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Zinc-positive afferents to the rat septum originate from distinct subpopulations of zinc-containing neurons in the hippocampal areas and layers

A combined Fluoro-Gold tracing and histochemical study

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Abstract. The purpose of the present study was to examine whether zinc-positive and zinc-negative hippocampal neurons in rats differed with respect to their projections to the septum. By combining retrograde axonal transport of the fluorescent tracer Fluoro-Gold with histochemical demonstration of zinc selenide complexes in zinc-containing neurons after intraperitoneal injection of sodium selenite, we were able to visualize the distribution of retrogradely Fluoro-Gold labeled neurons and zinc-containing neurons in the same sections. After unilateral injection of Fluoro-Gold into the rat septum a few retrogradely labeled cells were observed in layer IV of the ipsilateral medial entorhinal area, and numerous labeled cells were observed mainly in the superficial layers of the ipsilateral subicular areas and throughout the CA1 and CA3 pyramidal cell layers, as well as in the contralateral CA3 pyramidal cell layer. Zinc-containing neurons were observed in layers IV-VI of the medial entorhinal area, layers II and III of the parasubiculum, layers II, III and V of presubiculum, and in the superficial CA1 and deep CA3 pyramidal cell layers. Cells double-labeled with Fluoro-Gold and zinc selenide complexes were primarily located in distal (relative to the area dentata) parts of the superficial CA1 pyramidal cell layer and distal parts of the deep CA3 pyramidal cell layer and in layers II and III of presubiculum. Only a very few double-labeled cells were seen in the contralateral CA3. The result demonstrates that the hippocampo-septal projection of rats is a mixture of zinc-positive and zinc-negative fibers. Whereas zinc-negative fibers originate from neurons throughout the hippocampal and retrohippocampal areas, zinc-positive fibers originate from distinct subgroups of zinc-containing cells in different areas and layers.

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Key words: Retrograde tracing – Selenite – Zn-ergic – Glutamate – NMDA

Introduction

The zinc-containing neurons and their projections form one of the most extensive chemospecific systems in the mammalian forebrain (Slomianka et al. 1990; Slomianka 1992). Zinc has been shown to be released from presynaptic terminals and to modulate the effect of glutamate on glutamate receptors (Chadwick and Choi 1990; Hori et al. 1987; Nakanishi 1992; Peters et al. 1987; Westbrook and Mayer 1987; Wong and Kemp 1991). Moreover zinc has been implicated in the pathophysiology of epilepsy and ischemic brain damage (Fredrickson et al. 1988; Slomianka 1992; Tønder et al. 1990). Previous anatomical studies of the zinc-positive telencephalic pathways have relied on conventional lesion-degeneration methods in combination with histochemistry for zinc (Haug et al. 1971; Hjorth Simonsen 1973; Zimmer 1973). Recently it has, however, been shown that zinc-positive pathways of zinc-containing neurons can be traced by chemospecific retrograde transport of zinc selenide after intracerebral injection of sodium selenite or sodium selenide (Howell and Frederickson 1990; Christensen et al. 1992). The selenium ions precipitate the vesicular zinc, which then as zinc selenide is transported retrogradely to the nerve cell soma, where it can be visualized histochemically (Danscher 1982). Generalized retrograde labeling of telencephalic zinc-containing neurons can alternatively be obtained by intraperitoneal injection of sodium selenite (Slomianka et al. 1990). The chemospecific retrograde labeling of zinc-containing neurons by zinc selenite can be combined with the retrograde fluorescent tracer Fluoro-Gold (FG) (Garrett

et al. 1992). A previous study (Slomianka 1992) demonstrated that the zinc-containing neurons in the hippocampal formation are pyramidal cells located primarily in the superficial CA1 pyramidal cell layer and in the deep CA3 pyramidal cell layer as well as in the pre- and parasubiculum. This finding indicates that hippocampal pyramidal cells are a neurochemically heterogenous population and raises the question whether zinc-containing and zinc-negative pyramidal cells have parallel or diverging efferent connections. Some indirect evidence for diverging efferent connections can be found in a report by Chronister and DeFrance (1979), who reported that hippocampal pyramidal cells which project to the septum have rather specific locations along the radial axis of the cell layer. Furthermore, their placement superficially or deep in the pyramidal cell layer varied along the transverse axis of the layer. In the present study we have combined the generalized chemospecific retrograde labeling of zinc-containing neurons with the retrograde fluorescent tracer FG in order to examine the presumed differential distribution of zinc-containing neurons with septal projections.

