Zinc-enriched amygdalo- and hippocampo-cortical connections to the inferotemporal cortices in macaque monkey

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Abstract

Synaptic zinc (Zn), a co-factor in some glutamatergic synapses, has been implicated in plasticity effects, as well as in several excitotoxic and other pathophysiological conditions. In this study, we provide information about the distribution of Zn in inferotemporal cortex, a region at the interface of the visual and hippocampal networks. In brief, we found a lateral to medial increase in Zn, where TEad, a unimodal visual area, showed low levels of Zn; TEav, intermediate levels; and perirhinal cortex, a multimodal limbic area, high levels. The distribution of parvalbumin, a calcium binding protein, showed a reverse gradient to that of Zn. The neurons of origin of the Zn+ termination were identified by making intracortical injections sodium selenite (Na2SeO3). This substance interacts with Zn to form precipitates of ZnSe and in this form is transported retrogradely to the soma. A mixed population of labeled neurons was visualized, which included Zn+ neurons in CA1 of the hippocampus and in several amygdala subnuclei. In CA1, Zn+ neurons were restricted to the upper part of stratum pyramidale. Zn is thought to contribute to activity-dependent synaptic plasticity. The specifically high level in perirhinal cortex, and its origin from neurons in CA1 and the amygdala, may relate to cellular events involved in visual long-term memory formation.

Keywords: Parvalbumin; Gradient; Plasticity; Limbic; Theta rhythm

1. Introduction

Zinc-positive (Zn+) terminations occur widely throughout cortical areas, as has been shown in both rodent (Garrett and Slomianka, 1992; Perez-Clausell, 1996) and primates (Carmichael and Price, 1994; Franco-Pons et al., 2000; Ichinohe and Rockland, 2004). In the monkey, they are particularly dense in orbitofrontal and parahippocampal regions, and in much of the pre-Rolandic frontal cortex. In these and several other areas, a further specialization is seen in the form of distinct patches or honeycomb at the border of layers 1 and 2 (Ichinohe and Rockland, 2003, 2004). Variations in distribution of this Zn+ system are potentially important (1) as a marker of area or regional boundaries, and (2) as a clue to functional specialization. That is, Zn is believed to act in a neuromodulatory role, with significant influence on plasticity effects. It is released in an activity- and calcium-dependent fashion, and interacts with many...
receptors, ion channels, and neurotrophic factors (Manzerra et al., 2001; Kim et al., 2002; Rachline et al., 2005; Hwang et al., 2005; see for review: Cuajungco and Lees, 1997; Frederickson et al., 2000; Smart et al., 2004).

In this report, we provide further information about the distribution of Zn+ terminations in inferotemporal (IT) cortex, a region at the interface of the visual and hippocampal networks. In particular, we report a lateral to medial increase in Zn density, where TEad, a unimodal visual area, shows low levels of Zn; TEav, intermediate to medial increase in Zn density, where TEav, a unimodal hippocampal networks. In particular, we report a lateral cortex, a region at the interface of the visual and distribution of Zn+ terminations in inferotemporal (IT)

2. Materials and methods

2.1. Animals and tissue preparation

Nine adult macaque monkeys (Macaca mulatta and M. fuscata) were used (four for visualizing Zn+ terminals and five for sodium selenite injections). All experimental protocols were approved by the Experimental Animal Committee of the RIKEN Institute, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996). Every effort was made to minimize the number of animals used and any pain or discomfort experienced. Before perfusion, all animals were tranquilized with ketamine (11 mg/kg, i.m.) and deeply anesthetized with Nembutal (overdose, 75 mg/kg, i.p.).

