Supplementary data

Control of drug spread in intra-LBA infusions

Although we targeted the LBA, it is always possible that the effects were mediated by sites distal to the LBA because of uncontrolled drug diffusion. First, the differential effects of CNQX and ifenprodil could possibly be due to differential diffusion of the 2 drugs. This was not the case however since ifenprodil and CNQX had similar diffusion patterns measured in 3 different axes centered on the tip of the injector (Supplementary Fig. 3): both groups in each experiment overlap across all angles. In all comparisons (Repeated measure ANOVA on drug treatment over distance) there was no significant interactions for any of the 3 angles $F^\prime$s < 1. This demonstrates that there is no differential diffusion between ifenprodil and CNQX. Thus the reported effects cannot be attributed to CNQX and ifenprodil exhibiting differential diffusions.

Next, we asked if there was significant inhibition of protein synthesis in the central amygdala (Ce). In order to analyze the area of functional inhibition by anisomycin, we stimulated the brain, to induce a high expression of the immediate early gene c-fos. Pentylenetetrazol (PTZ)-induced seizure has been previously shown to significantly induce c-fos gene and Fos protein, in many brain regions, including the amygdala and hippocampus. We used this approach to enhance the overall Fos protein level, and against this high Fos background, to test the anatomical extent and the effectiveness of the anisomycin infused in the LBA. Animals received an intra-LBA vehicle infusion in one hemisphere and anisomycin in the other hemisphere, allowing within-animal comparisons. One hour later, Fos expression was decreased in the LBA by approximately 50% ($t (8) = 10, P <0.05$) and in the Ce by 18% ($t (8) = 5, P <0.05$; Supplementary Fig. 4). This suggests that anisomycin infused in the LBA does not massively affect Fos protein levels in the Ce.

In order to further assess the extent of protein synthesis inhibition due to anisomycin local infusion, we used an autoradiographic technique based on the incorporation of a radiolabeled amino acid into newly synthesized proteins, as previously described. Inhibition of protein synthesis by anisomycin in the region of the LBA was approximately 50% of controls ($n = 12$ sections from 3 animals; $t (22) =10, P <0.05$). In contrast, inhibition of protein synthesis in the region of Ce was only approximately 20% ($t (22) = 5, P < 0.05$; Supplementary Fig. 5). These values are consistent with the immunohistochemical analyses above.

Our results suggest that although anisomycin causes some degree of protein synthesis inhibition in other areas of the brain, such as the Ce, the level of inhibition is not very substantial, ranging from 18 to 20%, a level typically considered far too low to induce amnesia. In further support of the view that the Ce is not critical for these effects is the fact that lesions of the Ce have no effect on the acquisition of fear. However, we cannot completely rule out the contribution of Ce in the observed effects until a complete study comparing intra-Ce and intra-LBA drug infusions will specifically address the role of each nucleus in freezing expression and induction of lability. The one other area that is most likely to show high protein synthesis inhibition is the striatum, because it is positioned dorsal to the amygdala and drugs will always diffuse back up a cannula track. However, we have already performed tests to see if infusions of anisomycin, dorsal to the
amygdala, block reconsolidation and found no effect \(^7\). Thus, anisomycin is not acting dorsal to the amygdala either. In conclusion, it is most likely that the drug effects reported here are due to an action in the LBA.

