to –6 mm). Slabs are rinsed once into cold depc-phosphate buffer solution (depc-PBS) and once in 4% depc-paraformaldehyde (depc-PAF), and then fixed for at least 24 hours in depc-PAF at 4 °C. The slabs are cryoprotected before being stored at -20 °C: they are sunk in sucrose (30%) overnight and then kept into a cryoprotectant solution (50 % depc-PBS, 25% ethylene glycol and 25% glycerol).

Slabs are glued on the vibratome sectioning plate, covered with cold (6 °C) depc-PBS and sectioned at 120 µm. About 0.5 mm of tissue at the bottom of each slab was lost due to the diffusion of the glue inside the tissue. The caudal temporal cortices including the subiculum and entorhinal cortex were excluded because they could not be cut with reliable thickness (as they would detach from the rest of the brain during coronal slicing). Sections were rinsed in depc-PBS and incubated in proteinase K (10 µg / mL in depc-PBS containing 0.1% Tween-20) for exactly 30 min. They are then post-fixed in depc-PAF for 30 min, thoroughly rinsed and placed into a RNase free hybridization buffer (50% formamide, 5x SSC, 0.3 mg/mL yeast tRNA, 100 µg/mL heparin, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll-400, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA) at 60 °C for 2-5 hours. Sections are then hybridized in the same buffer at 60 °C for 12-16 hours with a cRNA digoxygenin-labeled probe.
(1 µg/mL) specifically binding c-fos mRNA. The next day, slices are washed with a probe-free hybridization buffer and a Tris-base buffer (TBST; 500 mM NaCl, 20 mM Tris, pH 7.5, 1% Tween-20) and then incubated with RNase A (50 µg/mL) to degrade the non-specifically bound probe. After blocking with 10% sheep serum/TBST, immunochemistry is performed overnight at 4 °C using antidiogoxigenin conjugated with alkaline phosphate (1:2000). The antibody is thoroughly washed (overnight) with TBST containing an endogenous alkaline phosphatase inhibitor (levamisole 2 mM) before the development step. The sections are first washed with an AP buffer (100 mM Tris pH9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween-20 and 2 mM levamisole) and then developed in the same buffer with BCIP (5-bromo-4-chloro-3-indolyl phosphate; 3.5 µg/mL) and NBT (Nitro blue tetrazolium; 1 µg/mL) for about 3 hours. The staining is fixed in 4% PAF overnight and then the sections are counterstained for 2 min (Nuclear Fast Red) to help delineate the brain areas. Finally, the sections are mounted in PBS/glycerol (1:3) onto Superfrost / Plus microscope slides. Throughout the protocol, sections are carefully transferred from one medium to another using mesh-cups and onto the slide using a bended spatula to optimally preserve morphology.

**Neuroanatomical analysis**

We used the so-called macroanalysis technique to get an overview of the distribution of cell profiles over the whole brain and to detect potential areas of differential activity in between groups. The main tool for this analysis is the computer program Neurolucida (microbrightfield.com). This program has a “Virtual slice” module which allows to capture pictures (through a CCD camera) at a given magnification and, automatically assemble them into a seamless mosaic using a computerized stage. A typical “virtual slice” picture of a mouse forebrain section (around 0.5 cm²) is composed of around 36 pictures taken with a 6x objective. This generates a large mosaic picture (around 1 m²) on which densely stained cell profiles can efficiently be thresholded (fig. 2D)
The principle of this analysis is the thresholding of blue cell profiles (the product of alkaline phosphatase as described above) through a series of image processing filters. Pixels are then transformed into vectors (markers) based on the average size and shape of cell profiles. In order to increase the speed of the macroanalysis and avoid human error, all the steps for transforming cell profiles into markers (vectors) are carried out automatically (using Automate, a software that controls Windows applications). First, the average luminance value of each section is verified using a densitometry tool and adjusted by modifying the brightness value of the video source tool. The goal is to acquire the virtual slices with as little luminance variability as possible to efficiently compare sections in between groups. Once a mosaic picture is acquired it goes through a series of filters (Histogram brighten, Noise removal, Kodalith transformation on the blue channel) which leads to a black and white image from which cell profiles are detected based on shape, fill and size (average 300 $\mu$m$^2$). Pixel clusters that do not correspond to these criteria are filtered out, while the others are assigned x, y and z coordinates (markers). Although this method allows detection of only the most stained cell profiles, it gives an accurate view of the cell distribution in any brain region (see Fig. 2D). The macroanalysis images were used to overlay 3 sections and analyze cell profiles distribution over 360 $\mu$m virtual sections.