Four monkeys were prepared for visualizing Zn+ termination, according to two perfusion methods (Danscher, 1996; Ichinohe and Rockland, 2004). (1) Two monkeys were perfused transcardially, in sequence, with saline containing 0.1% sodium sulfide (500 ml) for 5 min, and then 0.1% sodium sulfide and 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3, 4 l) for 30 min. The brains were removed from the skull, trimmed and postfixed for 12–15 h in 4% paraformaldehyde in 0.1 M PB. Then, brains were immersed in 0.1 M PB containing 30% sucrose until they sank (2–3 days). (2) Two other animals received intravenous injection of saline with 10% sodium sulfide (200 mg/kg). Two minutes after the injection, the animals were perfused transcardially, in sequence, with saline containing 0.5% sodium nitrite, with 4% paraformaldehyde in 0.1 M PB for 30 min, and chilled 0.1 M PB with 10, 20 and 30% sucrose. There was no obvious difference in quality of staining between these two methods. All brains were coronally sectioned by frozen microtomy (at 40–50 μm thickness). Tissue was collected in repeating series of three for Zn and parvalbumin (PV) histochemistry, and for cell body staining with thionin.

2.2. Zn histochemistry

Sections were washed thoroughly with 0.1 M PB, followed by 0.01 M PB. The IntenSE M silver Enhancement kit (Amersham International; Little Chalfont, Bucks, UK) was used to intensify Zn signals (Danscher et al., 1987; De Biarsi and Bendotti, 1988). A one-to-one cocktail of the IntenSE M kit solution and 33% gum arabic solution was used as a reagent. Development of reaction products was monitored under a microscope and terminated by rinsing the sections in 0.01 M PB and, subsequently, several rinses in 0.1 M PB. Selected sections were further processed for Nissl substrate using NeuroTrace 500/525 green fluorescent Nissl stain (Molecular Probes, Eugene, OR) according to the company’s protocol.

In addition to Zn, these methods potentially can reveal other metals such as copper and iron (see for review, Danscher, 1996). The specificity for Zn was evaluated by several means. First, EM data (Ichinohe and Rockland, 2005a) clearly show that the silver reaction product is localized to synapses, whereas iron is reported to be localized mainly to the nucleus of neurons and glia (Yu et al., 2001), and copper, mainly to glia (Szerdahelyi and Kasa, 1986). Second, we carried out controls in rat brains perfused and reacted under the same two conditions as the monkey material. In one control, intraperitoneal injection (1000 mg/kg) of the Zn chelating agent diethyldithiocarbamate 60 min before sacrifice (as per Danscher, 1996) resulted in unstained brain sections. Similarly, pretreatment of brain sections with 0.1 M HCl (Danscher, 1996) for 30 min or 15% trichloroacetic acid (TCA; Sigma, St. Louis, MO) was not effective for Zn and parvalbumin (PV) histochemistry, and for cell body staining with thionin.

2.3. Immunoperoxidase staining for PV

Sections were incubated for 1 h with 0.1 M phosphate buffer saline (PBS, pH 7.3) containing 0.5% Triton X-100 and 5% normal goat serum (PBS-TG) at room temperature, and then for 40–48 h at 4 °C with PBS-TG containing anti-PV monoclonal mouse antibody (Swant, Bellinzona, Switzerland; 1:50,000). After rinsing, the sections were placed in PBS-TG containing biotinylated anti-mouse IgG polyclonal goat antibody (Vector, Burlingame, CA; 1:200) for 1.5 h at room temperature. Immunoreactivity was
visualized by ABC incubation (one drop of reagents per 7 ml 0.1 M PB, ABC Elite kits; Vector, Burlingame, CA) followed by diaminobenzidine histochemistry with 0.03% nickel ammonium sulfate.