Materials and methods

Fluoro-Gold injection. Eight adult male Wistar-Kyoto rats, aged 2.5 months (approx. 300 g) were used. Four of these rats received a unilateral pressure injection of 0.2 µl 2.0% FG dissolved in deionized water into the septal area (coordinates AP: 0.0, L: 1.0, V: 5.0, relative to bregma and the dura with the incisor bar 3.3 mm below the interaural line). The remaining four animals had received a transplant of neonatal hippocampus tissue into their right mid-septotemporal hippocampal area on the day of birth. These animals were injected bilaterally into the septal nuclei with 0.2 µl 2.0% FG in distilled water, using the same stereotaxic coordinates as above. For all FG injections a 5-µl Hamilton syringe with an attached glass micropipette was used in order to minimize the injection trauma to cortex and septum.

Sodium selenite administration and perfusion. The animals were allowed to survive for 1 week after the FG injection before they were given an intraperitoneal injection of 8 mg/kg sodium selenite (Na_2SeO_3) dissolved in deionized water (0.8 mg/ml). After 24 h they were anesthetized with pentobarbital, followed by transcardial perfusion at 120 mmHg with approx. 500 ml of 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4. The brains were removed and immersed in a solution of 30% sucrose in PBS at 4° C. When the brains had sunk in the