We have performed semi-automated design-based stereology using the Stereoinvestigator software (MicroBrightField) and an Optical Fractionator probe, as previously described (Howard and Rose, 1998). Design-based stereology has been available for a while, but there are no published studies of c-fos mRNA mapping that have used this advanced neuroanatomical tool. The lack of appropriate computer instruments might be a justification for this. Most importantly, in situ hybridization techniques do not normally allow the application of the principles of stereology. First, the tissue has to be cut and mounted so that it is thick enough to perform stereology. Second, in situ hybridization staining often appears punctuated, rather than continuous, which makes it very difficult to identify single cells. The free-floating section in situ hybridization
technique used here allows cells to be thoroughly stained over 120 \( \mu \)m-thick slices. Figure 2G illustrates the quality of the staining at the 40x field of view used for cell counting. Cells were easily discriminated within the counting frame of a stereological probe as a section was optically scanned through its z-axis. The size of the counting bricks were generally: \( \Delta x=50 \) \( \mu \)m, \( \Delta y=50 \) \( \mu \)m, and \( h=60 \) \( \mu \)m. Sections cut at 120 \( \mu \)m shrank to 80 \( \mu \)m (T) after histological preparation. This gave us a rather large 10 \( \mu \)m-thick safety zone at the top and the bottom of the section where cells were not counted to avoid biases from blade artifacts. The sampling error, which takes into account the variability within sections and in between sections, was always equal to or less than 5% (Schaeffer test). Contours of brain areas acquired from a standard atlas (Paxinos and Franklin, 2001) were digitally fitted to the sample taking care to account for the differential tissue shrinkage. Contours could only be fitted locally (over areas 1 \( \text{mm}^2 \)) because of this differential tissue shrinkage. Average volumes for each measured brain areas, determined using Planimetry, were never significantly different between groups.


APPENDIX B

SUPPLEMENTAL DATA AND FIGURES

Table 1. Summary of the intensity of staining in various areas of the brains of mice exposed to the aversive ultrasound stimulus in their home cages vs. mice not exposed to any stimulus. The intensity of c-fos staining was semi-quantitatively divided into weak (+), moderate (++), and high (+++). The areas where differential activity has been found between naive (N) and sensitized (S) mice (N>S or S>N) are indicated by dots. Note that in some areas there is evidence of differential activity between N and S mice although the staining intensity range is the same. The areas showing moderate to strong activity, but no difference between groups (N*,S*) are also indicated by dots.
## Amygdala

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**Supplemental Figure 1**

Data indicating the absence of change in c-fos+ cells density in various periaqueductal gray (PAG) regions. Stereological counting (cells / mm³ +/- S.E.) was done in the caudal portion of A) the dorsolateral PAG (DLPAG) located at AP = -4.5 to -5.0 mm (vol. = 0.087 +/- 0.001 mm³), B) the dorsomedial PAG (DMPAG) located at AP = -4.5 to -5.0 mm (vol. = 0.094 +/- 0.001 mm³), C) the lateral PAG (LPAG) located at AP = -4.5 to -5.2 mm (vol. = 0.259 +/- 0.010 mm³), and D) the ventrolateral PAG (VLPAG) located at AP = -4.5 to -5.2 mm (vol. = 0.253 +/- 0.007 mm³). Student's t-tests did not reveal any significant differences between naive and sensitized mice in these areas.

**Supplemental Figure 2**

Photomicrographs illustrating the absence of change in monoaminergic nuclei. Well defined cluster of positive cells were observed in A) the median raphe (MnR), C) in the locus coeruleus (LC). B) Only few positive cells were observed in the ventral portion of the substantia nigra (SN).

**Supplemental Figure 3**

Photomicrographs illustrating that the superficial gray layer of the superior colliculus had the same moderate level of staining in naive and sensitized mice.
**Supplemental Figure 4**
Virtual sections illustrating the pattern of staining in the tuberal zone of the lateral hypothalamus and at the level of the rostral midline and the intralaminar portion of the thalamus. **A)** As indicated by the arrow, there was more staining in the dorsal part of LH in naïve mice. **B)** There were no differences in areas of the thalamus between naïve and sensitized mice. Note the particularly high density of positive cells in the paraventricular, central medial, mediodorsal and paratenial nuclei of the thalamus (PVA, CM, PT).

**Supplemental Figure 5**
Illustrations showing the distribution of staining in anterior cortical areas. The virtual sections show the absence of change in **A)** the infralimbic cortex (IL), and **B)** the orbital cortex (ventral and lateral, VO, LO).

**Supplemental Figure 6**
Virtual sections showing in **A)** the absence of differential activity in the ventrolateral periaqueductal gray (VLPAG) and the dorsal raphe (DR) and in **B)** a cluster of cell profiles observed at the boundary of the pedunculopontine nucleus (PPTg) and the cuneiform nucleus (CnF) which was more dense in naïve than in sensitized mice. There was some variability in the distribution of this cluster from section to section.
Supplemental Figure 7

Photomicrographs showing the distribution of staining in various cortical areas. Contrary to the hindlimb motor cortex C) there were no changes observed in A) area 1 and 2 of the nose component of the motor cortex (M1, M2), B) the temporal cortex (temporal association cortex, TeA, ectorhinal cortex, Ect, perirhinal cortex, PRh), D) the claustrum, the piriform and the endopiriform cortex (Cl, Pir, En), E) the parietal association cortex (medial and lateral, MptA, LptA), and F) area 1 of the sensory cortex (S1), and in G) the auditory cortex (ventral and dorsal, AuD, AuV).
Supplemental Figure 8.