2.4. Sodium selenite injection

For five of the nine monkeys, 0.5–0.7% Na₂SeO₃ in 0.9% NaCl was injected into temporal cortical areas (on the left side) in order to visualize Zn⁺ neurons giving rise to the Zn⁺ terminations. Surgery was carried out under sterile conditions after the animals were deeply anesthetized with barbiturate anesthesia (35 mg/kg Nembutal i.v. after a tranquilizing dose of 11 mg/kg ketamine i.m.). Cortical areas of interest were localized by direct visualization, subsequent to craniotomy and durotomy, in relation to sulcal landmarks (i.e. superior temporal, and anterior or posterior middle temporal sulci: sts, amts and pmts). Cortical sites of injection were made by a 10 μl Hamilton syringe in regions corresponding to TEO, TEm, TEd, TEav, and the border of TEav and perirhinal cortex (TEav/PRh, Fig. 3). Total volume injected was 1.0–1.5 μl, resulting in injection sites of 1.5–2.0 mm in diameter (Fig. 3). Sodium sulfide is toxic, and results in a central necrotic region of variable size (see for Fig. 3A). A post-injection survival 24 h was used. Short survival is standard for sodium selenite (Christensen et al., 1992; Casanovas-Aguilar et al., 1995, 1998, 2002). Moreover, in other experiments, we verified 24 h was adequate to visualize callosal and other long distance connections. The animals were re-anesthetized and then perfused transcardially, in sequence, with saline containing 50 mM sucrose, 30% sucrose in 0.1 M PB, ABC Elite kits; Vector, Burlingame, CA) followed by diaminobenzidine histochemistry with 0.03% nickel ammonium sulfate.

2.5. Nomenclature

Cortical areas were identified in part by reference to sulcal landmarks, in comparison with published maps, and by architectonic analysis of selected histological sections stained for cell bodies. Borders were further evaluated by analysis of laminar and density differences in the material stained by Zn histochemistry or immunohistochemistry for PV. We followed most closely: for the parahippocampal and inferotemporal cortex, the nomenclature proposed by Yukie et al. (1990) and Saleem and Tanaka (1996), with reference to that of Suzuki and Amaral (2003a,b); for the amygdala, the nomenclature of Price et al. (1987) and Amaral et al. (1992; see also Ichinohe and Rockland, 2005a,b); and for the hippocampal formation, that of Rosene and Van Hoesen (1987).

In referring to Zn-enriched terminals and neurons, we have for convenience used the shorter designation Zn⁺ (zinc-positive).

2.6. Data analysis

Retrogradely labeled cells were plotted using a computer-aided microscope system and the Neurolucida software package (MicroBrightfield, Williston, VT). The microscope (E800, Nikon, Tokyo, Japan) was equipped for fluorescent and darkfield microscopy. The outline of the section, laminar boundaries, and the borders of the amygdaloid nuclei and hippocampal formation were added to the plots by referring to fluorescence Nissl. Adjacent sections Nissl-stained by thionin were available for further confirmation of these features.

Photomicrographs were taken with an Olympus DP50 digital camera mounted on an Olympus BX60 and SZX-ILLD100 microscope. Images were saved in TIFF format and imported into Adobe Photoshop 5.5. Image brightness, contrast and color were adjusted as necessary to reproduce the original histological data.

3. Results

3.1. Zn⁺ terminal distribution in the IT cortex

Zn⁺ terminals show a conspicuous lateral-to-medial increase in density, from the sts to the rhinal sulcus (rhs) (Figs. 1 and 3A for orientation). In all areas, the middle layers (Figs. 3C and 4) are Zn-poor. These are the main target layers of pulvinocortical terminations (Benevento and Rezak, 1976; Baleydier and Mauguiere, 1985), and these, like all thalamocortical terminations, are Zn-poor. Layer 1a, another thalamorecipient layer, is also Zn-poor in these areas (Benevento and Rezak, 1976; Baleydier and Mauguiere, 1985). The highest density of Zn⁺ terminations is in the perirhinal cortex (areas 35 and 36). Here, Zn⁺ terminations form two distinct bands (Figs. 1 and 2A and B). The denser band is in the supragranular layers and comprises layers 1b, 2, and 3a,b. The second band corresponds to the infragranular layers 5 and 6. In area 35, layer 5 appears denser than layer 6 (Fig. 2A), but in area 36, the two layers are indistinguishable and of comparable density (Fig. 2B). No differences could be discerned along the AP axis. Perirhinal cortex is characterized as having a subtle, rather than conspicuous modularity in the uppermost layers (Fig. 2...
Fig. 1. Photomicrographs of adjacent coronal sections from the anterior part of the IT cortex, stained for Zn (A) and PV (B). Medial is to the left. Star in (A) indicates the border between area 35 (medial) and area 36 (lateral). Zn+ terminations become increasingly dense medially. Lines in (A) mark approximate borders. Compare (B), where PV density decreases medially, in several stages (indicated by arrowheads). For abbreviations, see list. Bar: 2 mm.

