Pathway-Specific Utilization of Synaptic Zinc in the Macaque Ventral Visual Cortical Areas

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Synaptic zinc is an activity-related neuromodulator, enriched in hippocampal mossy fibers and a subset of glutamatergic cortical projections, exclusive of thalamocortical or corticothalameric. Some degree of pathway specificity in the utilization of synaptic zinc has been reported in rodents. Here, we use focal injections of the retrograde tracer sodium selenite to identify zinc-positive (Zn+) projection neurons in the monkey ventral visual pathway. After injections in V1, V4, and TEO areas, neurons were detected preferentially in several feedback pathways but, unusually, were restricted to deeper layers without involvement of layers 2 or 3. Temporal injections resulted in more extensive labeling of both feedback and intratemporal association pathways. The Zn+ neurons had a broader laminar distribution, similar to results from standard retrograde tracers. After anterograde tracer injection in area posterior TE, electron microscopic analysis substantiated that a proportion of feedback synapses was colabeled with zinc. Nearby injections, Zn+ intrinsic neurons concentrated in layer 2, but in temporal areas were also abundant in layer 6. These results indicate considerable pathway and laminar specificity as to which cortical neurons use synaptic zinc. Given the hypothesized roles of synaptic zinc, this is likely to result in distinct synaptic properties, possibly including differential synaptic plasticity within or across projections.

Keywords: feedback, feedforward, subpopulations of pyramidal neurons, ventral visual pathway, zinc

Introduction

Neurochemically, the overwhelming majority of cortical pyramidal neurons use glutamate as neurotransmitter. A subset, however, is distinguished by using synaptic zinc as cofactor. The density of zinc-positive (Zn+) terminations is area dependent, being least in primary areas and greatest in limbic-associated areas (Pérez-Clausell 1996; Frederickson et al. 2000, 2005; Ichinohe and Rockland 2004). In all areas, the middle layer is zinc poor (Pérez-Clausell 1996; Ichinohe and Rockland 2005; Wong and Kaas 2008). This is consistent with the fact that thalamocortical terminations do not contain zinc (Brown and Dyck 2004; Ichinohe et al. 2006). An additional implication is that feedforward cortical connections terminating in layer 4 are zinc negative. From this, more particularly, the question arises of whether there is pathway specificity as to which neurons use synaptic zinc, for example, among the feedforward, feedback, and lateral corticocortical projection.

Synaptic zinc is an activity- and calcium-dependent neuromodulator, known to interact with receptors, ion channels, and neurotrophic factors (for review, see Smart et al. 2004; Frederickson et al. 2005; Nakashima and Dyck 2009, Paolletti et al. 2009; Sensi et al. 2009). Thus, a preferential association of synaptic zinc with specific projections or subpopulations would be expected to impact on synaptic properties, including synaptic plasticity.

In most cortical areas, Zn+ terminations form 2 dense bands, one in layers 1b, 2, and upper 3 and another in the deeper layers (Pérez-Clausell 1996; Ichinohe and Rockland 2005). This distribution at least partly corresponds to that of feedback connections (Rockland and Pandya 1979; Maunsell and Van Essen 1983; Felleman and Van Essen 1991). In rodents, furthermore, retrograde tracing experiments demonstrate that Zn+ neurons are concentrated in layers 2, upper 3, and 6 (Garrett et al. 1992; Casanova-Aguilar et al. 1998; Brown and Dyck 2005). The pathway-specific sources of Zn+ terminations are less investigated in the macaque cortex. We therefore set out to investigate whether synaptic is preferentially used by feedback projections.

Here, we used focal injections of sodium selenite to identify neurons giving rise to Zn+ terminations, within the ventral visual pathway of macaque monkeys, that is, V1, V4, TEO, and anterior temporal areas (see Materials and Methods, Sodium Selenite and BDA Injection). In fact, we find that feedforward projections, in contrast to feedback or lateral projections, tend to be zinc negative. More surprisingly, we also demonstrate a laminar dissociation within the early visual feedback projections, where projecting neurons in layer 6, but not in layer 2, are Zn+.

Materials and Methods

Experimental Subjects

Ten adult macaque monkeys (Macaca mulatta and Macaca fuscata) were used in this study: 9 were used for sodium selenite injections and one for electron microscopic (EM) analysis of anterogradely labeled Zn+ terminations. Three additional animals, used in other studies (in preparation), received sodium selenite injections in temporal cortex with variable survival times (40–48 h) in order to confirm the optimal postinjection survival times. All experimental protocols were approved by the Experimental Animal Committee of the RIKEN Institute and conformed to the National Institutes of Health (NIH) 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 by them.
Sodium Selenite and BDA Injection

There are 2 techniques for demonstrating Zn\(^+\) neurons. One is intraperitoneal injection of sodium selenite (Na\(_2\)SeO\(_3\)). This substance interacts with synaptic zinc to form precipitates of ZnSe, and in this form, it is transported retrogradely to the soma (Christensen et al. 1992; Casanovas-Agullar et al. 2002). After histological processing, it reveals all Zn\(^+\) neurons (Slomianka et al. 1990; Brown and Dyck 2005). An alternative second method, used here, is to make focal injections of sodium selenite. Focal injections are less toxic in primates and have the further advantage of selectively labeling only those Zn\(^+\) neurons that project to the injection site (Christensen et al. 1992; Casanovas-Agullar et al. 1995, 1998; Brown and Dyck 2005).

