Associative Pavlovian conditioning leads to an increase in spinophilin-immunoreactive dendritic spines in the lateral amygdala

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Abstract

Changes in dendritic spine number and shape are believed to reflect structural plasticity consequent to learning. Previous studies have strongly suggested that the dorsal subnucleus of the lateral amygdala is an important site of physiological plasticity in Pavlovian fear conditioning. In the present study, we examined the effect of auditory fear conditioning on dendritic spine numbers in the dorsal subnucleus of the lateral amygdala using an immunolabelling procedure to visualize the spine-associated protein spinophilin. Associatively conditioned rats that received paired tone and shock presentations had 35% more total spinophilin-immunoreactive spines than animals that had unpaired stimulation, consistent with the idea that changes in the number of dendritic spines occur during learning and account in part for memory.

Introduction

It has long been thought that learning involves the strengthening or formation of new connections between neurones that are engaged in the learning task (Ramón y Cajal, 1893). Although evidence for this proposal has been gathered in studies of invertebrates (Bailey & Kandel, 1993), attempts to study structural plasticity in the mammalian brain are hampered by poor understanding of the neural pathways that subserve learning. In recent years, however, considerable progress has been made in the elucidation of the key circuits and structures that mediate Pavlovian auditory fear conditioning, a behavioural task in which a neutral auditory stimulus [conditioned stimulus (CS)] acquires the ability to elicit fear responses following its association with an aversive unconditioned stimulus (US), such as an electric shock (LeDoux, 2000; Maren & Quirk, 2004; Tsvetkov *et al.*, 2004). Converging evidence from a variety of different kinds of studies points to the lateral amygdala (LA), and especially the dorsal subnucleus of the LA (LAd), as an important site of plasticity in fear conditioning (LeDoux, 2000; Lamprecht & LeDoux, 2004; Maren & Quirk, 2004; Tsvetkov *et al.*, 2004). Thus, the CS and US activate the same neurones in the LA (Romanski *et al.*, 1993; Li *et al.*, 1996), neural activity changes in the LA when the CS and US are paired (McKernan & Shinnick-Gallagher, 1997; Quirk *et al.*, 1997; Rogan *et al.*, 1997; Repa *et al.*, 2001), and damage to or pharmacological disruption of neural activity in the LA prevents fear conditioning (Schafe *et al.*, 2001).

Dendritic spines comprise the vast majority (∼88%) of postsynaptic partners for excitatory input into the LAd (Farb *et al.*, 1992), and are highly dynamic and motile structures (Fischer *et al.*, 1998; Engert & Bonhoeffer, 1999; Toni *et al.*, 1999; Sorra & Harris, 2000). Alterations in their shape or number might provide a cellular correlate for long-term memory (Greenough & Bailey, 1988; Bailey & Kandel, 1993; Lamprecht & LeDoux, 2004; Shors, 2004). Numerous studies support the idea that learning is accompanied by increases in the efficacy of neurotransmission at relevant excitatory synapses (McKernan & Shinnick-Gallagher, 1997; Rogan *et al.*, 1997; Geinisman, 2000; Tsvetkov *et al.*, 2002), alterations of synaptic structure (Geinisman, 2000), as well as increases in spine number or density (Moser *et al.*, 1994; Leuner *et al.*, 2003; Knafo *et al.*, 2001). Whether such changes occur in the LA is not known.

In the present study, we examined whether fear conditioning would produce alterations in dendritic spine number in the LAd. Spinophilin binds to F-actin and protein phosphatase-1 (Allen *et al.*, 1997; Feng *et al.*, 2000), which are all highly enriched in dendritic spine heads. Moreover, spinophilin has a remarkably distinct anatomical localization to dendritic spines in the brains of rodent and monkey (Allen *et al.*, 1997; Hao *et al.*, 2003; Tang *et al.*, 2004). We employed spinophilin as a marker of spines with high-resolution microscopy and stereological probes to obtain an estimate of the total spinophilin-immunoreactive (Sp-ir) spine number in the LAd and central nucleus of the amygdala (CeA) following fear conditioning.

Materials and methods

Animals

Adult male Sprague-Dawley rats (Hilltop Laboratories, Scottsdale, PA, USA) weighing 250–300 g at the beginning of the procedures were used. Animals were housed individually in plastic Nalgene cages and

maintained on a 12/12-h light/dark cycle. Food and water were provided *ad libitum*. All procedures were performed in accordance with the NIH Guide for the Care and Use of Experimental Animals, and were approved by the New York University and Mount Sinai School of Medicine Laboratory Animal Care and Use Committees.

Experiment 1

For the light and electron microscopic quantitative analysis of the total number of Sp-ir dendritic spines in the LAd and CeA, animals were randomly allocated to CS–US paired, unpaired and naive groups. On day 1, all rats were placed in the conditioning chamber (Colbourn Instruments, Allentown, PA, USA) for 30 min and allowed to habituate to the environment. On day 2 the rats underwent training. Animals in the paired group received five pairings of a tone (5 kHz, 80 dB, 20 s) CS that co-terminated with a footshock (1 mA, 0.5 s) US, with an average intertrial interval between pairings of 120 s. The unpaired group received non-overlapping CS–US presentations, such that the US was presented 1 min before the CS, with an intertrial interval that was random between unpaired presentations. The naive group was placed in the chamber but did not receive either the CS or US.

Experiment 2

In a separate experiment, rats were tested 1 day after conditioning to ensure that CS–US pairing resulted in associatively conditioned fear responses. Rats were again randomly assigned to paired, unpaired and naive groups. These animals were placed in a novel chamber on the day following conditioning and behaviour was recorded on videotape for later manual scoring of freezing behaviour offline (e.g. Lamprecht *et al.*, 2002).