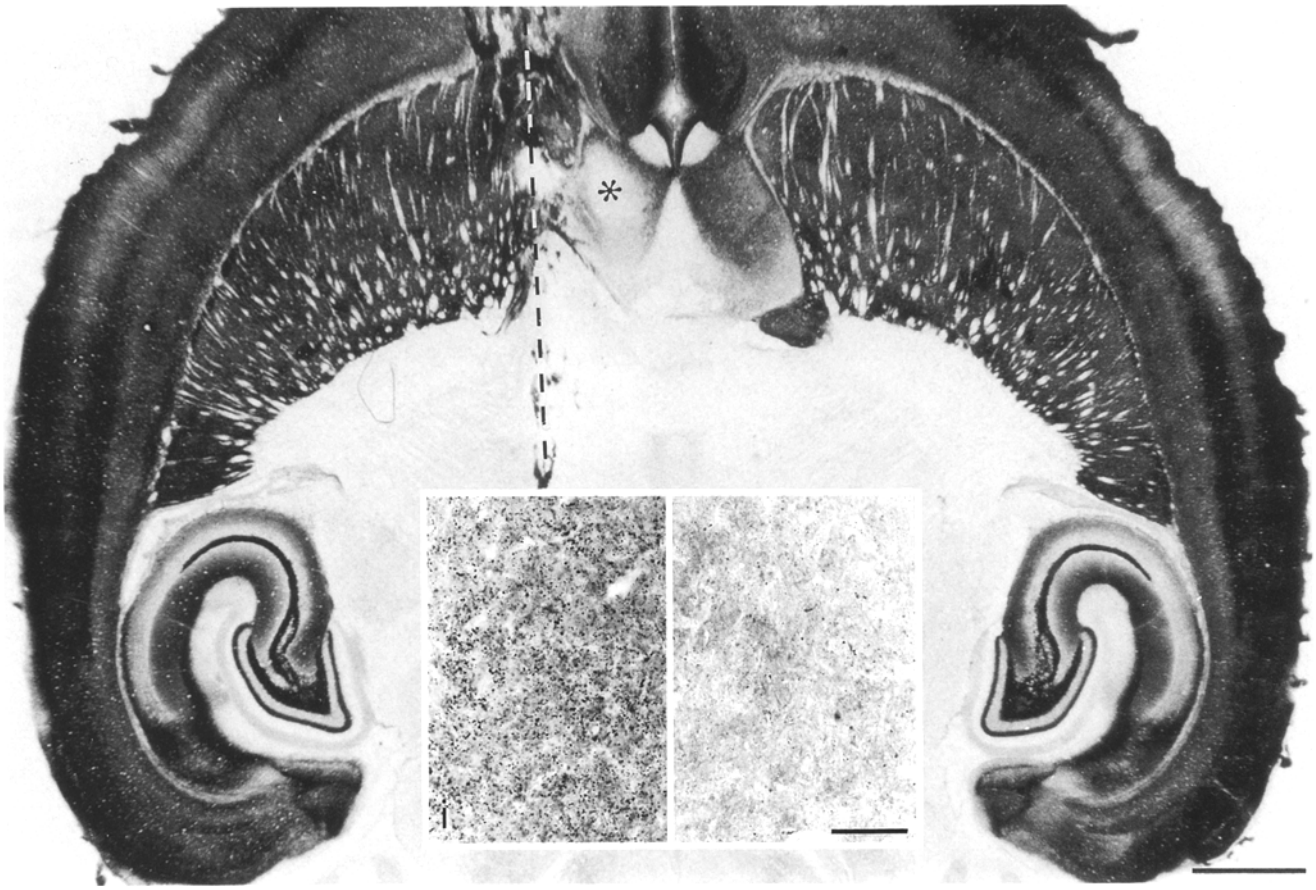

Fig. 1. Horizontal section through the mouse telencephalon (*Apodemus* sp.) showing septum and midseptotemporal part of hippocampus, histochemically stained for zinc selenide. Lateral to the septum and extending into the thalamic nuclei a knife cut (*punctuated line*) has transected the fimbria fornix and disconnected the left septal nuclei from the temporal three-quarters of the left hippocampal formation. The lesion-induced degeneration of hippocampal afferents to the septum is seen to cause a bleaching of the neuropil staining in the lateral septal nucleus (*asterisk*). Bar 1 mm. The *inset* (*i*) shows the lateral septal nucleus in high magnification from the left and right side in an adjacent section histochemically stained with the Fink-Heimer method for degenerating axon terminals. Bar 75 µm

sucrose the following day, they were frozen by means of gaseous CO_2, cut into 30-μm-thick horizontal sections, thawed onto gelatinized slides, air dried, and stored at −25° C until staining.

Histochemical procedures. Series to be stained for zinc selenides were fixed in 96% ethanol for 15 min, rehydrated, and rinsed in deionized water. The silver amplification procedure was similar to that described by Danscher (1982). Briefly, slides were immersed in a silver lactate/hydroquinone developer containing a protective colloid (gum arabic) and pH-adjusted with citrate buffer. Sections were developed for 60 min at 26° C in a dark chamber. After development, the slides were submerged in a 5% sodium thiosulfate solution for 12 min, rinsed in deionized water, postfixed for 30 min in 70% ethanol, dehydrated, cleared, and coverslipped with Entellan. Adjacent series were Nissl-stained or left unstained and coverslipped with Entellan. Sections were examined and photographed in a Zeiss Axiophot epifluorescence microscope.

Results

Zinc-containing neurons

The chemospecific retrograde labeling of neurons after intraperitoneal injection of sodium selenite concurred entirely with that previously described (Slomianka 1992). Briefly, the labeled cells were located as follows: layers IV–VI of both medial and lateral entorhinal area, layer II of the lateral entorhinal area, layers II, III, and V of the presubiculum and parasubiculum (Fig. 4a), in the superficial CA1 pyramidal cell layer (Figs. 5a, 6a) and deep in the CA3 pyramidal cell layer (Figs. 7a, 8a). In the dentate hilus, cells could not be observed due to the high level of neuropil staining. Granule cell staining was more distinct than described previously, which is probably related to the better preservation of histological detail afforded by the fixative as compared to the unfixed material used in earlier studies (Slomianka et al. 1990; Slomianka 1992).

Fluoro-Gold injection sites

In most cases the FG injection was centered in the septum (Fig. 2), covering the septum from the dorsal lateral septal nucleus to the anterior commissure, above the bed nucleus of stria terminalis (BNST), ventrally. Medially, the injections involved the medial septal nucleus and extended laterally throughout the lateral septal nucleus to the lateral ventricle. In the rostrocaudal direction, the FG injections were also centered in the septum. In three cases with unilateral septal FG injections, the injection site was confined to the ipsilateral side. In one case, siphoning of FG up the cannula tract gave a small amount of labeling in the overlying cingulate cortex. In one unilaterally injected animal, the injection site did not involve the posterior parts of the septal complex, but involved a minor part of the anterior contralateral septum. In none of the eight animals did the FG injection site include the BNST.