Illustration of the neural pathways highlighted in the present study in relation with the observed c-fos pattern induced by the aversive ultrasound. Auditory input transiting through the inferior colliculi (IC), the medial geniculate (MG) and the auditory cortex reach the lateral amygdala (LA), the caudate putamen, the hippocampal formation, and the retrosplenial cortex. Blue and red arrows represent pathways arising from regions preferentially activated in N and S mice, respectively. Flatmaps are modified from Swanson (1998) Brain Maps, Elsevier, Amsterdam. See text for details and references below: i (Yukie, 1995), ii (McGeorge and Faul, 1989), iii (Clugnet et al., 1990), iv (Brog et al., 1993; Petrovich et al., 1996), v (Kunishio and Haber, 1994), vi (Petrovich et al., 2001), vii (Heimer et al., 1991), viii (Simerly and Swanson, 1988), ix (Bandler et al., 2000), x (Brog et al., 1993; Cassell and Wright, 1986), xi (Gonzalo-Ruiz and Morte, 2000), xii (Caffe et al., 1987), xiii (Pikkarainen et al., 1999), xiv (Nitecka, 1981; Ottersen, 1980), xv (Whishaw et al., 1972), xvi (Vertes et al., 1995), xvii (Floyd et al., 2001), xviii (Brog et al., 1993), xix (Abrahamson and Moore, 2001; Vertes et al., 1995), xx (Floyd et al., 2001), xxi (Risold and Swanson, 1997; Tsukahara and Yamanouchi, 2001), xxi (Jakab and Leranth, 1995; Staiger and Nurnberger, 1989, 1991), xxii (Jaksb and Leranth, 1995; Risold and Swanson, 1997), xxiii (Shipley et al., 1996; Canteras et al., 1992; Bandler and McCulloch, 1984; Risold and Swanson, 1997).


APPENDIX C

SUPPLEMENTAL DISCUSSION

Neural correlates of USS-induced flight vs. freezing

Within each section, the reader may link to numbered sections of the on-line Appendix ([AQ#]) for further details and literature citations. More detailed anatomical connections drawn on flatmaps (Fig. S8) are indicated by lower-case Roman numerals in brackets.

A cortico-amygdalo-striatal stream of neural activity mediating motile defense via the mesencephalon

In N mice, the pattern of c-fos expression in cortical, striatal and amygdaloid areas is consistent with their known functions in programming, coordinating and motivating active defensive behaviors. As expected (Castro-Alamancos et al., 1992), in the cortex we observed increased c-fos expression in the limb motor regions of these animals. However, the largest area of enhanced activity in N animals was in the cingulate cortex, an area involved in complex aspects of avoidance behaviors [AQ1] (Gray and McNaughton, 2000). The coordination of innate motor programs is most likely the product of the striatum (Hikosaka, 1994). We observed elevated c-fos expression in subregions of the caudate putamen (Cpu) [v] and especially the shell of the nucleus accumbens (Acb) [x] which both receive input from the cingulate cortex [AQ2]. The Acb in particular is thought to motivate active defensive behaviors (Gustavo and Pazo, 1995). These striatal regions also receive input from the amygdala [iv], and there was preferential c-fos activation in a subset of amygdaloid nuclei linked to the striatum [AQ3]. Increased c-fos expression in the amygdala of N mice was observed in basomedial and corticomedial nuclei, which have more motor features than the lateral amygdala. This is consistent with previous studies
implicating these amygdalar regions in flight and learned escape behaviors [AQ4].

In the mesencephalon, the dorsal periaqueductal gray region (PAG) is an area believed to be the final common pathway for the integration of flight behaviors (Bandler and Depaulis, 1991). Although activated, the dorsal PAG did not show preferential $c-fos$ expression in N mice, consistent with a function in motor pattern initiation rather than in frequency control [AQ5]. However, N mice showed preferential $c-fos$ activity at the border of the nucleus cuneiform (CnF) and the pedunculopontine tegmentum (PPTg). Interestingly, low-level stimulation of the PPTg / CnF boundary can induce freezing while repeated activation induces flight [AQ6]. Thus this brainstem area may regulate the balance between flight and freezing, depending on input from forebrain regions such as the striatum [vii].

*Competing septo-hypothalamic circuits may control the balance between motile and immobile defense behaviors*

N mice exhibited preferential activity in the dorsolateral posterior zone of the hypothalamus, and strong (but not preferential) activity in the medial behavioral control column nuclei generating defense (Canteras, 2002) [AQ7]. Stimulation of the dorsolateral posterior hypothalamus is known to produce flight reactions [AQ8]. This dorsolateral posterior hypothalamic interconnected zone [xix] receives excitatory projections from cortical motor programming areas such as the cingulate cortex [xvii]. In turn, it projects back to the septal VDB, which is one of the most strongly preferentially activated areas in N mice. The VDB, in response to these hypothalamic inputs, controls electrical activity in the hippocampus and RS cortex [AQ9][AQ13]. These afferent and efferent connections of prelimbic and cingulate cortex with the lateral and posterior hypothalamus and the VDB may, therefore, form a positive-feedback loop that reinforces activity in the cortico-amygdalo-striatal motor processing stream (Fig. 8A, blue arrow).
In S mice, the only regions of the forebrain that showed preferential *c-fos* expression were the LSV and the medial periventricular zone of the hypothalamus (Fig. 8B). These regions are heavily interconnected (Risold and Swanson, 1997) (Fig. 8C) [AQ10], raising the possibility that they participate in a positive-feedback loop. In principle, such a circuit could either inhibit flight behavior, promote freezing or both. Stimulation and lesion data strongly suggest that the lateral septum is involved in mediating the effects of anxiety to inhibit ongoing behaviors, including active defense responses [AQ11]. Some of this ‘behavioral inhibition’ would in turn be controlled by hippocampal regulation of lateral septal outputs (Gray and McNaughton, 2000). Although we saw no evidence of differential *c-fos* expression in the hippocampus of S vs. N mice, the hippocampus is likely to play some role in the sensitization of fear or in anxiety [AQ12].