Perfusion and tissue processing

To ensure that the analysis was performed blind, each animal was coded by an independent observer prior to perfusion and the code was not broken until the analysis was completed. On day 3, 24 h after conditioning, rats were anaesthetized with a lethal dose chloral hydrate. They were then perfused transcardially with cold 1% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 1 min, followed by fixation for 10–12 min with cold 4% paraformaldehyde containing 0.125% glutaraldehyde in PBS at a flow rate of 55 mL/min. Brains were dissected and postfixed overnight at 4 °C in the same fixative. Brains were sectioned serially on a Vibratome (Leica, Vienna, Austria) in a bath of cold PBS at 50 μm throughout the rostrocaudal extent of the amygdala.

Spinophilin immunohistochemistry

Sections were thoroughly rinsed in 0.01 M PBS (pH 7.4) containing 0.3% Triton X-100, and then incubated in the blocking buffer containing 0.3% Triton X-100, 0.1% cold water fish gelatin (Electron Microscopy Sciences, Fort Washington, PA, USA), 0.5% bovine serum albumin (Sigma, St Louis, MO, USA) and 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The spinophilin antibody (graciously provided by Drs Patrick Allen and Paul Greengard of Rockefeller University) recognizes residues 367–390 of the 140-kDa spinophilin protein and has been affinity purified as described in Allen *et al.* (1997). Antibody specificity has been established in spinophilin knockout mice (Feng *et al.*, 2000). Free-floating sections were incubated with a rabbit anti-spinophilin primary antibody (diluted 1 : 240 000 in the above-described blocking buffer; Hao *et al.*, 2003; Tang *et al.*, 2004) for 72 h at 4 °C, washed and incubated in secondary antibody labelled with immunogold (goat anti-rabbit IgG, ultrasmall EM grade, Electron Microscopy Sciences) in the above diluent for 3 h at room temperature. Sections were washed and postfixed with 2% glutaraldehyde in 0.01 M PBS for 10 min, and rinsed with distilled water until silver enhancement was performed with an Aurion R-Rent-Lm kit (Electron Microscopy Sciences) for 15–25 min at room temperature. All animals used in Experiment 1 (for light and electron microscopic analyses) were processed together and reacted in the silver-enhancement reagent for the same length of time. After washing, the sections were mounted, dehydrated through ascending series of ethanol and xylene, and coverslipped with Biomount (Electron Microscopy Sciences).

Pre-embedding electron microscopy

Silver-enhanced nanogold localization of spinophilin, similar to that used for light microscopy, was prepared for cryosubstitution electron microscopy in order to verify the appropriateness of spinophilin as a spine marker in the LAd following paired ($n = 2$) and unpaired ($n = 2$) conditioning. Although several studies have validated that spinophilin is localized to dendritic spines in the rodent and monkey brain (Allen *et al.*, 1997; Hao *et al.*, 2003; Tang *et al.*, 2004), its localization to dendritic spines of the LAd was verified in CS–US paired and unpaired animals. After processing for spinophilin immunoreactivity as described above, freeze substitution and low-temperature embedding of specimens were performed as described previously (van Lookeren Campagne *et al.*, 1991; Hjelle *et al.*, 1994; Chaudhry *et al.*, 1995; Hao *et al.*, 2003; Tang *et al.*, 2004). Slices were cryoprotected by immersion in increasing concentrations of glycerol in phosphate buffer (10, 20 and 30%) and plunged rapidly into liquid propane cooled by liquid nitrogen (−190 °C) in a Universal Cryofixation KF80 (Reichert-Jung, Vienna, Austria). The samples were immersed in 1.5% uranyl acetate (for en-bloc fixation) in anhydrous methanol (−90 °C, 24 h) in a cryosubstitution Automated Freeze Substitution unit (Leica). The temperature was increased in steps of 4 °C/h from −90 to −45 °C. The samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences) at −45 °C with a progressive increase in the ratio of resin to methanol for 1 h each, followed by pure Lowicryl (overnight). Polymerization was performed with ultraviolet light (360 nm) at 45 °C for 48 h, followed by 24 h at room temperature. The dorsal portion of the LA was identified and sectioned. Serial ultrathin sections (70–80 nm thick) were cut with a diamond knife on a Reichert-Jung ultramicrotome and mounted on formvar-coated slot grids for analysis. A total of 100 asymmetric synapses per animals were analysed in single ultrathin sections for Sp-ir particles. In the three-dimensional reconstruction of Sp-ir synapses, a total of 100 axospinous synapses were collected and imaged through series of five ultrathin sections to determine the total percentage of Sp-ir synapses. A synapse was considered immunoreactive if it contained silver-intensified product in any one section within the series of five sections. The sections were analysed on a 1200EX electron microscope (JEOL, Tokyo, Japan). Images were captured using a high-resolution Advantage CCD camera (Advanced Microscopy Techniques Corporation, Danvers, MA, USA). Control experiments omitting the primary antibody were performed and no labelling was observed.

Histology

The boundaries of the LAd and CeA were defined by counterstaining adjacent sections for cresyl violet and acetylcholinesterase. Briefly, the

acetylcholinesterase stain was carried out by incubating slide-mounted sections in 50 mM sodium acetate buffer (pH 5.0), 4 mM copper sulphate, 16 mM glycine, 4 mM acetylthiocholine iodide and 0.1 mM ethopropazine for 6 h. After incubation, the slides were immersed into a developing solution (1% sodium sulphide, pH 7.5) for 3–5 min, rinsed in dH$_2$O, dehydrated and coverslipped with Permount.