For 9 of the 10 monkeys, 0.5-0.7% of the retrograde tracer sodium selenite (Na\(_2\)SeO\(_3\); Sigma; diluted in 0.9% NaCl) was injected into V1 (n = 2), V4 (n = 2), TEO (n = 1), or temporal cortical (n = 4) areas in order to visualize neurons giving rise to the Zn\(^+\) terminations in each of these areas. The 4 animals with temporal injections have been used in a previous report (Ichinohe and Rockland 2005), and injection localization is mapped in figure 3 of that report. Designations of subdivisions within temporal cortex are further described below and under "Nomenclature."

Surgery was carried out under sterile conditions after the animals were deeply anesthetized with barbiturate anesthesia (35 mg/kg Nembutal, intravenously [i.v.], after a tranquilizing dose of 11 mg/kg ketamine, intramuscularly [i.m.]). Cortical areas of interest were localized by direct visualization, subsequent to craniotomy and durotomy, in relation to sulcal landmarks (i.e., inferior occipital, lunate, superior temporal, and anterior or posterior middle temporal sulci). Pressure injections of sodium selenite were made by a 10-μL Hamilton syringe in regions corresponding to V1, V4, TEO, posterior dorsal TE (TEpd), anterior dorsal TE (TEad), anterior ventral TE (TEav), and the subdivisions within temporal cortex are further described below and under "Nomenclature."

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In one monkey, biotinylated dextran amine (BDA; Molecular Probes) was injected in area TEpd for subsequent EM analysis of pathway-specific Zn\(^+\) boutons, anterogradely labeled by BDA (see below). Two injections (1.0 μL each) of 10% solution (1:1 mixture of 3000 and 10 000 MW) in 0.0125 M phosphate-buffered saline (PBS: pH 7.4) were made.

Visualization of Zn\(^+\) Projection Neurons

A postinjection survival of 24 h was used, consistent with previous experiments in rodents (Christensen et al. 1992; Casanovas-Agullar et al. 1995, 1998) and primates (Ichinohe and Rockland 2005). The efficacy of the survival time was evaluated by several features of the resulting projections. 1) We successfully identified, in our material, projections that were 1.8-2.1 cm from the injection site; for example, Zn\(^+\) neurons in the posterior hippocampus were labeled by injections in TEav (see also, Ichinohe and Rockland 2005), and neurons in the temporal pole were labeled after injections in TEpd (case 66). 2) As a further control, longer survivals (40-48 h) were tested in 3 animals used in other experiments. The longer postinjection survivals produced more necrosis and less intense labeling around the injection site, with no detectable enhancement in either the local or long distance connections. Because a negative trend was already evident with longer survivals, we did not extend postinjection survival times beyond 48 h, but the expectation is that, like horseradish peroxidase, the retrogradely transported sodium selenite would be cleared from the neurons with longer survival times.

After injection and recovery, the animals were re-anesthetized (11 mg/kg, i.m. ketamine; ketamine followed by 75 mg/kg, intraperitoneally Nembutal) and then perfused transcardially, in sequence, with saline containing 0.5% sodium nitrite; 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min; and chilled 0.1 M PB with 10%, 20%, and 30% sucrose. The brains were removed from the skull, blocked, and immersed into 30% sucrose in 0.1 M PB. The brains were cut in the coronal plane by frozen microtomy (at 50 μm thickness), and tissue was collected in repeating series. The series consisted of: zinc, thionin, and parvalbumin, with 1 or 2 sections reserved or discarded. Sections were washed thoroughly with 0.1 M PB, followed by 0.01 M PB. The IntenSE silver enhancement kit (Amersham International) was used, I: with 33% gum arabic solution, to intensify zinc signals (Danscher et al. 1987; De Blasi and Bendotti 1998). 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. Precipitate, interpreted as the reaction product (zinc selenite) of injected selenium with Zn\(^++\) at the injection site, was formed within cell somata, consistent with previous reports (Christensen et al. 1992;
EM Analysis of Boutons Containing Zn and BDA

After the BDA injections, the animal was allowed to recover and survived 22 days. As a terminal procedure, the animal was deeply anesthetized and then injected i.v. with saline containing 10% sodium sulfite (200 mg/kg). Two minutes after the injection, the animal was perfused transcardially, in sequence, with saline containing 0.5% sodium nitrite, 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M PB for 30 min, and chilled 0.1 M PB with 10% and then 20% sucrose. The brain was removed from the skull, and 2 small cortical blocks were excised and kept in 0.1 M PB. Block 1, trimmed in an oblique coronal plane, included areas TEO and V4, respectively, ventral and dorsal to the inferior occipital sulcus. Block 2 was slightly posterior, oriented in the horizontal plane, and included V1, V2, V4, and the adjacent posterior bank of the superior temporal sulcus (STS).

Vibratome-cut sections (50 μm thick) were prepared and washed thoroughly with 0.1 M PB, followed by 0.01 M PB. For each block, a repeating series of 4 consecutive sections was collected in 4 groups (A-D). Group C was first prepared for light microscopic examination of BDA only in order to identify regions with dense BDA label. These sections were incubated in 0.5% Triton-X 100 in 0.1 M PBS for 30 min, followed by avidin-biotin complex (VECTASTAIN Elite ABC, Vector Labs) containing 0.5% Triton-X 100 overnight (17–24 h). In the final step, BDA was demonstrated by 3,3'-diaminobenzidine tetrahydrochloride (DAB) histochemistry.