There are several sites where the LSV might inhibit activity in the descending cortico-amygdalo-striatal motor pathway. The LSV generates an extensive series of GABAergic projections to the VDB [xxii], and this is consistent with the reduced activity in the VDB of S mice. The lateral septum may also suppress activity in the VDB indirectly, via inhibitory projections to the dorsolateral posterior hypothalamic zones [xxi] that, in turn, exert an excitatory action on the VDB (Fig. 8B). As discussed above, the VDB may serve as an important node in a positive-feedback circuit involving the cingulate cortex (Fig. 8A). Inhibition of VDB activity by the LSV could therefore serve to break this positive-feedback loop, as part of the “behavioral inhibition” command leading to freezing (Fig. 8B) [AQ13] [AQ14]. Interestingly, the VDB sends reciprocal GABAergic collaterals back to the LSV [xxii]. The bi-directional inhibitory connections between the VDB and LSV suggest that this circuit could form part of a septal switch mechanism that controls flight vs. freezing responses.

It was surprising to find enhanced expression of *c-fos* in medial periventricular hypothalamic nuclei of S mice, because these nuclei are mainly implicated in sexual behaviors (Canteras, 2002). Our results suggest that distinct
subregions of these nuclei are activated by either sexual or fearful stimuli [AQ15]. The medial periventricular hypothalamic subnuclei involved in defense may inhibit flight behavior by reinforcing activity in the LSV (Fig. 8B, C). However there are at least two other potential pathways through which their influence to inhibit flight (and/or promote freezing) might be exerted: one involving the amygdala, and the other the Acb [AQ16]. In addition to promoting freezing indirectly through influences on the amygdala, this region of the hypothalamus may also contribute more directly to freezing behavior. For example, lesions of the dorsal premammillary nucleus (PMd), which was strongly and equally activated in S and N mice, inhibit both flight and freezing reactions to a predator (Canteras et al., 1997).

Role of the extended amygdala in USS-induced freezing behavior

Studies of the circuits that mediate freezing behavior have focused on the central nucleus of the amygdala (CeA) and its efferents to the ventral PAG (Goosens and Maren, 2001; De Oca et al., 1998). Surprisingly, we found no evidence for higher levels of c-fos expression in either the CeA or ventral PAG of S animals, although both regions showed elevated activity over baseline. There are several possible explanations for this apparent discrepancy [AQ17A][AQ17B]. On the one hand, the fact that there was a clear induction of c-fos in the same medial sector of the La and BLA of both N and S mice suggests that the amygdala is somehow involved in freezing (as well as flight) reactions to the USS [AQ18]. On the other hand, the LSV-hypothalamic circuit discussed earlier may constitute a separate pathway for the sensitization of freezing released by innately fearful stimuli, one that does not require the precise temporal pairing between a CS and a US that the La, the BLA and the CeA presumably mediate (Maren and Fanselow, 1996).

Consistent with the latter view, there is evidence that the sensitization of acoustic startle (which correlates with freezing), involves a circuit distinct from that involved in conditioned fear (Walker and Davis, 1997). This circuit has been
suggested to require activity in the commissural component of the BNST (CST) (Gewirtz et al., 1998). However we did not observe increased \( c-fos \) levels in the CST of S mice (although higher levels of activity in BNSTa were consistently correlated with flight [AQ19]). Instead, we observed the greatest enhancement of \( c-fos \) activity in S mice in the LSV and ADP, which are immediately adjacent to the CST [AQ20]. This raises the possibility that the effects of lesions aimed at the CST were actually due to impairments of function in LSV and/or ADP. More precisely localized functional perturbations in these septal and hypothalamic nuclei, as well as in the CeA, will be required to distinguish these possibilities.

Potential role of \( c-fos \) neuromodulators in sensitization of the freezing response to USS

How might stress or anxiety shift the balance of activity from the circuit mediating flight, to that mediating freezing (Fig. 8A vs. 8B)? Pharmacological studies on \( c-fos \) expression (Imaki et al., 1993; Bardgett and Henry, 1999) suggest at least two loci at which stress-induced changes in neuromodulators could influence the proposed circuit. One possibility is that levels of corticotropin-releasing hormone (CRH; or related peptides), which are elevated by stress, disinhibits LSV activity through an inhibitory action on the LSD, and thereby promotes freezing (Bakshi et al., 2002) [AQ21]. Because of the putative reciprocal inhibition between VDB and LSV (Fig. 8A, B), this bias towards LSV activity could tip the balance of activity towards the S circuit. Another, non-exclusive, possibility is that a stress-induced reduction in dopaminergic transmission in the shell of the Acb could contribute to an inhibition of locomotor activity [AQ22]. Consistent with this notion, reduced dopamine release in Acb shell is correlated with enhanced freezing to an auditory fear stimulus (Gambarana et al., 1999). It will be interesting to examine the effects of manipulating these neuromodulators on the responses to the USS.


What are the respective roles of different subregions of the cingulate cortex in programming motor and fear-related aspects of defense?