Stereological analysis

All quantitative analyses were performed by an observer unaware of the behavioural treatment. A stereological method, the optical disector, and Cavalieri principle (Gundersen *et al.*, 1988; Schmitz & Hof, 2005) were used to estimate the total number of Sp-ir spines in the LAd and CeA. All quantitative analyses were performed using a computer-assisted morphometry system consisting of a Zeiss Axioplan 2 photomicroscope equipped with an Applied Scientific Instrumentation MS-2000 XYZ computer-controlled motorized stage, a DAGE-MTI DC-330 video camera, a Gateway microcomputer, and STEREO INVESTIGATOR morphometry and stereology software (MicroBright-Field, Wiliston, VT, USA). The contours of the LAd and CeA were traced at 2.5× magnification using adjacent series of Nissl- and acetylcholinesterase-stained sections, and were based on the rat atlas of Paxinos & Watson (1986) and other previous work (Pitkanen *et al.*, 1997). Optical disector counting frames were placed in a systematic-random fashion in the delineated regions of the sections with constant intervals in the *x*- and *y*-axes. The *x* and *y* distances between sampling frames were set at 90 μm for the LAd. The counting frame width and height were 1.5 μm, and the *z*-axis thickness of the counting frame was 3 μm. The area sampling fraction was $(1.5 \times 1.5 \, \mu m)/(90 \times 90 \, \mu m) = 9/32\,400$. An oil-immersion objective (100×/1.4 NA) was used for counting. A 0.5-μm 'guard zone' was placed at the top surface of the sections. Counting was performed with the optical disector technique through a depth of 3 μm (the height of the disector). The total Sp-ir spine number in each area was calculated by using the density value obtained from the disector multiplied by the volume for the LAd ($N_v \times V_{REF}$), which was estimated using the Cavalieri principle (Gundersen *et al.*, 1988; Schmitz & Hof, 2005). Moreover, the LAd and CeA volumes were assessed independently to verify that there was no treatment effect on volume.

Statistical analysis

The group data for Sp-ir spine density, volume, and total numbers in the LAd and CeA were compared with a one-way ANOVA, followed by posthoc pairwise comparisons using a Bonferroni correction. The coefficients of variation (coefficient of variation = SD/mean) and error for the stereological estimates were calculated as previously described (Schmitz & Hof, 2000). Differences were considered significant at $P < 0.05$ for the F omnibus and $P < 0.0167$ (α level for Bonferroni) for each posthoc comparison. All values were expressed as mean ± SEM.

Results

Neuronal distribution of spinophilin in the dorsal subnucleus of the lateral amygdala

Adjacent series of sections were processed for spinophilin immuno-staining, acetylcholinesterase histochemistry and Nissl as the latter two delimit the anatomical boundaries of the LAd (Paxinos & Watson, 1986; Pitkanen *et al.*, 1997). Light microscopic examination of silver-enhanced immunogold labelling for spinophilin revealed a diffuse pattern of staining, with more sparsely stained regions in the dorsal-most portion of the LAd, which is located under the vertex formed between the amygdalostriatal area and external capsule. At higher magnification, Sp-ir spines appeared in an abundant distribution of numerous small dark puncta throughout the LAd (Fig. 1). Cell bodies (10–15 μm diameter) were apparent at varying focal planes through-out the Sp-ir profile by their lack of punctate staining. Qualitative analysis of the light microscopic materials from the paired CS–US and unpaired CS–US control animals did not reveal any frank anatomical differences between them in staining pattern or intensity.

Ultrastructural distribution of spinophilin in the dorsal subnucleus of the lateral amygdala is predominantly in dendritic spines

The pre-embedding immunoelectron microscopic analysis of spino-philin revealed a distinct localization to dendritic spines in the LAd (Fig. 2). Spinophilin was found to be present mainly in the region of the spine cytoplasm, either adjacent or subjacent to the postsynaptic specialization. Frequently, spinophilin appeared as a clustering of silver-coated ultrasmall gold particles within the spine neck and especially the spine head and disappeared abruptly at the junction of the spine neck and dendritic shaft. Previous work examining the proportion of excitatory synapses in the LA in single ultrathin EM sections established that ∼88% of glutamatergic terminals synapse onto dendritic spines and the remaining 12% synapse primarily onto small dendritic shafts (Farb *et al.*, 1992). Careful quantitative analysis of multiple fields in single ultrathin EM sections suggested that the great majority (73%) of visible spines were immunoreactive for spinophilin. Axodendritic synapses were seldom observed in the LAd (1 : 5 ratio relative to axospinous synapses). Nonetheless, of the axodendritic synapses that were identified in the LAd, 22% were immunoreactive for spinophilin. There were occasional single gold particles present in dendritic shafts but these were below the level of detection by the light microscope and would not have been included in the stereological Sp-ir spine counts. No presynaptic labelling of spinophilin was evident in any of the EM material examined. A quantitative, serial section analysis of 100 randomly chosen axospinous synapses was carried out to determine the percentage that contained spinophilin immunoreactivity in CS–US paired and unpaired animals. Although some spines were observed to lack spinophilin immunoreactivity in one plane of sectioning, 89% of the synapses in this analysis contained robust spinophilin labelling in at least one section in the series, suggesting that the overwhelming majority of spines in the LAd contained spinophilin (Fig. 2D). In these analyses, no differences were observed in the percentage of Sp-ir axospinous or axodendritic synapses in the LAd between CS–US paired and unpaired groups.

Higher spinophilin-immunoreactive spine density and number in the dorsal subnucleus of the lateral amygdala in paired vs. unpaired groups