After we identified BDA-dense regions, we selected several adjoining sections in the B and D groups. These were double-reacted, first for zinc and then for BDA. The IntenseM silver enhancement kit, mixed 1:1 with the same amount of 33% gum arabic, was used to intensify zinc histochemistry for BDA as described above, but with Triton-X 100 at 0.03% instead of 0.5%. The sections double-labeled for zinc and BDA were then osmicated, dehydrated, and flat-embedded in Araldite resin (TAAB). From the plastic sections, we identified and further trimmed out small areas where dense BDA-labeled terminals overlapped with a dense stratum of Zn+ terminals. Semiserial silver sections were collected on formvar-coated, single-slot grids; contrasted with 4% uranyl acetate; and examined with an electron microscope (JEM 2000-EX; JEOL). Five to 10 ultrathin sections from selected brain areas (TEO, V4, STS, and V1; see Table 1) were surveyed for BDA and Zn+ profiles at low magnifications (×5000 to ×10 000). When candidate BDA+/Zn+ or BDA+/Zn-negative profiles were identified, electron micrographs were taken at magnifications of ×50 000 for detailed observation.

Nomenclature

Areas V1, V4, and TEO were identified by reference to sulcal landmarks, in comparison with published maps, and by architectonic analysis of selected histological sections stained for cell bodies or parvalbumin (Saleem and Logothetis 2007; Ungerleider et al. 2008; Borra et al. 2010). Subdivisions within the inferotemporal region are more variable between individual monkeys and tend not to have sharp borders (Zeki 1996). In placing injections, we targeted 3 major subdivisions, defined in relation to the posterior and anterior middle temporal sulci, namely TEp, TEa, and TEav. A forth injection was at the border of TEav and perirhinal cortex. This nomenclature follows that of Yukie et al. (1990), Saleem and Tanaka (1996), Saleem et al. (2007), and Saleem and Logothetis (2007), with reference to that of Suzuki and Amaral (2003a, 2003b). Foci of Zn+ neurons are described within area TF, TH, TG, and smaller subdivisions of TE within or near the STS (Tem, TEa, and TPO). These are designated with reference to Saleem and Logothetis (2007) and Seltzer and Pandya (1989) and marked on the section outlines in Figures 5, 6, 7, and 9.

In referring to zinc-enriched terminals and neurons, we have for convenience used the shorter designation Zn+ (= zinc positive). We avoided the descriptor “zincergic” as we are taking a conservative stance that further criteria are needed before these populations can be accepted as distinct types.

Results

Zn+ Neurons Nearby the Injection Sites

Injections in all our targeted areas produced Zn+ neurons in the vicinity of the injection, but marked area-specific differences were apparent in the laminar distribution and density (Fig. 1). In area V1, there were relatively few Zn+ neurons. These were mainly in layer 2 and were restricted to the immediate vicinity of the injection site (less than 0.7 mm from the injection edge; Fig. 1A,B). A few Zn+ neurons were observed in layer 6 (Fig. 1C). Injections in V4 or TEO also produced Zn+ neurons mainly in layer 2 (Fig. 1D,E,G,H), but, compared with the labeling after injections in V1, the Zn+ neurons extended further from the edge of the injection (up to 1.0 mm). Some Zn+ neurons occurred in layer 6, in the immediate vicinity of the injections (Fig. 1D,G,I,D).

The distribution of Zn+ neurons was conspicuously different in the temporal cases (Fig. 1J,K,L). First, more layers were involved. Numerous Zn+ neurons occurred not only in layer 2 but also in layer 6 and, to a lesser extent, in upper layer 3 and layer 5. Second, the bands of Zn+ neurons extended further from the edge of the injection, up to 1.5–2.0 mm. None of these Zn+ intrinsic connections had any discernible patchiness. We note, however, that the sparse label in layer 3 of temporal areas exhibited some periodic fluctuation. The number of labeled

Data Analysis

Retrogradely labeled cells were plotted using a computer-aided microscope system and the Neurouloida software package (Micro-Brightfield). The microscope (E800; Nikon) was equipped for fluorescent and dark field microscopy. The outline of the section and laminar boundaries was added to the plots by referring to fluorescence Nissl or adjacent thionin-stained sections.

Photomicrographs were taken with a digital camera (Axiscop2 and Axioacam; Carl Zeiss Vision). Images were saved in TIFF format and imported into Adobe Photoshop Cvi. Image brightness, contrast, and color were adjusted as necessary to reproduce the original histological data.

Additional experimental tissue from several previous experiments was available. This included 2 brains perfused for synaptic zinc (Ichinohe and Rockland 2005), 3 brains with injections of choleratoxin B-subunit conjugated to Alexa 488 or Alexa 594 in temporal areas (Borra et al. 2010), and 4 brains injected with BDA (Ichinohe et al. 2008).