Fig. 2. Photomicrographs of sections stained for Zn. Coronal sections of the border between area 35 and entorhinal cortex (A), area 36 (B), TEav (C), TEad (D), and TEm (E). Semitangential section of TEav, in the lower bank of the ams (F). Tangential section of perirhinal cortex through the layer 1/2 border (G). Note reticular, honeycomb pattern in both (F) and (G). Bar: 1 mm (A–F), 500 μm (G).
in Ichinohe and Rockland, 2004). Accordingly, patches of Zn+ terminations at the border of layers 1 and 2 can be seen in tangential section (Fig. 2E), but may not be easily discernible in coronal sections (Fig. 2B).

The region immediately ventral to amts is variously called perirhinal cortex (Suzuki and Amaral, 2003a,b) or TEav (Yukie et al., 1990; Saleem and Tanaka, 1996). This region shows two bands, similar to more medial cortex, but the density is not uniform (Figs. 1A and 2C). Especially in the supragranular layers, a very dense band in layers 1b and 2 can be distinguished from a moderately dense underlying band consisting of layer 3a,b. In an appropriate section, where tissue is cut tangentially (for example, in amts), we can recognize a patchy pattern at the layer 1,2 border (Fig. 2G).

In TEad, the region lateral to amts, the overall density is again less, except in the supragranular layers, corresponding to 1b and 2, and uppermost layer 3 (Figs. 1A and 2D). A moderately dense, deeper band corresponds to layer 6. In addition, there are two weaker bands: one corresponding to layer 3a,b and the other to layer 5a. Layer 4 is very Zn-poor.

Finally, the cortex at the lip of the sts, sometimes referred to as TEm (Seltzer and Pandya, 1978), has an even lower density of Zn+ terminations. This forms trilaminar pattern, where layers 1b and 2 are moderately dense, and layers 3a,b and 6 are only weakly dense (Fig. 2E). In TEm, as for TEad, there is no evidence of a patchy conformation in layers 1, 2 in either coronal or tangential sections.

The cortex posterior to TEa (TEp and TEO) shows a progressive decrease in Zn density (not illustrated). However, a similar medial-to-lateral gradient (with greater density near the occipitotemporal sulcus, ots) can be observed. (The Zn distribution more anteriorly, in entorhinal cortex, is complex, and will be described in a separate communication; Ichinohe and Rockland, 2005b.)
Although we are not primarily concerned with area borders, parallel sections reacted for PV showed a reverse gradation, generally complementary to the Zn distribution. That is, from perirhinal cortex to TEm, there is gradual decrease of PV density, opposite to Zn. In TEp and TEO, the Zn dense area around the ots is PV weak. According to other studies, it is likely that PV terminations in layers 2 and 3 are mainly inhibitory, although these in layers 3 and 4 may be a mix of local GABAergic and thalamocortical excitatory terminations (Williams et al., 1992; Del Rio and DeFelipe, 1994; Melchitzky et al., 1999).

3.2. The origin of Zn+ terminals in the IT cortex: hippocampal formation

By injecting sodium selenite into several regions of IT cortex, we were able to investigate the origin of the Zn+ terminations. This was found to be mixed, with some neurons nearby the injection sites, some in more distant cortical areas, and others in the hippocampus, amygdala and claustrum. For the sake of clarity, we will here concentrate only on the Zn+ connections from the hippocampus and amygdala.

After injections in TEm, TEad, or more posterior areas (TEp/TEO), no labeled cells were apparent in the Hp; but injections in TEav or TEav/PRh both resulted in labeled neurons. These occurred in CA1, throughout its rostrocaudal extent, except for the uncal portion. Neurons were concentrated at the distal portion (close to prosubiculum) of CA1 and, in the most densely labeled fields, extended for 2.5–3.0 mm medio-lateral. Labeled neurons had pyramidal-shaped somata, and were restricted to the pyramidal cell layer (Fig. 4). Double labeling for Zn and fluorescence Nissl showed that Zn+ neurons selectively clustered in the upper part of the pyramidal cell layer (Fig. 4). A few neurons occurred in the prosubiculum, but only when the injection involved perirhinal cortex.