**Methods for the supplementary data**

**Dye spread:** In order to compare the spread of CNQX and ifenprodil, Neutral red dye (62.5 g per l), was added to the solutions of interest. The infusions were of the exact compound and parameters used in the behavioral studies. In order to visualize the spread of the drug in single infusions, a first group of rats received an intra-LBA infusion of CNQX+dye (2.5 g per 0.25 l per side) in one hemisphere and ifenprodil+dye (1 g per 0.25 l per side) in the contralateral LBA. To visualize the spread of the receptor antagonists in double infusions, a second group received an infusion of ifenprodil+dye in one LBA and CNQX+dye in the contralateral side, followed with bilateral infusion of anisomycin 10 min later. A third group was administered the identical treatment, with the difference that dye was added to the anisomycin solution to test the spread of anisomycin solution in double infusions. Animals were sacrificed 10 min after the last infusion and their brains were cut in 50 m coronal sections and slide-mounted for histological evaluation of dye diffusion. The optical intensity of the dye was taken along 3 different axes, 1- along axis of cannula penetration into the LBA, 2- 60 degrees from that axis, and 3- 120 degrees from cannula axis. Data were acquired for 1250 m on both sides of the tip of the cannula. The data were analyzed at every 100 m with a repeated measures ANOVA comparing drug treatment by distance from the cannula center for each angle.

**PTZ-induced seizure and Fos immunohistochemistry:** We performed immunohistochemical evaluation of anisomycin’s inhibition of an activity-induced protein, Fos. Each animal received vehicle in one LBA and anisomycin in the contralateral LBA. Rats were then systemically administered PTZ (Pentamethylenetetrazole, Sigma-Aldrich, 50 mg per kg) 10 minutes after the last infusion and returned to their home cages to be monitored for seizure \(^1\). Rats were transcardially perfused one hour later with 200 ml PBS (pH 7.4), followed with 400 ml 4% PFA in 0.1 M PB (pH 7.4). Brains were removed and post-fixed overnight in the same fixative and then processed for Fos immunohistochemistry using a standard protocol \(^8\). Brains were cut into 50 µm-thick free floating sections on a vibrating microtome (Leica Microsystems). Sections were treated for 20 min with 0.3 % H\(_2\)O\(_2\) in PBS, then washed in PBS, incubated for 1 hour with blocking solution (2 % normal serum, 2 % BSA, 0.5 % Triton X-100, in PBS) and then incubated overnight with the primary antibody at room temperature (anti-Fos, Oncogene). After 3 washes in PBS, tissue sections were incubated for 2 hr with the biotinylated secondary antibody (Vector Laboratories). After 3 washes in PBS, sections were incubated for 2 hr in avidin-biotin-peroxidase complex (ABC Elite kit, Vector Laboratories). Peroxidase was revealed by incubating sections for 2 min with 3-3' diaminobenzidine (Sigma-Aldrich; 0.5 mg per ml) and 0.002 % H\(_2\)O\(_2\) in PBS. Sections were then mounted on glass slides, dehydrated in ethanol solutions and toluene, and coverslipped (DPX Mountant, Fluka). The specificity of the signal was attested in control experiments by the absence of signal when the
primary antibody was omitted. Sections were examined at the microscope (Model IX81, Olympus, Japan) equipped with a CCD monochrome camera (Model ICX-285, Sony, Japan) and labeled cells were counted in the LBA and Ce using the Image Pro Plus software (MediaCybernetics) by observers blind to the origin of the sections. The background levels were set such that only unequivocally positive, darkly stained cells were counted. Anisomycin-induced inhibition of Fos expression in the LBA and Ce was assessed by comparing the number of Fos-positive nuclei in the anisomycin-infused LBA vs. vehicle-infused ones.

**Autoradiographic estimation of protein synthesis inhibition.** We evaluated anisomycin-induced protein synthesis inhibition using autoradiographic quantification of radiolabeled-leucine incorporation. Rats received intra-amygdala infusion of anisomycin in one hemisphere (62.5 g per 0.50 l Saline), and vehicle in the contralateral amygdala. Ten minutes later, rats were systemically injected with L-[1-14C]-Leucine (100 Ci per kg, GE Healthcare), administered through the jugular vein, under deep anesthesia. Rats were decapitated 60 minutes later; their brains were rapidly removed and frozen on dry ice. Brains were cut on a cryostat, and 30 m sections were collected on microscope slides and dried on a hot plate. These slides were apposed for 5 days to a Phosphor Screen (GE Healthcare) that was subsequently scanned with a Storm Scanner (GE Healthcare). The data were quantified using ImageQuant software (Amersham Biosciences).