Fluoro-Gold labeling and zinc-containing neurons

Description of the FG retrograde labeling in relation to the distribution of zinc-containing neurons, i.e., neurons that contain silver precipitate, will follow the allocortical areas at a mid-septotemporal level from the medial entorhinal area (MEA), over the subicular areas, to the hippocampus proper (CA1 and CA3) and the area dentata, using the terminology of Blackstad (1956). No qualitative differences in the distribution of FG labeled cells were observed in the hippocampal septo-temporal axis, but the number of FG-labeled hippocampal pyramidal cells were increased at mid-septo temporal levels. The description of differences in the ipsilateral and contralateral FG retrograde labeling will be based on the four animals with unilateral FG injections. In the four bilaterally FG-injected animals, the fluorescent and histochemical labeling was only assessed on the "normal" side contralateral to the grafted side. No qualita-

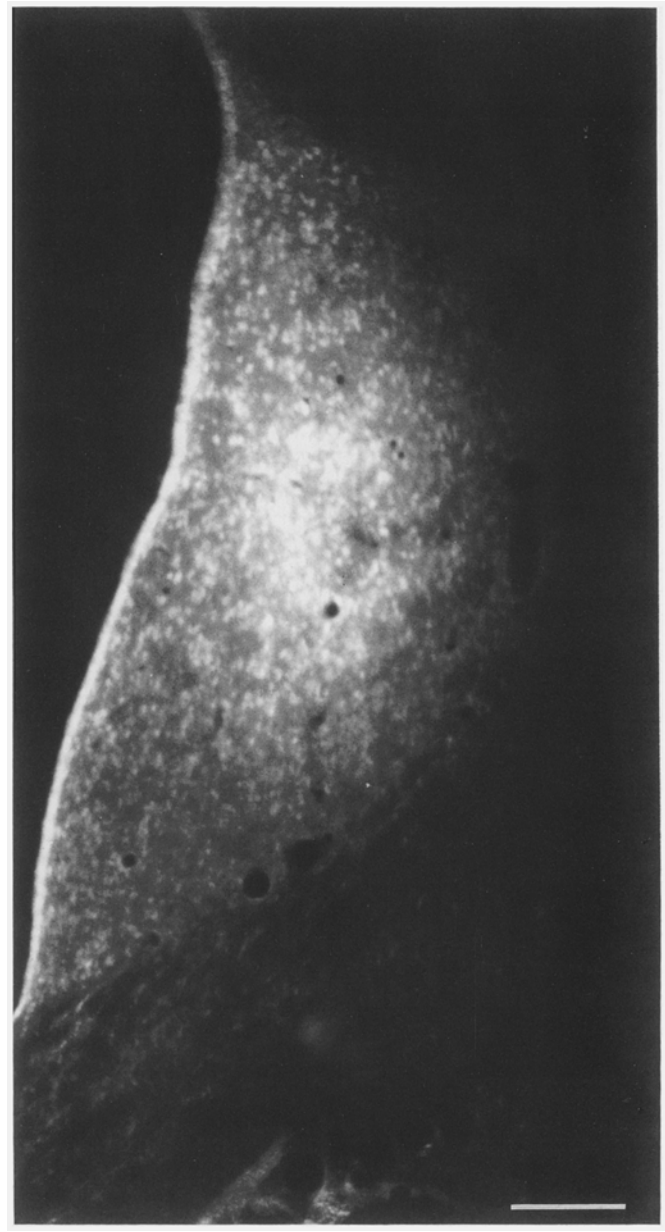

Fig. 2. FG injection site in the lateral septal nuclei of the rat at a mid-dorsoventral level. Midline along right edge of the micrograph. Bar 200 μm