Table 1

| EM block | Dimension of surveyed field (L1/L2 [mm²]) | Number of ultrathin sections analyzed | Location | Total | BDA+/Zn+ | BDA+/Zn-negative |
|----------|------------------------------------------|--------------------------------------|---------|-------|---------|-----------------|
| 105a (3 H) | 1 × 0.5 | 10 | TEO | 5 | 1 | 4 |
| 105b (2.5 H) | 0.7 × 0.35 | 10 | TEO | 7 | 3 | 4 |
| 185a (2.5 H) | 1 × 0.75 | 5 | TEO | 7 | 3 | 4 |
| 120a (3 H) | 1 × 0.5 | 10 | V4 | 15 | 6 | 9 |
| 283 (2.5 H) | 0.5 × 0.5 | 5 | V1 | 5 | 4 | 1 |
| 206a (3 H) | 0.75 × 0.6 | 8 | STS | 10 | 6 | 4 |

*The first number is length, measured parallel to the pia; second number is depth from pia.
*Incubation time for zinc enhancing.
neurons near the injection site increased from V4 and TEO to TEad (see Fig. 1).

**Zn**⁺ Extrinsic Cortical Projections: Areas V1, V4, and TEO

In this section and the following section, we begin with a brief summary of the main projections, as reported by others from standard retrograde tracers, that is, wheat germ agglutinin conjugated with cholera toxin, fluorochromes, horseradish peroxidase, fluorescent dyes, or cholera toxin conjugates, see also Figure 11. This is intended as aid in the interpretation of zinc-specific patterns. We continue to use the nomenclature of "feedforward" projections to designate projections originating mainly from layer 3 and terminating mainly in layer 4. “Feedback” projections designate those avoiding layer 4, terminating mainly in layer 1, or in layers 1 and 6, and originating mainly from neurons in layers 6, 2, and upper 3 (Rockland and Pandya 1979; Maunsell and Van Essen 1983; Felleman and Van Essen 1991; Born and Bradley 2005; Sinich and Horton 2005; Ungerleider et al. 2008). As repeatedly discussed in the literature, classification criteria are not always clear, and especially, connections between higher order areas can be hard to classify with the available criteria. In general, what have been called “association,” “intermediate,” or “lateral” connections originate from and terminate in more layers (Rockland and Pandya 1979; Maunsell and Van Essen 1983; Felleman and Van Essen 1991; Ungerleider et al. 2008).

**Standard Tracers (Area V1)**

Cortical projections to area V1 originate from areas V2, V3, V4, MT, several smaller areas in the STS and intraparietal sulcus (IPS), and sporadically from areas TEO, TE, parahippocampal gyrus, and frontal eye fields (Maunsell and Van Essen 1983; Distler et al. 1993; Rockland et al. 1994; Barone et al. 2000). These are all feedback projections. Labeled neurons are in layers 2, upper 3, and 6, or in layer 6 alone for the more distant foci, such as in TEO and TE (Doty 1983; Rockland and Van Hoesen 1994; Barone et al. 2000).

**Sodium Selenite (Area V1)**

Of areas reviewed above, only 2 foci contained Zn⁺ neurons, one in the depth of the lunate sulcus (area V2: Fig. 2A,B) and the second in the lower bank of the STS (area MT: Fig. 2C,D). In marked contrast to standard tracers, labeled neurons were only in layer 6 and not in layers 2 and 6.

**Standard Tracers (Area V4)**

Cortical projections to V4 differ slightly, depending on whether the injection is placed in the central or peripheral visual field representation (for review, see Barone et al. 2000; Ungerleider et al. 2008). Major projections are from area V2 (feedforward, with neurons predominantly in layer 3); from areas V3A, V4t, MT, and DP (intermediate or lateral, originating from neurons in layers 3 and 5); and feedback from anterior areas in TE, TEO, the STS, IPS, and area TF in the parahippocampal gyrus. These latter originate from neurons in the deeper layers, with some involvement of layers 2 and uppermost 3 (Rockland and Pandya 1979; Felleman and Van Essen 1991). In addition, neurons in the frontal eye field project to V4 (Barone et al. 2000).

**Sodium Selenite (Area V4)**

Sodium selenite injections in area V4 produced label mainly in a subset of feedback connections (Figs 3 and 4), namely the most posterior STS (Fig. 3, section 81; Fig. 4A,B) and area TEO in the lower bank of the STS and the lateral bank of the occipitotemporal sulcus (OTS: Fig. 3, section 53). Surprisingly, Zn⁺ neurons were limited to layer 6. As remarks above, in contrast, standard tracers produce labeled neurons in the upper layers as well. After the more dorsal injection in V4, Zn⁺ neurons also occurred in the parahippocampal gyrus (not illustrated). These were in the deeper layers, in this instance, consistent with previous reports (Felleman and Van Essen 1991; Ungerleider et al. 2008).

The more ventral injection in V4 resulted additionally in a few neurons at the lateral edge of the annectant gyrus (area V3A: Fig. 3, sections 81 and 90; Fig. 4C,D). This has been described as an “intermediate-type” projection (Ungerleider et al. 2008), although the Zn⁺ neurons were mainly in layer 6.

**Standard Tracers (Area TEO)**

Cortical projections to area TEO (Rockland and Pandya 1979; Distler et al. 1993) include those from areas V2, V3, V4, and, more sparsely, MT (feedforward); from FST in the STS and from parietal area LIP (intermediate); and from area TE, areas in the anterior STS, parahippocampal area TH, perirhinal area 36, and TG (feedback: Distler et al. 1993; Lavenex et al. 2002). The peripheral field representation, located more ventrally (and not injected by us), receives several additional projections (Distler et al. 1993).