There was a main effect of behavioural treatment on total Sp-ir spine numbers in the LAd ($F_{2,9} = 4.9$; $P = 0.04$). As illustrated in Fig. 3A, fear conditioning (paired group, $4.6 \pm 0.1 \times 10^8$ spines) resulted in a 35% increase in Sp-ir spine number in the LAd relative to unpaired controls ($3.4 \pm 0.8 \times 10^8$ spines; $P = 0.01$). However, animals in the naive group that were exposed to the conditioning chamber and received no stimuli did not significantly differ from either paired
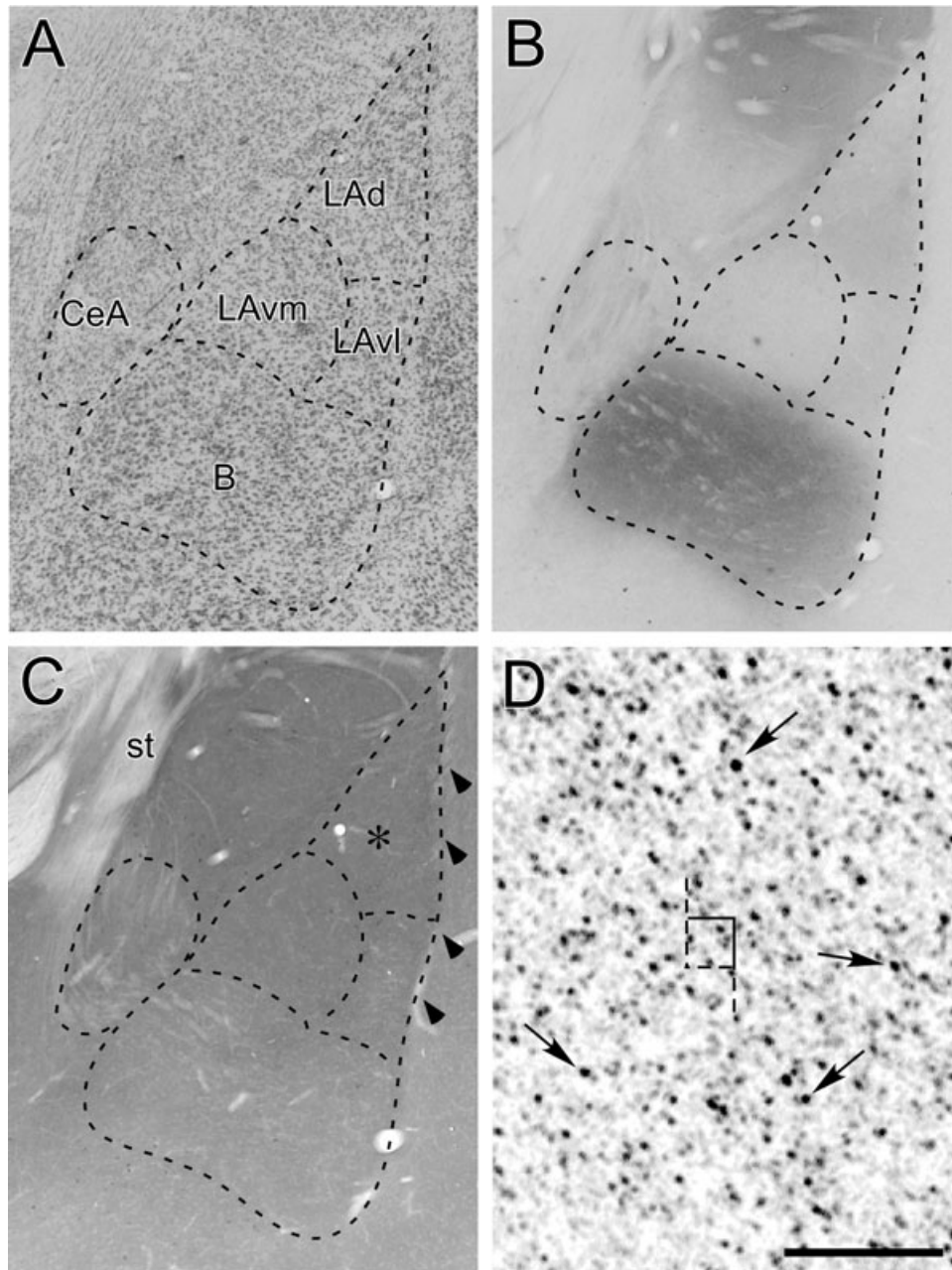
FIG. 1. Spinophilin immunolabelling in the dorsal subnucleus of the lateral amygdala (LAd) under light microscopy. Photomicrographs of Nissl (A) and acetylcholinesterase (B) staining in the rat basolateral amygdala. The boundary for the LAd was delineated based upon previous work (Paxinos & Watson, 1986; Pitkanen *et al.*, 1997; Repa *et al.*, 2001). These borders were overlaid on adjacent series of spinophilin-immunostained sections for quantification of Sp-ir spine numbers at high magnification. LAvm, lateral nucleus of the amygdala, ventral medial subdivision; LAvl, lateral nucleus of the amygdala, ventral lateral subdivsion. (B) Basal nucleus of the amygdala. CeA, central nucleus of the amygdala. (C) Photomicrograph of spinophilin immunolabelling at low magnification. There is intense spinophilin staining throughout the neuropil but none in the white matter [e.g. stria terminalis (st); external capsule, arrowheads]. The asterisk in C corresponds to the region of higher magnification in D. (D) High resolution image showing spinophilin-immunoreactive profiles in the LAd at the level of magnification used for stereological analysis. An unbiased counting frame is shown. The dashed and solid borders represent the exclusion and inclusion lines, respectively. Spines not visualized at their optimal focal plane appear blurred. Spines were counted as they came into focus within the height of the optical disector and within the counting frame when moving the focal plane continuously through the section. A spine was considered to be within the counting frame if it was entirely or partially within the counting frame, while neither touching nor intersecting the exclusion line when in focus. Arrows point to examples of spines that are in the plane of focus. Scale bar: 0.5 mm in A–C and 5 μm in D.