The cingulate cortex is divided into the anterior (Cg) and the posterior cingulate regions (or retrosplenial cortex; RS). These areas have distinct and important functions for the perception of innately aversive stimuli: Cg is primarily involved in 'executive functions' related to emotional motor control, while RS subserves 'evaluative functions' such as the monitoring of sensory events (Vogt et al., 1992). Discriminated active avoidance experiments have shown that both of these cortical modules are necessary elements of a GO/STOP motor system (Gabriel, 1990). RS receives information about auditory stimuli directly from the auditory cortex [i] and monitors behavior in relation with memories and context, while the Cg has sensori- and viscero-motor components involved in preparing innate motor plans (Gray and McNaughton, 2000).


Which striatal areas are most likely to be engaged by a fearful auditory stimulus? The striatum can be divided into a limbic and a non-limbic part: the dorsolateral neostriatum, where \( c-fos^+ \) cells were sparse, receive projections mainly from sensorimotor areas, while the ventral and dorsomedial striatum, where \( c-fos^+ \) cells were found most abundant in N mice, have numerous and topographically organized interconnections with the limbic system. The auditory cortex sends specific projections to the CPu mediodorsal where c-fos activity was most intense [ii]. Non-cortical sensory inputs are also of interest as auditory outputs from the medial geniculate nucleus are sent to the posterior CPu [iii]. These auditory and limbic connections are interesting because lesions of either mdCPu or pCPu decrease fear to auditory cues (LeDoux et al., 1986). At the level of the ventral striatum, flight was correlated with more \( c-fos^+ \) cells in the shell of the Acb. An increased activity there is consistent with the notion of the Acb acting as a motivation motor center in aversion. Under certain conditions, stimulation of the ventral part of the striatum can even induce flight responses (Gustavo and Pazo, 1995).


What are important amygdalo-striatal interactions relevant to the present data?
The amygdala, which is merged with the striatum (Swanson and Petrovich, 1998), has received more attention in relation to emotions than to motor coordination. A cognitively perceived threat is evaluated at the level of the amygdala under the control of descending projections from the prefrontal cortex [x]. The lateral amygdala is the locus of convergence of various sensory modalities, including auditory stimuli. From there, sensory information about danger is transmitted to interconnected amygdalar nuclei such as the medial and the basal amygdala. This processed information is then sent via the basal amygdala to the striatum for the expression of coordinated emotional reactions (Fig. 8A). The shell of the Acb is innervated by the basal amygdala while sectors of the lateral and basolateral amygdala, where c-fos staining was denser, project to the dmCPu [iv]. However, there were no group differences in the lateral and the basolateral amygdala. This suggests that the increased striatal activity in N vs. S mice is not due to increased amygdalar sensory input.

What is the evidence implicating the amygdala in flight or learned escape responses?

In areas of the amygdala having motor features, there was greater activity in the basomedial and the corticomedial nuclei of N mice. It has long been known that stimulation of some ill-defined amygdaloid areas produces flight and active defense behaviors, while lesions of these areas prevents defense behaviors in cats and wild rats (Ursin, 1965; Fernandez de Molina and Hunsperger, 1959; Kemble et al., 1984; Kemble et al., 1990). Furthermore, a recent study indicates that the basal, but not the central, nucleus of the amygdala is important for the expression of a learned escape response (Amorapanth et al., 2000).


What evidence implicates the dorsal PAG in flight behaviors and how do we explain the absence of differential activity in this region?

Electrical or chemical stimulations of the dorsal PAG are well known to induce explosive flight reactions, while lesions of the dorsal PAG prevent flight reaction induced by stimulation of other areas of the brain (Graeff, 1990). However, quantification did not reveal any change in activity within these columns in mice displaying more flight. In previous studies, expression of Fos was found in the dorsal PAG of animals displaying flight, but no correlation has been made between the amplitude of Fos expression and the intensity or the frequency of flight. Similarly, the present results do not negate the involvement of the dorsal PAG in the integration of flight behaviors because mice displaying defense responses always had more activity in that area than mice not exposed to any aversive stimuli. These data instead support the notion that the PAG is a motor pattern initiator rather than a motor pattern controller (Swanson, 2000). The data further suggest that aversion-induced activity in the dorsal PAG occurs in an all-or-none fashion, rather than following a linear relationship with the frequency of flight.


Which observations suggest that the central tegmentum might regulate flight and freezing?

The CnF/PPTg receives direct projections from the PAG (Sandner et al., 1992)[ix]. A previous study (Mitchell et al., 1988) has shown that a single microinjection of glutamate into the CnF produces freezing whereas a second or a third injection produces flight. This pattern of behavioral reactions following CnF stimulation is not caused by a cumulative effect of glutamate in that area, but rather by some form of neuronal plasticity. This fact, taken together with the observation of a decreased activity in the CnF / PPTg of animals displaying freezing, suggests that this caudal area regulates the balance between flight and freezing.


How do we see the present results in the context of previously established hypothalamic columns controlling defense behaviors?

The medial hypothalamic column that has been suggested to mediate defense, based on prior studies (Swanson, 2000; Canteras, 2002), includes the anterior nucleus (AHN), the ventromedial nucleus dorsomedial (VMHdm) and the nucleus premammillary dorsal (PMd). In the present study, a substantial amount of USS-induced activity was observed in this hypothalamic column, but there was no evidence of differential activity between N and S mice (Fig. 8C, black arrows and letters). In any case, there is no evidence of differential involvement of the AHN, the VMH or the PMd in flight vs. freezing behaviors. For example, lesion of the PMd decreases both flight and freezing reactions in rats exposed to a natural predator (Canteras et al., 1997).