3.3. The origin of Zn+ terminals in the IT cortex: amygdaloid nuclei

All five cases resulted in retrogradely labeled Zn+ neurons in the amygdala (Figs. 4 and 5). In the basal nucleus, more laterally situated injections tended to produce more dorsal labeling (Figs. 5A, A’ and 6). That is, injections in TEm and TEad showed more labeled neurons in the magnocellular and intermediate portions, while injections in TEav and TEav/PRh revealed more neurons in the intermediate and parvicellular portions. The dorsal portion of the lateral nucleus contained labeled

![Fig. 4. Darkfield photomicrographs of coronal sections through CA1, showing Zn+ neurons retrogradely labeled by the sodium selenite injection in Fig. 1E. (A’) is higher magnification, from the white box in (A). (B) and (B’) are merged darkfield and fluorescence Nissl images, to show more clearly the stratification of the labeled neurons in the CA1 pyramidal layer. Bar: 1 mm (A, B), 500 μm (A’, B’).](image)
neurons, except after the TEm injection (Figs. 4B and 5). In all cases, the accessory basal nucleus contained labeled neurons (Fig. 5). Only TEav/PRh injection cases, however, resulted in any substantial number of labeled neurons in the periamygdaloid cortical nuclei (Figs. 6C and 7D). TEp/TEO injection showed retrograde labeling similar to TEm. That is, labeling was only found in the magnocellular and intermediate portions of the basal nucleus. Labeled somata are in general oval, triangular, or polymorphic in shape (Fig. 6A').

Fig. 5. Coronal sections through the Hp with Neurolucida plotting of Zn+ retrogradely labeled neurons in CA1, after sodium selenite injection into TEav (A) and TEav/PRh (B). Labeled neurons (filled circles) are located superficially in stratum pyramidale. Medial is to the right, and rostral at the top. Bar: 2 mm.

Fig. 6. Darkfield photomicrographs of coronal sections through the amygdala, showing Zn+ neurons retrogradely labeled by the sodium selenite injection into TEav/PRh (see Fig. 1E). (A') is higher magnification, from the white box in (A). See arrows in Fig. 7 for general location of the foci of labeled neurons. Bar: 1 mm (A), 200 μm (A'), 400 μm (B, C).
4. Discussion

We have demonstrated differences in the distribution of Zn+ terminations, such that medial and anterior temporal areas (that is, area 36) are denser than areas situated more laterally (TEa) or more posteriorly (TEp, TEO). By making cortical injections of sodium selenite, we further established that neurons in the amygdala and/or hippocampus are one source of the Zn+ terminations. These results relate to several major issues.

4.1. Subtypes of projection neurons to the IT cortex

One is the question of subtypes within projection neuron populations. Evidence for subtypes of excitatory neurons has so far been more fragmentary than that for interneurons.
et al., 2004). There is increasing recognition, however, of complex and important interactions (Phelps, 2004); and the amygdala, visualized by intraperitoneal injection of sodium selenite shows a distinct stratification of later-born, Zn+ neurons in the upper part of CA1 (Slomianka, 1992).

In monkeys, there are fewer data concerning subdivisions of CA1, and these are based mainly on connections. For temporal areas, conventional tracers produce labeling throughout the full depth of CA1 (Iwai and Yukie, 1988; Blatt and Rose, 1998; Figs. 7 and 9 in Yukie, 2000; Insausti and Munoz, 2001). However, for prefrontal areas, some reports describe that hippocampal projections selectively originate from the deeper parts of CA1 (Barbas and Blatt, 1995; Cavada et al., 2000). Further work is necessary to determine whether these deeper neurons are positive or negative for Zn.