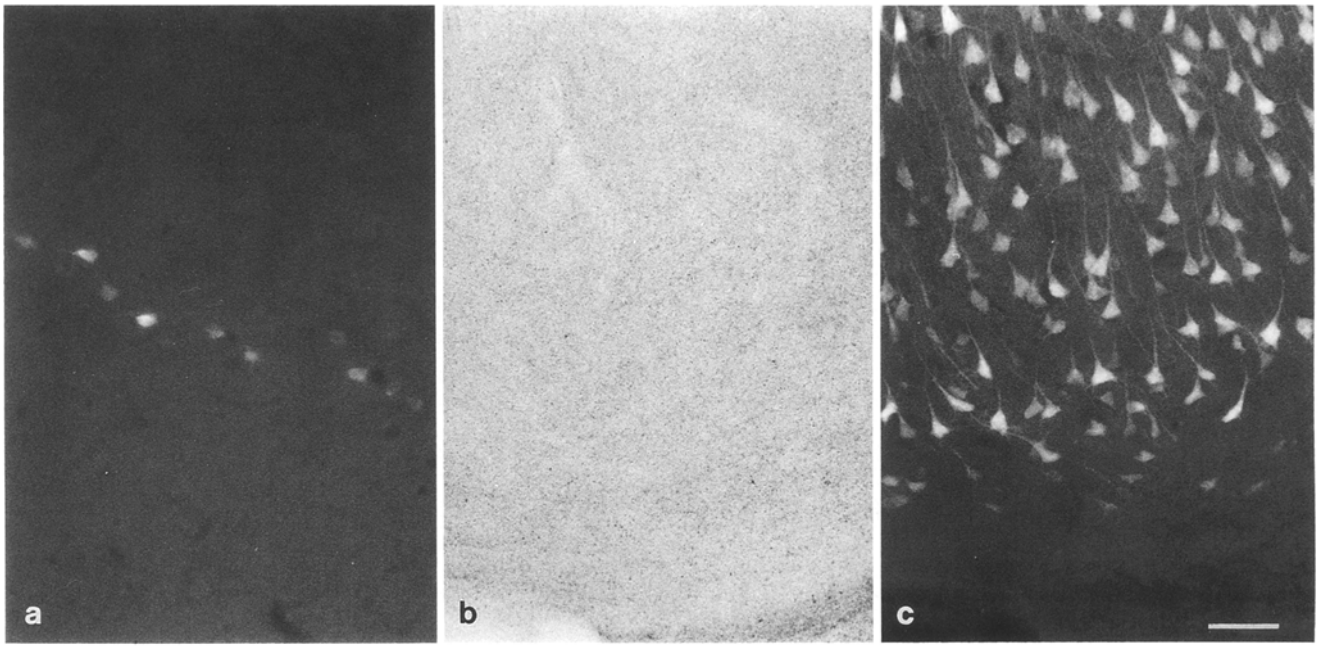

Fig. 3. **a** Neurons in the medial entorhinal cortex layer IV retrogradely labeled with FG. **b** No zinc selenide-labeled cells are seen in the proximal part of the subiculum. **c** FG-labeled zinc-negative neurons in same part of subiculum as shown in **b**. Bar 40 μm