**Sodium Selenite (Area TEO)**

In contrast with standard tracers, it is again apparent that only a subset of projections are Zn⁺, and these corresponded closely to the feedback projections. A small focus of Zn⁺ neurons was found in the perirhinal cortex (Fig. 5, section 118). Other foci were detected in the lower bank of the anterior STS (TEad and possibly IPa: Fig. 5, section 92) and in the parahippocampal gyrus (area TEad: Fig. 5, sections 63 and 82). Except for the parahippocampal gyrus, these had a bilaminar distribution but were denser in layer 6. From the vicinity of the injection, dense label extended about 10 mm anterior, well into area TE and adjoining perirhinal cortex (Fig. 5, sections 74, 82, 92, and 118). At these levels, consistent with previous descriptions of feedback connections, label was predominantly in layer 6. Posterior to the injection, the field of labeled neurons extended 4.0 mm, within what is probably still area TEO (Fig. 5, section 44). Labeled neurons were in both the supra- and infragranular layers, as would be expected for intrinsic or lateral connections.

**Zn**⁺ Extrinsic Cortical Projections: Temporal Areas

**Standard Tracers**

The projections to perirhinal cortex and the various subdivisions of area TE have been extensively investigated (Yukie et al. 1990; Baizer et al. 1991; Webster et al. 1991, 1994; Distler et al. 1993; Suzuki and Amaral 1994; Saleem et al. 1993, 2000, 2007; Saleem and Tanaka 1996; Kondo et al. 2005) and will only be summarized here. Main projections include those from posterior unimodal visual areas (V4 and TEO), from several areas in the STS, from temporal polar cortex, and (for TE) from perirhinal cortex. Projections from the unimodal visual areas can be considered feedforward. Other projections have a bilaminar distribution of efferent neurons in the supra- and infragranular layers, a pattern consistent with intermediate or lateral connections (Yukie et al. 1990; Webster et al. 1991, 1994;
Four injections were placed in different locations within TE. The distribution of Zn\(^{+}\) neurons in case 299, with an injection at the TEav/perirhinal border, is similar to that in case 295, where the injection was more lateral, confined to TEav without involvement of perirhinal cortex. The projections in case 295 are described but not illustrated.

In our material, there were few or no Zn\(^{+}\) neurons posterior to the injections, in what would correspond to feedforward projections (Figs 6–9). Notably, the expected feedforward projections from V4 and TEO were not labeled in any of the 4 cases. Major projection foci were detected in the OTS, in the

**Sodium Selenite**

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**Figure 2.** Two fields of Zn\(^{+}\) neurons, retrogradely labeled by an injection of sodium selenite in area V1. (A) Neurons in layer 6 of the lunate sulcus (area V2). An image is from rectangle part in (E) section 32. (B) Higher magnification from the arrow in (A). (C) Neurons in layer 6 of the STS (area MT). An image is from rectangle part in (E) section 71. (D) Higher magnification from the arrow in (C). (E) Schematic right hemisphere (case R63) and 2 coronal section outlines (where larger numbers are more anterior) showing the location of the injection site in area V1 (red spot) and the location of Zn\(^{+}\) neurons (in red; one dot = one neuron). The anterior-posterior position of the sections is indicated by the interrupted lines drawn on the right hemisphere schematic, at the top of the figure. The lines are numbered to correspond to the respective sections. IOS, inferior occipital sulcus; LS, lunate sulcus; V1, area V1; V2, area V2; MT, area MT. Scale bar: A, C, 500 \(\mu\)m; B, D, 100 \(\mu\)m; E, 5 mm.

**Figure 3.** Schematic right hemisphere (case 286) and 4 coronal section outlines (where larger numbers are more posterior) showing the location of the injection site in area V4 (red spot and arrowhead in section 71) and the location of Zn\(^{+}\) neurons (in red; one dot = one neuron). Other conventions are the same as in Figure 2E. LF, lateral fissure; PMTS, posterior medial temporal sulcus; TEO, area TEO; V3A, area V3A; V4, area V4; others, see Figure 2E. Scale bar, 5 mm.
parahippocampal gyrus, in several fields in the STS, and anterior to the injections.

**Sodium Selenite: Projection Foci Posterior to the Injections**

Some Zn⁺ neurons were detected in areas posterior to the injection (>4.0 mm), but these are likely to be within a subdivision of TE (Figs 6-9). In case R62 (TEad injection), there was a prominent posterior focus in the medial bank of the OTS (TEpv: Fig. 9, section 91). In case 299 (TEav/perirhinal), there was also a posterior focus in the OTS, but in both its lateral and its medial banks (TEpv: Fig. 7, section 68; Fig. 8A). In case 295 (TEav injection, not illustrated, but see fig. 3 in Ichinohe and Rockland 2005), labeled neurons were similarly located in the depth and lateral bank of the OTS (TEpv). In these foci in the OTS, labeled neurons were predominantly in a bistratified distribution, but with a bias for the upper layers. In case R66 (TEpd injection), there was extensive bistratified labeling for about 2.0 mm posterior to the injection (Fig. 6, section 94). Because this is relatively close to the injection, this can be "intrinsic" and/or lateral (i.e., remaining within TEpd).