($P = 0.2$) or unpaired ($P = 0.1$) treatments (Table 1, Fig. 3A). Analysis of the Sp-ir spine density in the LAd obtained from the optical disector also yielded a main effect ($F_{2,9} = 5.1$; $P = 0.03$). Fear conditioning resulted in a significant increase in Sp-ir spine densities (paired group, 0.77 ± 0.02 spines/μm^3) compared with unpaired controls (0.62 ± 0.3 spines/μm^3; $P = 0.01$; Table 1, Fig. 3B). Sp-ir spine densities in the LAd for the naive group were not significantly different from either paired ($P = 0.2$) or unpaired ($P = 0.1$) treatments (Fig. 3B). No effect was observed for LAd volume as a function of treatment condition ($F_{2,9} = 0.5$; $P = 0.6$; Table 1, Fig. 3C).
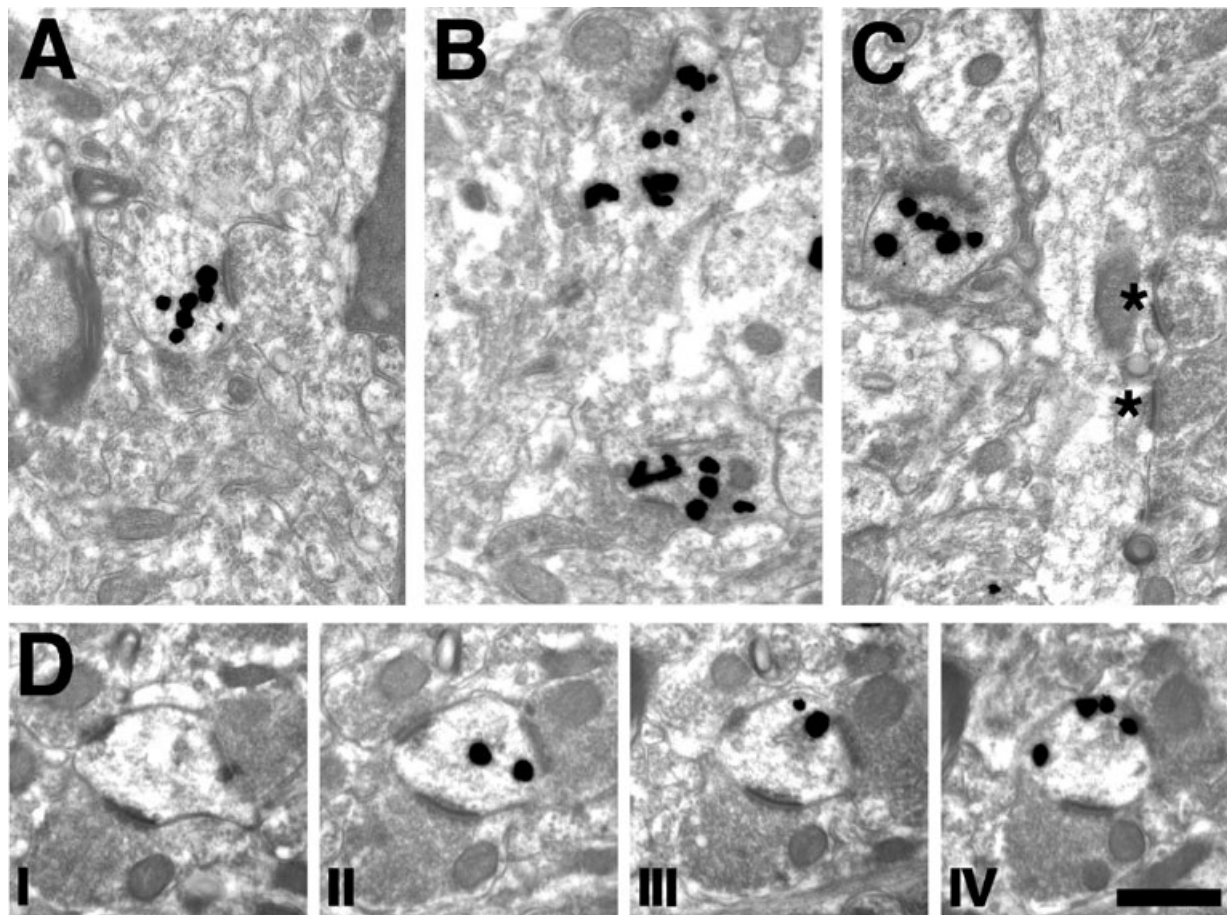
FIG. 2. Ultrastructural localization of spinophilin to dendritic spines. Pre-embedding silver-intensified electron microscopic (EM) image demonstrating spinophilin immunoreactivity in the dorsal subnucleus of the lateral amygdala. (A–C) Images of spinophilin immunoreactivity within and throughout axospinous synapses of both conditioned stimulus–unconditioned stimulus paired and unpaired animals. Silver-intensified particles are seen throughout spines and in close apposition with the postsynaptic density. Asterisks indicate spinophilin-immunonegative axodendritic synapses in C. (D) (I–IV) Serially sectioned EM images demonstrate that, although most sections through a single axospinous synapses contain spinophilin-immunoreactive particles, a small population is revealed to be immunonegative. Scale bar, 0.5 µm.

Estimates of total Sp-ir spine numbers were also made in the CeA to assess whether the effect of fear conditioning is specific to the LAd. No overall or group difference for total Sp-ir spine numbers ($F_{2,18} < 0.1$; $P = 0.9$), density ($F_{2,18} = 0.4$; $P = 0.7$) or volume ($F_{2,18} < 0.1$; $P = 0.9$) in the CeA was present after fear conditioning (Table 1, Fig. 3).

Paired presentation of tone and foot shock led to associatively conditioned fear responses

To verify that our procedures produced behavioural differences between the groups, animals in the fear testing groups received five presentations of CS alone at 24 h after paired, unpaired or naive training. Paired animals showed a conditioned freezing response of $61.5 \pm 4.9\%$ during the 20-s CS presentation. Unpaired and naive animals showed a conditioned freezing response of 6.5 ± 5.2 and $0.25 \pm 0.25\%$, respectively, during the 20-s CS presentation. Statistical tests revealed a significant difference in the paired group relative to the unpaired and naive animals ($F_{2,9} = 65.46$; $P < 0.0001$), thus confirming that the training conditions were capable of inducing an associatively conditioned memory to the CS tone (Fig. 3D).