What is the evidence for a dorsolateral posterior hypothalamic zone subserving flight?

The dorsomedial nucleus (DMH) is the area where flight is induced with the lowest electrical current (Graeff, 1990). Although US-induced c-fos expression was strong in the DMH, there was no indication of differential activity in that area, but the dorsal part of the lateral hypothalamus (LH), adjacent and interconnected to the DMH, displayed enhanced activity in N mice. More caudally, the posterior hypothalamus (PH), interconnected to LH [xix], also had more activity in N mice. This is interesting because stimulation of either LH or PH produces flight reactions; electrical stimulation of the LH produces flight reactions and alteration of cholinergic transmission in LH occurs as a function of escape behaviors (Rada et al., 1998; Rada and Hoebel, 2001; Schwartzbaum and Leventhal, 1990). Furthermore, a dorso-ventral trend in the aversive effect of LH stimulation was previously reported (Cazala and Schmitt, 1987). More PH activity is consistent with the fact that GABAergic receptor-blockade in PH elicits flight reactions in rats (Shekhar and DiMicco, 1987).


[AQ9]

How would the dorsolateral posterior hypothalamus reinforce active defense via the septum?

Afferents from LH and PH modulate the cholinergic output of the VDB [xv][xvi]. It is well known that pacemaker cells of the medial septum/diagonal band nuclei drive hippocampal theta rhythm (Vertes and Kocsis, 1997). Fear stimuli elicit slow wave theta-type-2 hippocampal activity in conjunction with alert immobility in rats, while a higher frequency theta-type-1 rhythm is associated with movements (running, rearing, swimming) (Sainsbury et al., 1987a; Sainsbury et al., 1987b). Hypothalamic areas such as the dorsal LH and the PH project to the medial septum/diagonal band nuclei and thereby control the frequency of hippocampal theta (Oddie and Bland, 1998; Vertes et al., 1995; Whishaw et al., 1972). Interestingly, stimulation of these hypothalamic areas produces running behaviors whose intensity is proportional to the frequency of theta-rhythm type-1 (but not of alert immobility-related theta-rhythm type-2).


Which hypothalamic nuclei of the medial and periventricular zones are implicated in sensitization?

Many areas with preferential c-fos activity in S mice were found in the medial and periventricular zones of the hypothalamus. As illustrated in Figure 8C, these medial periventricular hypothalamic nuclei have important interconnections with the LSV [xxiii]. The pattern of reciprocal connections between the medial or anterodorsal preoptic nuclei (MPO, ADP) and the LSV corresponds to areas where US-induced c-fos activity was observed (Risold and Swanson, 1997). Furthermore, staining at the boundary of the arcuate and the nucleus premammillary ventral (Arc/PMv) matches the input/output pattern of the LSV in that region (Canteras et al., 1992). There are projections from the LSV surrounding the paraventricular nucleus (Pa), while the core of Pa projects exclusively to the LSV (Jakab and Leranth, 1995).


Why do we think that the lateral septum is crucial in exerting behavioral inhibition and anxiety?

It has been proposed that the septum and the hippocampus work as an integrated system exerting behavioral inhibition when an animal is exposed to conflicts or anxiety. In this view (Gray and McNaughton, 2000), the septo-hippocampal system works to inhibit any ongoing prepotent behaviors, such as feeding, but only when motor programs are tagged as inadequate to cope with a given situation. In the present model, we view motile defense reactions such as flight as the prepotent responses to the USS in N mice, and these behaviors are inhibited by signals reaching the hippocampus about anticipatory fear. The lateral septum, as a hippocampal output, is likely to play an important role in mediating this function because: 1) Stimulation of the lateral septum inhibits the active defense responses produced by hypothalamic stimulation (Thomas and Evans, 1983), 2) Lesion of the lateral septum produce hyperdefensiveness, hyperactivity and reduces freezing behavior (Albert and Chew, 1980; Mattingly et al., 1979), 3) Lesions of the lateral septum have anxiolytic-like effects in various animal paradigms (Mattingly et al., 1979; Menard and Treit, 1996).


What are the arguments involving the hippocampus in anxiety and the sensitization of fear?

The hippocampus is believed to play an important role in the sensitization of fear (Kalynchuk et al., 2001). Sensitization of freezing to an innate fear stimulus might occur as circulating stress hormones alter hippocampal physiology (Korte, 2001). Prolonged acute periods of stress down-regulate, on the next day, glucocorticoid and mineralocorticoid receptor-mRNAs in all fields of the hippocampus (Liberzon et al., 1999). Furthermore, fear-induced hippocampal electrical activity is altered following foot-shock sensitization (Sainsbury et al., 1987a). A large body of evidence also indicates that low frequency hippocampal theta rhythm is associated with behavioral inhibition and anxiety. Furthermore, lesions of the septo-hippocampal system decrease anxious-like behaviors, and all classes of anxiolytic drugs tested so far decrease theta activity associated with alert immobility or anxious-like behaviors (Gray and McNaughton, 2000). Changes in patterns of hippocampal activity, especially those related to theta rhythm, are unlikely to be detected by the c-fos method.


What links the blunted activity in RS of S mice to the activity of the septo-hippocampal system?