**Sodium Selenite: Projection Foci in the Parahippocampal Gyrus**

All 4 injections resulted in label in the parahippocampal gyrus. In case R62 (with the most anterior injection), this was...
relatively posterior, in TF and TH, and restricted to layer 6 (Fig. 9, sections 77 and 91). In case 299 (TEav/perirhinal injection), the focus was slightly more anterior, with the same layer 6 distribution but also upper layer involvement (Fig. 7, sections 54 and 68). In case 295 (TEav injection; not illustrated), the focus was similarly anterior (near the anterior tip of the OTS), but neurons were restricted to layer 6. In the posterior case of this series (R66), only a few scattered neurons occurred in area TF, but there was a distinct focus in area TH in layer 6 (Fig. 6, sections 94 and 102).

Sodium Selenite: Projection Foci in the STS
Projections from the STS were visualized from 2 foci, likely to be multimodal areas. In case 62 (with the most anterior injection), Zn⁺ neurons occurred in the upper bank of the STS (area TPO) and continued for about 6.0 mm (Fig. 9, sections 25, 35, and 45). These were located in the supra- and infragranular layers, except at the posterior fringe, where they were restricted to layer 6. After a gap of about 4.0 mm, a second group of Zn⁺ neurons appeared in the lower bank of the STS, in a bistratified distribution (Fig. 9, section 91; Fig. 10C). In case 299 (TEav/perirhinal injection), at anterior levels of the STS, Zn⁺ neurons were found in the lower bank (TEa), mainly in the upper layers, with a few neurons in layer 6 (Fig. 7, sections 26, 35, 54, and 68; Fig. 8B). There were also a few neurons in layer 3 of the adjoining lip of the STS (TEm: Fig. 7, section 26). More posteriorly, Zn⁺ neurons occurred in the upper bank (TPO), depth (IPa), and lower bank (TEa: Fig. 7, section 68). These were mainly in the upper layers. In case 295 (TEav injection; not illustrated), there was a comparatively smaller number of Zn⁺ neurons. These were in the lower bank, anterior (TEa) and more posterior (TEm). Neurons in both foci were mainly in the upper layers. In the most posterior case (R66), the injection was immediately adjacent to the STS. This resulted in abundant
labeling in the STS, in a bistratified distribution, both anterior and posterior to the injection (Fig. 6, sections 49, 64, 74, 84, and 94). Given the proximity of the injection site, this projection could be mainly intrinsic but encompassing a further extrinsic component (Fig. 6, sections 84 and 94).

Sodium Selenite: Projection Foci Anterior to the Injection

In all 4 cases, Zn⁺ neurons were found anterior to the injection sites. In the immediate vicinity of the injections, these could be considered association connections, as described with standard retrograde tracers (Figs 6–9, 10B; Yukie et al. 1990; Fujita I and Fujita T 1996; Lavenex et al. 2004). In addition, labeled neurons occurred ventrally in the temporal pole (Figs 6, 7, 9, 10, mainly in layers 2 and 6 (TEav/TGv and TGsts: Kondo et al. 2003; Saleem et al. 2007). The injection at the TEav/perirhinal border (case 299) produced Zn⁺ neurons more dorsomedially in the temporal pole (TGa: Kondo et al. 2003; Fig. 7, section 14). The anterior most injection (case R62) produced neurons dorsally (area Tgd of Saleem et al. 2007; Fig. 9, section 12). The injection in area TEav (case 295, not illustrated) resulted in only a small number of Zn⁺ neurons in TGv.

EM Identification of Zn⁺ BDA-Labeled Cortical Projections

We prepared one brain with BDA injections in TEpd. The intention was to corroborate by another technique (BDA labeling), the finding that feedback and association projections can be Zn⁺. We did not carry out the same experiment for feedforward projections because of the evidence that these would be largely negative for zinc. That expectation was based on 1) the present sodium selenite results and 2) the relative absence of Zn⁺ terminations in the principal layers targeted by feedforward terminations.

Zn⁺ synapses were identified by the presence of large silver grains within a vesicle-containing profile (Fig. 12D,E). These Zn⁺ synapses derive from multiple sources, including callosal connections, extrinsic cortical projections not labeled by our BDA injection, intrinsic cortical connections, and amygdalo-cortical connections (Casanovas-Aguilar et al. 1995, 1998; Brown and Dyck 2005; Miyashita et al. 2007). Thus, Zn⁺ synapses alone were not included in our analysis.

BDA-labeled profiles were identified by the standard dark DAB reaction product. Synapses judged to be positive for both zinc and BDA were required to have an unambiguous colocalization of silver grains and DAB reaction product, together with distinct synaptic vesicles (Fig. 12F–H). Profiles where the vesicles were obscured or where the presence of DAB was ambiguous were not counted. Thus, some degree of undercounting is likely. Fields were selected from the densest regions of BDA label, at the border of layers 1 and 2 (Fig. 12A–C). Some retrogradely filled neurons resulted from the BDA injections.

Figure 8. Two fields of Zn⁺ neurons, labeled by the injection in case 299. (A) Zn⁺ neurons in layers 2, 3, 5, and 6 in the medial bank of the OTS (area TEpv; Fig. 7, section 68). The ventral surface of the brain is at the top and medial to the left. (B) Zn⁺ neurons in layers 2, 3, and 6 of the lower bank of the STS (area TEa; Fig. 7, section 58). Scale bar: A, 100 μm; B, 200 μm.