For the amygdala, it is more difficult to assess whether the Zn+ neurons constitute a distinct subpopulation. The distribution of our retrogradely labeled neurons is basically similar to published observation based on standard retrograde tracers (Iwai and Yukie, 1988; Stefanacci et al., 1996; Amaral et al., 2003). The proportion of Zn+ neurons may be important. In rodents, the proportion of Zn+ neurons in the amygdala, visualized by intraperitoneal injection of sodium selenite, is reported as 0–70%, depending on the subnuclei and on the antero-posterior level (Christensen and Geneser, 1995). In larger animals, however, a similar procedure would be difficult, because of the toxicity of this compound.

### 4.2. Amygdalo-hippocampal interactions within IT cortices

A second issue concerns amygdalo-hippocampal interactions. These structures are frequently considered separately, as primarily involved in emotion (amygdala; e.g., LeDoux, 2003) or episodic memory (hippocampus; Squire et al., 2004). There is increasing recognition, however, of complex and important interactions (Phelps, 2004); and the occurrence of a Zn+ population of cortical projection neurons from both structures adds another dimension. Previous results on connections to temporal areas have shown that the full complement of amygdala projections densely terminates in layers 1, 2, 5, and 6 (Amaral and Price, 1984), while those from CA1 project densely to layers 3 and 5 of perirhinal cortex and, less densely, to the same layers of what some authors call TEav and TEpv (Saunders and Rosene, 1988; Iwai and Yukie, 1988; Suzuki and Amaral, 1990; Blatt and Rose, 1998; Zhong and Rockland, 2004). Consistent with the wider area distribution of amygdalo-cortical projections, the Zn+ band in the upper layers is relatively uniform across areas. The density of the Zn+ deeper band correlates well with the distribution of hippocampal connections, being particularly dense in perirhinal cortex, but presumably this deeper band reflects a combination of connections from both the amygdala and hippocampus, as well as other cortical sources. Further work might address whether the Zn+ terminations target similar neuronal populations, at different locations of the dendritic tree, or, alternately, different populations (see Ichinohe et al., 2003; Ichinohe and Rockland, 2004).

### 4.3. Functional significance of synaptic Zn in the IT cortices

A third issue is the functional significance of the synaptic Zn. This is the subject of considerable ongoing debate (e.g.: Cole et al., 2001; Cuajungco and Faget, 2003); but evidence from in vitro preparations and deprivation experiments consistently suggests a role in activity-dependent synaptic plasticity (Frederickson et al., 1990; Li et al., 2001; Takeda et al., 2004). Among various possible mechanisms are: translocation into postsynaptic components (Li et al., 2001); interaction with NMDA receptors (Rachline et al., 2005); biphasic effect on NMDA receptors (acute block, followed by Src family kinase-mediated up-regulation, Manzerra et al., 2001); or activation of Trk receptors (Hwang et al., 2005). The influence of Zn on Trk receptors is potentially significant in the context of up-regulation of BDNF, a neurotrophic factor associated with TrkB receptors. In particular, perirhinal cortex of monkeys trained to learn a pair-association task exhibits up-regulation of BDNF (Tokuyama et al., 2000), and it may be that activity-dependent Zn release influences this phenomenon. This possibility is supported by the laminar co-localization of Zn+ terminations and BDNF expressing neurons, in layers 2, 3, 5, and 6 (Tokuyama et al., 2000).

Finally, one might wonder about the reverse gradients of Zn and PV. Several previous reports (reviewed in Fujita, 2002) have identified a strong anterior–posterior (AP) gradient from area V1 to entorhinal cortex, which parallels the AP increase in Zn. In part, this may be related to regional characteristics, such as degree and kind of plasticity (Murayama et al., 1997), or degree and kind of oscillatory rhythms. An interesting recent study of striatal neurons in rats observed opposing gradients where dorsal/lateral neurons, including a higher density of PV-positive GABAergic interneurons, entrained to high-voltage spindle oscillations (7–14 Hz), and ventral/medial, entrained to
hippocampal theta rhythm (5–9 Hz, Berke et al., 2004). Distinctive rhythmic activity has already been characterized in perirhinal cortex of cats (Collins et al., 1999), and further work might investigate the repertoires of synchronized neuronal events across these interconnected structures, and their neural substrates.

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