Discussion

The aim of the present study was to examine whether the number of dendritic spines in the LAd is altered following auditory fear conditioning in rats. We observed a 35% increase in the number of Sp-ir dendritic spines following fear conditioning in the paired CS–US animals compared with unpaired CS–US animals. Furthermore, we found that fear conditioning did not result in any change in Sp-ir spine number in the CeA, suggesting specificity within the amygdala for learning-induced structural plasticity. The naive control group (placed in the conditioning chamber receiving neither CS nor US) was also expected to yield Sp-ir spine estimates in the LAd similar to the unpaired group. However, the estimates of Sp-ir spine numbers in the LAd of these animals were not significantly higher than in unpaired groups and lower than in paired groups. Nonetheless, these results lend support to the possibility that at least one of the long-term structural changes in the brain that underlies associative Pavlovian conditioning is a change in the number of axospinous synapses in the LAd.

Methodological considerations

The results of the present study are based upon a high-resolution light microscopic analysis and quantification of individual spines as
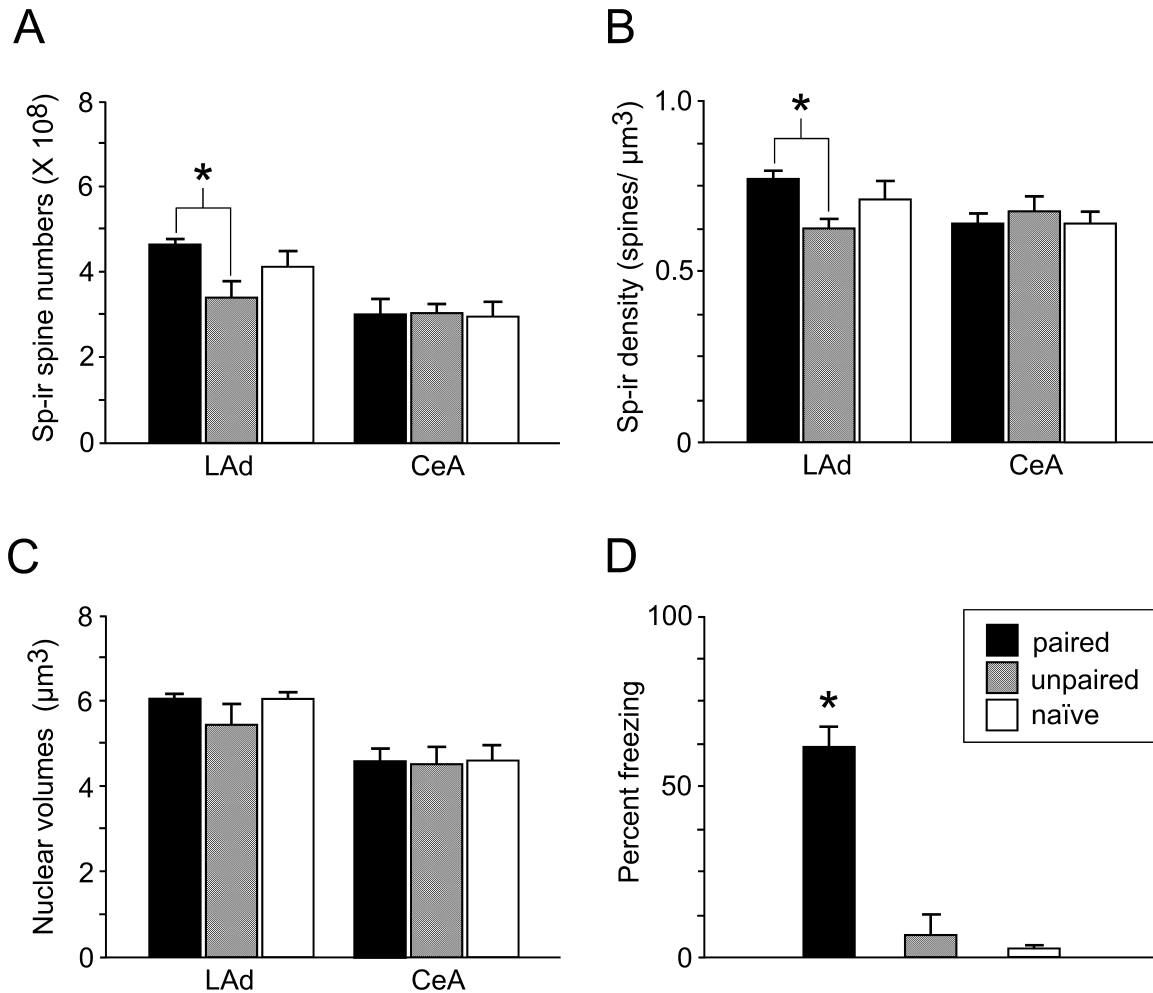
FIG. 3. Fear conditioning results in a significant increase in spinophilin-immunoreactive (Sp-ir) dendritic spine density and number in the dorsal subnucleus of the lateral amygdala (LAd) 24 h after fear conditioning. The light microscopic quantitative analysis showed that fear conditioning significantly increased LAd Sp-ir spine number (A) and density (B) in animals perfused 24 h after a training session of five conditioned stimulus–unconditioned stimulus (CS–US) pairings compared with unpaired controls, whereas no effects of fear conditioning on Sp-ir spine number and density were evident in the central nucleus of the amygdala (CeA). There was no effect of treatment on volume for either the LAd or CeA (C). (D) The same conditioning also results in memory for the CS tone in paired but not in unpaired and naive animals when tested 24 h following CS–US training. Asterisks in A and B denote a statistically significant difference ($P = 0.01$) between the paired and unpaired groups. Asterisk in D denotes $P < 0.0001$. Values represent mean + SEM.

visualized by spinophilin immunogold labelling followed by the silver enhancement method, a technique that is traditionally employed in EM studies. The immunogold labelling procedure offers a fundamental advantage over immunoperoxidase staining for certain types of quantitative anatomical studies because it allows for a more discrete localization and quantification of small proteins that are densely packed, such as observed within dendritic spines. The punctate immunostaining profile revealed under light microscopy allows for the estimation of total numbers of Sp-ir spines in anatomically predetermined regions using unbiased stereology. As the LAd has received much attention as a putative site of plasticity for Pavlovian fear conditioning (LeDoux, 2000; Lamprecht & LeDoux, 2004; Maren & Quirk, 2004; Tsvetkov et al., 2004), this method provides a tractable means of addressing the relationship between learning and spine number that may otherwise be impossible with Golgi or EM approaches.