Figure 9. Schematic left hemisphere and ventral portion of 7 coronal sections showing the location of the injection site anterior in TEad (case R62) and the distribution of Zn⁺ neurons. Larger numbers are more posterior. Other conventions are the same as in Figure 2E. Tgd, area Tgd; others, see Figure 7. Scale bar, 5 mm.
Scale bar: main in the lower layers of the STS, with a few in the upper layers (Fig. 9, section 45). Neurons mainly in layers 5 and 6 of the lower bank of the anterior medial temporal sulcus.

Within this smaller sample in V1, there was a distinct bias for terminations in layer 6, and scattered Zn+ neurons in layer 2. Presumably, the intrinsic Zn+ population more closely coinciding with the distribution of Zn+ terminations would be more likely to be Zn+.

Discussion

In general, primary and early sensory areas have low levels of zinc, whereas limbic areas in the temporal lobe, medial interhemispheric surface, and orbitofrontal areas contain higher levels of Zn+ terminations (Carmichael and Price 1994; Pérez-Clausell 1996; Frederickson et al. 2000; Ichinohe and Rockland 2004, 2005). In area V1, Zn+ terminations in layers 3 and 4A form patches that are complementary to thalamocortical terminations, as visualized by cytochrome oxidase (Dyck and Cynader 1993; Dyck et al. 2003).

By using focal injections of sodium selenite, we have determined intrinsic and extrinsic cortical sources of Zn+ terminations to specific cortical sites. For the early visual areas (V1, V4, and TEO), Zn+ projections are correlated with feedback projections. Surprisingly, however, only the component originating from layer 6, but not that from layer 2 or 3, is Zn+. For temporal areas, association intratemporal connections as well as feedback projections were observed to be Zn+. Laminar distribution of neurons in this case appeared similar to what has been reported after standard retrograde tracers, namely layers 2 and 6 for feedback and layers 2, 3, 5, and 6 for associational connections (Rockland and Pandya 1979; Maunsell and Van Essen 1983; Felleman and Van Essen 1991; Suzuki and Amaral 1994; Saleem and Tanaka 1996; Saleem et al. 2000; Ungerleider et al. 2008).

All the injections resulted in intrinsic Zn+ neurons in layer 2. In V1, where the density of Zn+ terminations is lowest, these were limited to the immediate vicinity of the injection. Presumably, the intrinsic Zn+ connections in V1 are predominantly vertical, so that most of the Zn+ neurons in deeper layers would be obscured within the injection site. In V4 and TEO, Zn+ neurons also concentrated in layer 2 but extended further laterally. In these areas, Zn+ neurons were notably absent from layer 3 or 5, although these layers contain abundant intrinsically projecting pyramidal neurons after injections of standard tracers. Finally, the temporal injections all produced extensive label in layer 2 (for 1.5–2.5 mm), a secondary band in layer 6, and scattered Zn+ neurons in layers 3 and 5. None of these intrinsic connections had any discernible patchiness, except that the sparse label in layer 3 of temporal areas showed some periodic fluctuation.

Technical Considerations

Evaluation of these results depends on the reliability and sensitivity of sodium selenite as a tracer. That is, could the absence of Zn+ neurons in certain projections or layers be due to a simple failure of transport? We cite 3 factors that argue against this possibility. First, sodium selenite has been extensively vetted in rodents, with no reports of technical artifacts (Christensen et al. 1992; Casanovas-Aguilar et al. 1995, 1998; Brown and Dyck 2005). Second, Zn+ neurons are detected in distant sites as well as nearby the injection (Ichinohe and Rockland 2005; this study). Third, our results...
are consistent with the known pattern of Zn+ terminations (Pérez-Clausell 1996; Ichinohe and Rockland 2005). These are low in layer 4, the recipient layer of feedforward terminations (Rockland and Pandya 1979; Maunsell and Van Essen 1983; Felleman and Van Essen 1991), and our injections in fact revealed absence of Zn+ neurons among feedforward projections. Similarly, Zn+ terminations are dense in layers that receive feedback projections. Both our retrograde labeling and EM results, however, indicate that only a subset of the feedback connections are Zn+. This is consistent with the fact that BDA-labeled feedback terminations extend beyond the zone of Zn+ terminations, which avoid layer 1a. Because we have scanned sections at a relatively close interval (200 μm), we consider that the absence of supragranular labeling is real and not due to observer oversight (see Supplementary Fig. 1). Moreover, comparison of labeled foci after injections of sodium selenite or injections of conventional tracers did not reveal marked differences in density of labeled neurons in temporal area injection cases (see Fig. 11). Thus, we suggest that the absence of retrogradely transported zinc selenite can be taken as a reliable indicator that those neurons do not have Zn+ synapses at the injection site.

**Functional Significance**

The functional significance of our results depends on what happens at particular Zn+ synapses. From the limited experimental data on the physiological properties of long distance cortical synapses, we can expect that Zn+ synapses have distinct voltage dependence, decay kinetics, and receptor subunit-specific pharmacology (in rodent: Kumar and Huguenard 2003). Corticocortical synapses, as assayed in vitro, exhibit either paired-pulse facilitation or inhibition, depending on the combination of pre- and postsynaptic subtypes (see for review: Thomson and Lamy 2007). In the case of Zn+ synapses, the synaptic response may be further influenced by zinc concentration and target identity (Paoletti et al. 2009).