The RS was included in the septo-hippocampal system because it displays the same characteristic theta rhythm as other elements of this system (Gray and McNaughton, 2000). It is an important output station of the hippocampal formation and a reduced activity in the RS of S mice can be linked with change in hippocampal electrical activity (Wyss and van Groen, 1992;Colom et al., 1988;Kang and Gabriel, 1998). Neuronal activity in RS is particularly dependent on cholinergic afferents arising from septal nuclei such as the VDB. Lesion of the medial septum/diagonal band abolishes all theta activity, but in RS theta persists following medial septum lesions that spare the VDB (Borst et al., 1987;Colom et al., 1988;Gonzalo-Ruiz and Morte, 2000). Therefore, the reduction in RS neural activity in S mice might be related to a concomitant change in the VDB.


How could decreased limbic cortical activity lead to freezing?

The diminished activity in the anterior cingulate cortex (Cg) of S mice could result from a decreased activity in its posterior portion (RS). RS has less motor features than Cg and is linked with sensory and association cortex areas. An interesting possibility is that the decreased activity in the RS of S mice reflects reduced cholinergic input from the VDB [xi] (Fig. 8B). This would indirectly lead to less activity in rostral areas such as the dorsal prefrontal cortex involved in higher order programming of innate fear reactions. Behavioral experiments indicate that inactivation of the dorsal prefrontal cortex with electrolytic lesions increases timidity and produces a general increase in fear, leading to enhanced freezing (Holson, 1986; Morgan and LeDoux, 1995). Discrete lesions of the prelimbic cortex were also shown to produce anxiogenic-like effects in the open-field and the elevated-plus-maze models of anxiety (Jinks and McGregor, 1997). Our results are thus consistent with the notion that hypofunction of the dorsal prefrontal cortex decreases motile responses to aversive stimuli. One view, therefore, is that freezing is facilitated by a decreased excitatory action of the prefrontal and the cingulate cortices on the amygdalo-striatal sites generating motile defense.


Can hypothalamic nuclei, known to be involved in sexual behaviors, also be involved in defense?

The MPO has mainly been implicated in the control of reproductive behaviors, but it extensively innervates regions of the PAG involved in defense and stimulation of the MPO produces a Fos activation pattern in the PAG comparable to that of aversive stimuli (Shipley et al., 1996). The Pa and the Arc are involved in neuroendocrine secretomotor systems controlling both sexual and defense behaviors (Swanchenko et al., 1996; Merali et al., 2001). Although premammillary regions are involved in sexual behavior, lesions of the PMd produce severe reduction in both flight and freezing to a natural predator (Canteras et al., 1997), while lesions of the PMv increase aggressive behaviors (van den Berg et al., 1983). Rats exposed to an innately aversive odor show a pattern of neuronal activity remarkably similar to the one observed in mice exposed to the innately aversive ultrasounds – that is, a dense Fos expression in the PMd and some staining at the PMv/Arc boundary (Dielenberg et al., 2001).


[AQ16]

How might the medial periventricular hypothalamus inhibit flight behavior?

Two potential pathways, through which the hypothalamus inhibits motile defense, can be postulated: Many nuclei of the medial periventricular zone (MPO, Pa, Arc, and PMV) send projections to central and medial amygdalar nuclei [xiv]. It is possible that these hypothalamic areas exert a suppressing effect on amygdalar regions mediating flight, or alternatively activate restricted amygdalar sites important for freezing. Second, MPO or ADP could exert behavioral inhibition by acting on regions of the Acb motivating aversive locomotor behavior (Fig. 8B). This could occur via the known inhibitory action of the MPO on dopaminergic cells of the ventral tegmental area (VTA) that in turn innervate the shell of Acb, and which can induce Fos in that area (Maeda and Mogenson, 1980; Sandner et al., 1992) [viii].


How does the present data relate to the involvement of the ventral vs. dorsal PAG in freezing?

Conditioned fear was shown to increase Fos expression more in the caudal portion of the ventrolateral PAG than in other columns of the dorsal PAG (Carrive et al., 1997), while chemical lesions of the caudal, but not the rostral, regions of the ventral PAG decreases conditioned freezing (LeDoux et al., 1988). Furthermore, stimulation of the ventral PAG consistently produces freezing, but no flight (Bandler and Depaulis, 1991). These data have led to the idea that freezing is mediated primarily by the ventral PAG. This may seem at odds with our observation that the ventral PAG as much as the dorsal PAG were activated similarly in both N and S mice. However, it is noteworthy that stimulation of the dorsal PAG also induces freezing in certain conditions (Schenberg et al., 1990), and that this dorsal PAG-mediated freezing is not blocked by lesion of the ventral PAG (Vianna et al., 2001). It is possible that the aversive ultrasound elicited freezing behaviors initiated by the dorsal PAG. Our assay may not detect, however, a qualitative difference in the activity of the dorsal PAG associated with the initiation of either flight or freezing behaviors.


How do we explain the absence of preferential activity in CeA of S mice?

The involvement of the central nucleus of the amygdala (CeA) in the expression of conditioned freezing is well established based on lesion evidence (Goosens and Maren, 2001). However, our study clearly shows no increase in the activity of the CeA in S vs. N mice. Nevertheless, this is not inconsistent with the idea that CeA mediate freezing in S mice as freezing often does not correlate with neural activity in CeA as measured by elevated c-fos expression (Campeau and Watson, 1997; Schettino and Otto, 2001). Indeed anxiolytic drugs, which decrease freezing or increase locomotion, increase c-fos expression in the CeA (Beck and Fibiger, 1995; Hitzemann and Hitzemann, 1999). Therefore, increased freezing may actually involve decreased activity of CeA. Consistent with this, stimulation of the CeA produces fast inhibitory responses at the level of the PAG columns that initiate freezing behaviors (Costa Gomez and Behbehani, 1995). Therefore, while our data do not provide evidence in support of a role for CeA in USS-induced freezing in S mice, they do not exclude it either.