One caveat of our study is the degree to which spinophilin labels all dendritic spines and only spines. Spinophilin binds to proteins that are highly enriched in dendritic spine heads, such as F-actin and protein phosphatase-1 (Allen et al., 1997; Feng et al., 2000). Spinophilin is also important for the formation of dendritic spines and regulation of glutamatergic synaptic transmission (Feng et al., 2000), suggesting that it is essential for mature spine function. One possibility is that spinophilin may be regulated locally within spines as a function of the behavioural treatment, thus confounding our interpretation of structural shifts in spine numbers in the LAd following Pavlovian fear learning. In paired and unpaired groups that were examined in the EM analysis, no differences were observed in the subcellular distribution of spinophilin and neither did the behavioural treatment result in changes in the proportion of Sp-ir axospinous or axodendritic synapses in the LAd. Our pre-embedding EM analysis demonstrated that clustered gold particles rarely label profiles other than spines, with labelling typically ending abruptly at the junction of the spine neck and dendritic shaft. Most importantly, the serial section analysis that was performed on material equivalent to that used for the quantitative light microscopic analysis showed that a majority (89%) of LAd spines contain abundant spinophilin. Finally, other studies support our interpretation that learning is correlated with an increase in spine number and not in spinophilin levels in spines (Greenough & Bailey, 1988; Moser et al., 1994; Leuner et al., 2003; Knafo et al., 2001).

That our data provide further evidence of the specificity of spinophilin to dendritic spines in the mammalian brain does not

TABLE 1. Estimates of total spinophilin-immunoreactive (Sp-ir) spine numbers[†]

Animals	LAd				CeA			
	N_V	V_{REF} (×10^8)	Numbers (×10^8)	CE	N_V	V_{REF} (×10^8)	Numbers (×10^8)	CE
CS–US paired								
1	0.80	6.00	4.8	0.03	0.61	4.23	2.58	0.05
2	0.72	6.30	4.54	0.03	0.60	4.06	2.43	0.05
3	0.77	5.90	4.53	0.04	0.65	4.94	3.21	0.05
4	0.79	6.00	4.74	0.04	0.70	5.29	3.7	0.05
Mean	0.77 (0.05)*	6.05 (0.03)	4.65 (0.03)*	–	0.64 (0.07)	4.63 (0.13)	2.98 (0.20)	–
CS–US unpaired								
1	0.66	6.60	4.36	0.04	0.76	3.60	2.75	0.05
2	0.64	5.10	3.26	0.03	0.72	4.43	3.2	0.04
3	0.64	5.40	3.46	0.05	0.64	5.40	3.44	0.05
4	0.55	4.60	2.53	0.05	0.59	4.70	2.78	0.05
Mean	0.62 (0.08)	5.43 (0.16)	3.40 (0.22)		0.68 (0.11)	4.53 (0.16)	3.04 (0.11)	
Naive								
1	0.69	6.10	3.58	0.05	0.68	4.65	3.16	0.05
2	0.82	6.00	4.92	0.03	0.60	4.20	2.52	0.04
3	0.74	5.70	4.22	0.03	0.71	5.42	3.85	0.05
4	0.59	6.30	3.72	0.05	0.58	4.09	2.37	0.05
Mean	0.71 (0.14)	6.03 (0.04)	4.11 (0.15)		0.64 (0.10)	4.59 (0.14)	2.97 (0.23)	

[†]Total spine numbers are the product of the estimated density (optical disector) of Sp-ir puncta and estimated volume (Cavalieri estimator) for the region analysed ($N_V \times V_{REF}$). The numbers in parentheses are the interindividual coefficients of variation; CE, coefficient of error for each estimate; CeA, central nucleus of the amygdala; LAd, dorsal subnucleus of the lateral amygdala. *$P = 0.01$, compared with conditioned stimulus–unconditioned stimulus (CS–US) unpaired.

preclude its distribution to other synapse types or non-synaptic elements. For instance, we found that 22% of axodendritic synapses examined in the LAd contained spinophilin (analysis performed in single EM sections). As the overwhelming majority of excitatory synapses in the LA are axospinous (~88%; Farb *et al.*, 1992), even if a serial section analysis revealed spinophilin labelling in as much as half of the entire population of axodendritic synapses, it would still comprise a small minority compared with the total population of Sp-ir axospinous synapses. Moreover, we did observe an occasional single spinophilin particle in dendrites that seemed not to be associated with any postsynaptic specialization. It could not be ruled out that this dendritic representation of spinophilin was non-synaptic, as it may have been associated with axodendritic synapses that would only be revealed by serial sectioning. Although we did not observe any spinophilin labelling in axonal or glial elements, one study has shown some such localization in the primate prefrontal cortex (Muly *et al.*, 2004).

Relationship between dendritic spine numbers in the dorsal subnucleus of the lateral amygdala and associative Pavlovian conditioning

Partly consistent with our hypothesis, CS–US paired animals showed a significant increase in dendritic spine numbers in the LAd compared with only unpaired but not naive animals. It was thought that a follow-up study would address this variability in the naive group results, and also replicate the effect between paired and unpaired treatments. However, in our repetition of this experiment, the total Sp-ir spine number in the LAd reflected the same trend as the first experiment (data not shown), as the naive group remained in the middle range between the paired and unpaired groups. Our interpretation is that the naive group may provide a baseline value and that the paired and unpaired conditions vary about this baseline. Whether this outcome reflects a natural variation in the rat LAd dendritic spine number or the failure of our technique to reveal statistical significance between naive-paired and naive-unpaired animals cannot be ascertained at the

present time. Another possible interpretation of this study is that there was not an increase in spine number in the paired group but instead a reduction in the unpaired group. Such a reduction in dendritic spine number in the LAd could perhaps result from the stressful nature of the unpaired presentation of the CS and US. However, the available evidence suggests that acutely stressful experiences *per se* actually increase spine density in the amygdala and hippocampus (Shors *et al.*, 2001; Mitra *et al.*, 2005).