Evidence from in vitro preparations and deprivation experiments has consistently suggested that synaptic zinc has a role in activity-dependent plasticity (Frederickson et al. 2005; Nakashima and Dyck 2009). The potential mechanisms are numerous and varied, including translocation into postsynaptic neurons (Li et al. 2001), interaction with NMDA receptors (Rachline et al. 2005), activation of Trk receptors (Huang et al. 2008), and, still hypothetical, an important role in the modulation of the postsynaptic density (Gundelfinger et al. 2006). Finally, although most discussions have emphasized the potential positive influences of zinc on synaptic plasticity, another idea is that the release of zinc may help to stabilize synapses, which are otherwise predisposed to modifications in synaptic strength, by a high density of NMDA receptors or other factors (Slomianka 1992; Ueno et al. 2002; Paoletti et al. 2009).

The location of Zn+ neurons and the distribution of Zn+ terminations suggest a preferential association of zinc with feedback and lateral connections; and our combined EM-BDA results corroborate that a subpopulation of feedback synapses are Zn+. Feedback and lateral connections have consistently been differentiated from feedforward, layer 4-targeting inputs, as

![Figure 11](https://academic.oup.com/cercor/article-abstract/20/12/2818/362754)
exhibiting more rapid or differential plasticity (Crair and Malenka 1995; Feldman et al. 1998; Glazewski et al. 1998; Trachtenberg et al. 2000; Jiang et al. 2007). In this respect, it is interesting to note that feedback inputs from higher visual areas to V1 have been discussed as accelerating and strengthening perceptual learning (see for review: Gilbert and Sigman 2007; Kiper et al. 2007). Feedback signals from medial temporal perirhinal areas are thought to mediate the visual associative mnemonic codes of inferotemporal neurons (Higuchi and Miyashita 1996).

More unexpected is the selective absence of synaptic zinc in feedback-projecting neurons from layer 2 to early visual areas. This is particularly puzzling because layer 2 contains abundant intrinsically projecting Zn+ neurons. The most plausible explanation is that layer 2 contains a mixed population of Zn+ intrinsically projecting and Zn negative extrinsically projecting neurons. This dissociation seems to occur preferentially in the early visual areas, in contrast with temporal association areas.

Another indication of important distinctions between feedback-projecting neurons in layers 2 and 6 is from investigations of collateralization. That is, after retrograde dye injections in areas V1 and V4, a few neurons in layer 6 of V2...
distribution of amygdalocortical and Zn+ terminations, there is tight correspondence between the laminar negative (Garrett et al. 1992). The finding that Zn+ projections from the cingulate, retrosplenial, perirhinal, and prefrontal cortex revealed that all the inspected BDA-labeled amygdalocortical terminations has confirmed that these are Zn+ (Miyashita et al. 2007). EM analysis of amygdalocortical projections to medial prefrontal cortex revealed that all the inspected BDA-labeled terminations, not just a subset, were Zn+. Consistent with this finding, there is tight correspondence between the laminar distribution of amygdalocortical and Zn+ terminations, both of which target layer 1b and the layer 1/2 border (Freese and Amaral 2006). The proportion of Zn+ neurons may be species specific as a comparable study in the rat reported that only 35% of basolateral neurons, projecting to the medial prefrontal cortex, contained zinc (Cunningham et al. 2007).

Previous studies, using other criteria than the presence or absence of synaptic zinc, have reported subpopulations within a projection system. Thicker myelinated versus thinner unmyelinated axon subpopulations are commonly identified within a given projection, for example, there is a recent report concerning corticocellular projections in the cat (Fuentes-Santamaría et al. 2009). Particularly relevant to the present results, V2 feedback axons to V1 are reported as thick, myelinated, heavily branched, and bearing clusters of terminations or thin, unmyelinated, and uniformly covered with boutons (Anderson and Martin 2009). An earlier study, where retrogradely labeled neurons were double-reacted for neurofilament protein, reported that neurofilament-containing feedback-projecting neurons in areas V4 and MT are more frequent in layers 5 and 6 than in layers 2 and 3 (Hof et al. 1996).

The observation that some projections are entirely Zn negative is borne out by studies in rodent, for example, projections from the cingulate, retrosplenial, perirhinal, and lateral entorhinal cortices to mouse visual cortex are Zn negative (Garrett et al. 1992). The finding that Zn+ terminations can originate from only some of the layers giving rise to a given projection has also been repeatedly described in rodent. In the rat visual cortex, Zn+ callosally projecting neurons occur in layers 2, 3, and 6 but not in layer 4 or 5 (Casanovas-Aguilar et al. 1995). Sodium selenite injections in mouse barrel cortex result in Zn+ neurons in layers 2, 3, and 6. This is a more limited distribution than that seen after injections of cholera toxin (Brown and Dyck 2005). Zn+ cortical projections from hippocampal CA1, in both rodents (Slomianka 1992) and monkeys (Ichinohe and Rockland 2005), preferentially originate from pyramidal neurons in the upper stratum. It is currently unknown whether Zn+ and Zn-negative components have separate postsynaptic targets.

Subpopulations within specific projections are an excellent target for future functional investigations into synaptic properties and connectional interactions (Cardin et al. 2009; Petreanu et al. 2009).

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**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

**Notes**

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