How might the amygdala control flight vs. freezing?

The medial amygdala sends vasopressinergic projections to GABAergic interneurons of the lateral septum [xii] (Caffe et al., 1987; Koolhaas et al., 1998). Infusion of vasopressin into the septum was shown to have an anxiolytic-like effect (Appenrodt et al., 1998). Therefore, the medial amygdala might promote flight in N animals by inhibiting activity in LSV via this vasopressinergic input. If so then reduced activity in MeA could promote freezing in S mice by disinhibiting the LSV. Other septo-amygdalar interactions are well known to modulate defense: For example, amygdalar lesions abolish hyperdefensiveness to threats produced by septal lesions (Blanchard et al., 1979). As shown in figure 8B, the LSV could indirectly limit motile defense mediated by the amygdala, via its action on hypothalamic nuclei of the medial periventricular zone.


What is the evidence implicating the bed nucleus of the stria terminalis in flight reactions?

In their pioneering work, Fernandez de Molina and Hunsperger (1959) reported that stimulation of either the BNST or the amygdala elicits flight reactions in the cat and the same observation has since been reported in rats (Casada and Dafny, 1991). The anterior portion of the BNST is contiguous with the Acb, which is also more activated in N vs. S mice. Neural activity in both the BNST and the Acb is regulated by dopaminergic terminals (Carboni et al., 2000) and by projections from the basomedial amygdala [iv]. Therefore, the anterior portion of the BNST and the ventral striatum might share complementary functions.


The BNST, the LSV or the ADP; which is most crucial in foot-shock sensitization?

Davis and colleagues have studied the role of the BNST in long-term sensitization of startle. This form of sensitization produces a similar potentiation of the startle reflex as CRH. A series of lesion and microinjection studies suggested that both CRH and long-term sensitization enhance startle via the BNST (Gewirtz et al., 1998; Lee and Davis, 1997; Davis et al., 1997; Davis, 1998; Walker and Davis, 1997). However, the region of the BNST targeted (the CST) lies immediately adjacent to the LSV and the ADP (which are collectively called the septohypothalamic nucleus in some rat atlases). In light of our results, one should consider a role for the LSV and ADP in the sensitization of startle, in addition to or instead of the BNST based on the extent covered by the lesions and the diffusion area of drugs injected into the CST in the studies discussed above.


How might elevated levels of CRH increase anxiety and activity in the LSV?
CRH is known to be anxiogenic in mice (Stenzel-Poore et al., 1994) and sensitization increases levels of CRH in the Pa (Bruijnzeel et al., 2001). There are CRH-like immunoreactive fibers from the hypothalamus in the lateral septum (Sakanaka et al., 1988) and intracerebroventricular (i.c.v.) injection of CRH produces a major increase in Fos expression in the LSV, but not in the LSD (Imaki et al., 1993) (Fig. 8C). Freezing reactions that occur immediately after an aversive US are decreased following blockade of CRH receptors at the level of the lateral septum (Bakshi et al., 2002). Freezing might be enhanced by activation of the CRH-2 receptors located specifically in the LSD (as imaged by autoradiography) that inhibit neural activity at this level (Bakshi et al., 2002). The presence of dorso-ventral GABAergic inhibitory collaterals in the lateral septum (Jakab and Leranth, 1995) suggests that the increased c-fos expression observed in the LSV in response to the USS (or i.c.v. CRH; Imaki et al., 1993) might be due to reduction of a tonic inhibitory action of the LSD over the LSV. An elevation of CRH levels, caused by anticipatory anxiety, could therefore tip the balance in the VDB-LSV switch circuit in favor of the LSV.


How could changes in Acb dopaminergic transmission be related to the switch between flight vs. freezing?

The Acb is an important dopamine (DA) projection area which, as a convergence site between the amygdala and the hippocampus, is well placed to regulate behavior via its connections with the mesencephalon. The LH has direct projections to Acb [xviii] and aversive stimulation of LH causes release of DA in the Acb whose amplitude is proportional to the frequency of LH-induced-flight behavior (Rada et al., 1998). In contrast, DA release in the shell of Acb is blunted in animals displaying escape deficits following inescapable stress and in animals displaying enhanced freezing in response to an auditory fear stimulus (Gambarana et al., 1999; Pezze et al., 2002). Blunted DA transmission in S mice could thus account for the reduced expression of c-fos in the shell of the Acb compared to N mice, as could a decreased activity in glutamatergic inputs to this structure from the hippocampus. Indeed, hippocampal stimulation elevates Fos expression in the Acb together with motor hyperactivity, and both of these effects are prevented by DA receptor blockade (Bardgett and Henry, 1999), while stimulation of DA cells in the VTA produces hyperactivity and Fos expression in the Acb (Sandner et al., 1992). A pathway linking the septo-hippocampal system to the Acb, modulated by DA, could thus determine the nature of the defense behaviors elicited by the USS in different contexts.