We also assessed whether a learning-induced alteration in Sp-ir spine number in the LAd would be accompanied by a corresponding change in the CeA. The CeA controls the expression of fear responses (LeDoux *et al.*, 1988; Davis, 1992) and has also been implicated as a site of plasticity for Pavlovian fear conditioning (see e.g. Sah & Lopez de Armentia, 2003; Paré *et al.*, 2004). That we did not observe any significant difference in Sp-ir spine density or total numbers for the CeA suggests that the specificity of the effects of the paired vs. unpaired groups is limited to the LAd. Based on the stereological data of Sp-ir spine counts, the interpretation that spine number in the CeA remains unchanged following auditory fear conditioning must be made with caution. Although the principal cell types in the LAd are very spiny large pyramidal-like neurones, the CeA contains neurones that more closely resemble stellate neurones in the striatum, with long dendrites that branch sparingly and have a moderate number of spines (McDonald, 1982a,b). Although we did not validate the use of spinophilin as a spine marker for the CeA in an EM analysis, previous work suggests that it is also specific to dendritic spines in such neurones (Allen *et al.*, 1997; Yan *et al.*, 1999).

It has previously been shown that neurones in the ventral LAd exhibit long-latency responses to an auditory CS that may contribute to the storage of fear memories, whereas neurones in the dorsal LAd exhibit short-latency responses and may be involved in their initiation (Repa *et al.*, 2001; Radwanska *et al.*, 2002). To assess whether learning-induced plasticity was limited to a portion of the LA, a separate quantitative analysis was performed whereby the LAd was divided into a dorsal and ventral region. Our data suggest that the ventral LAd was the critical subregion that showed

an increase in Sp-ir spine number in paired relative to unpaired animals, whereas no effect was evident in the dorsal tip of the LAd (data not shown). Unfortunately, the lack of precise cytoarchitectonic criteria for the dorsal and ventral LAd did not allow for their presentation.

Functional implications

Although not without controversy (for review see Yuste & Bonhoeffer, 2004), much data indicate that spines are constantly in a labile state (Kirov et al., 1999; Yuste & Bonhoeffer, 2004; Petrak et al., 2005). Spine numbers change rapidly in response to oestrus, temperature, oxygen availability and long-term potentiation (Woolley & McEwen, 1994; Kirov et al., 2004; Yuste & Bonhoeffer, 2004; Zhang et al., 2005). That our data demonstrate an increase in the number of spines in the LAd following associative Pavlovian conditioning could provide a structural analogue of the hypothesis of Hebb (1949) that connection strengths are increased between correlated units and decreased for uncorrelated units. However, it is still unclear whether these structural changes are long-lasting and representative of memory storage or temporary. Although some evidence supports the idea that structural changes in synapse number or density might provide a correlate for learning (Greenough & Bailey, 1988; Bailey & Kandel, 1993; Moser et al., 1994; Leuner et al., 2003; Knafo et al., 2001), others have found structural modifications in excitatory synapses following learning but no overall change in synapse number (Rusakov et al., 1997; Geinisman, 2000). One possibility that would explain these discrepancies is that learning may be accompanied by a net rearrangement of excitatory synapses rather than a lasting increase in their number. In this regard, O'Malley et al. (1998, 2000) showed that hippocampal-dependent learning is accompanied by transient increases in synapse number in the dentate gyrus at 6 h after training that subsided after 72 h. Despite these advancements in understanding of the relationship between structural plasticity and learning, it is still unclear whether learning-induced synapse formation mediates information storage or represents a more general property of the engaged parts of the brain circuitry underlying these behaviours (e.g. see Moser, 1999).

Another possibility is that a memory trace may not necessarily be driven by an increase in spine numbers above basal levels but rather as a modification in their number relative to learning. In this case, our results are consistent with the idea that dendritic spines may be regulated in such a way that spines with strengthened synapses are preserved, whereas spines that are activated but not strengthened are reduced (Sorra & Harris, 2000). A homeostatic mechanism may thus operate to limit dendritic spine numbers, such that differential changes in spine numbers in concert with changes in spine morphology may encode memory (Harris et al., 2003). If all learning were driven by an increase in spine number then saturation would be reached. Therefore, memory may be encoded by the relative change in spine numbers, whereby differences in numbers of spines may reflect the direction of learning experienced by the animal as reflected by our data. In this regard, the present data may reflect a subtle increase in spine number during CS–US pairing and a correspondingly small decrease during CS–US unpairing. Although neither trend is significantly different from the naive group, such differences are evident when they are compared directly.

One study to date has shown learning deficits in spinophilin knockout mice (Stafstrom-Davis et al., 2001), which supports a role for spinophilin in learning-dependent cytoskeletal changes involved in spine formation. The RhoGTPases are another example of actin-binding proteins whose activity-dependent regulation of the spine cytoskeleton may be important in spine formation during fear conditioning (Nakayama et al., 2000; Lamprecht & LeDoux, 2004). Inhibition of the downstream Rho-associated kinase in the LA prevented long-term fear memory formation (Lamprecht et al., 2002). The observation of a 35% increase in dendritic spine number in the LAd after fear conditioning and the known role of spinophilin, as well as other cytoskeletal-associated spine proteins for learning, strongly support the possibility that one type of structural change that accompanies learning is the formation of Sp-ir dendritic spines.

Abbreviations

CeA, central nucleus of the amygdala; CS, conditioned stimulus; EM, electron microscopy; LA, lateral amygdala; LAd, dorsal subnucleus of the lateral amygdala; PBS, phosphate-buffered saline; Sp-ir, spinophilin-immunoreactive; US, unconditioned stimulus.

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