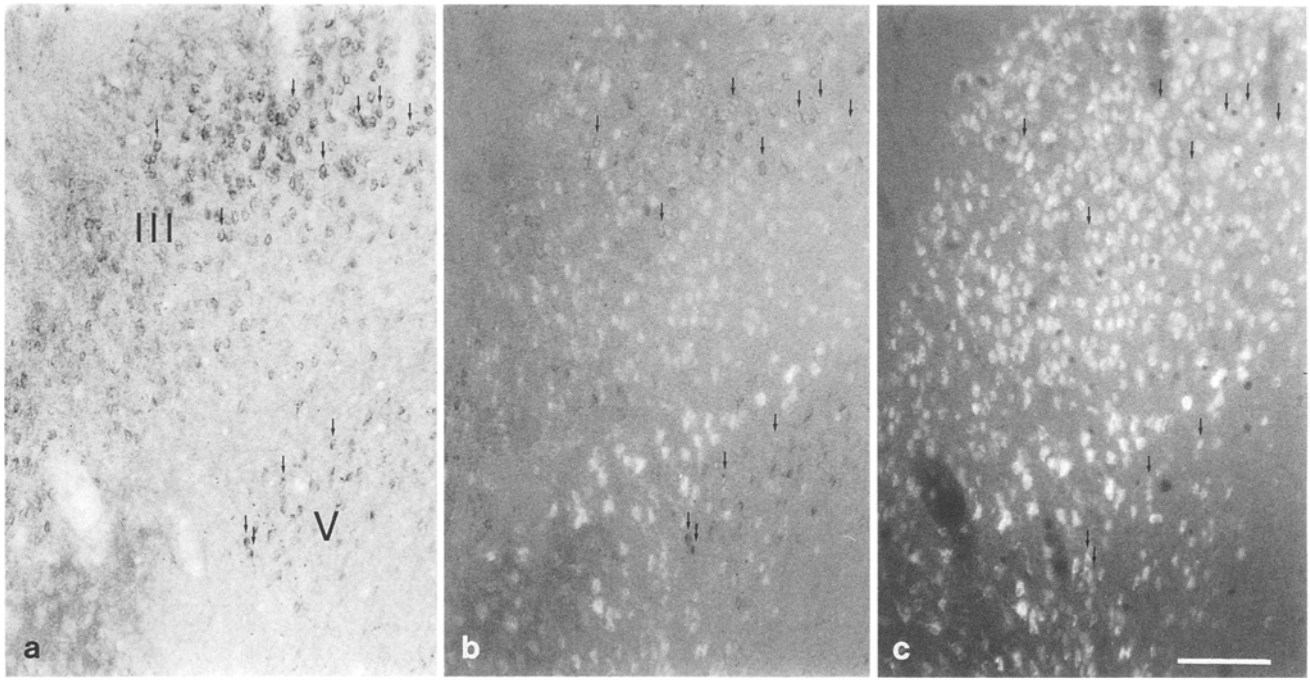

Fig. 4a–c. Zinc selenide and FG labeling in the presubiculum. **a** zinc-containing neurons are predominantly seen in layers II and III while they are fewer in layer V. *Small arrows* indicate neurons double-labeled with zinc selenide complexes of FG. **b** Zinc selenide and FG-containing neurons viewed in brightfield and UV illumination. **c** FG-labeled neurons seen in UV illumination. Bar 80 μm

tive differences in the FG-labeling pattern were observed between this "normal" side in the grafted animals and the side ipsilateral to the septal FG injection in the unilaterally FG-injected animals. The description of retrograde FG labeling and zinc-containing neurons in the hippocampal grafts will follow in a separate study.

Medial entorhinal area

Ipsilateral to the injection site, a few FG-labeled neurons were observed in layer IV of the medial entorhinal area (MEA) (Fig. 3a). Occasionally one of the fluorescent cells was also labeled with silver precipitate. In the MEA

contralateral to the septal FG injection, no neurons retrogradely labeled with FG were observed.

Subicular areas

In the ipsilateral *parasubiculum* many FG-labeled neurons were observed in the superficial layers II and III. None of these contained silver precipitate in spite of many adjacent zinc-containing neurons. In the ipsilateral *presubiculum* many FG labeled cells were present in layers II, III and V (Fig. 4c). Numerous cells in layers II and III were also zinc-containing, whereas only a few double-labeled cells were seen in layer V (Fig. 4b). FG-labeled cells were only seen in the three cases in which the FG injection site included the posterior region of the septal complex (triangular nucleus and septofimbrial nucleus). In the part of the ipsilateral *subiculum* adjacent to the presubiculum, FG-labeled cells were observed mainly in the superficial cell layer, whereas the FG labeling spread throughout the cell layer in the part of subiculum close to the CA1 area (Fig. 3c). Even though the subiculum contains abundant zinc-positive afferents, there are no zinc-containing cells (Fig. 3b) and, consequently, no double-labeled cells. Contralateral to the injection site, no FG labeling was observed in the subicular areas.

Hippocampus

CA1 (regio superior). The distal (relative to the dentate area) CA1 close to the subiculum and ipsilateral to the septal FG injection, contained many FG-labeled cells throughout the pyramidal cell layer (Fig. 5b, c). In the superficial part of the distal CA1 pyramidal cell layer, the majority of the FG-labeled cells also contained silver precipitate and vice versa (Fig. 5b). The pyramidal cell layer in the proximal CA1 area, close to CA3, was distinctly layered, with cells containing silver precipitate in the superficial part of the layer, and FG-labeled cells in the deep part of the layer (Fig. 6a–c). The transition in the labeling pattern, from distal to proximal CA1, was gradual. A few double-labeled cells were also present in the deep part of the pyramidal cell layer at the CA1 to CA3 interface. No FG-labeled cells were observed in the contralateral CA1, except in one animal where a minor part of the FG injection had spread to the contralateral side, resulting in a few FG-labeled pyramidal cells in the deep pyramidal cell layer of CA1. No double-labeled cells were observed.

CA3 (regio inferior). In distal CA3 *ipsilateral* to the injection site, a tri-laminar staining pattern was observed. Superficially the mossy fiber zone showed an intense neuropil staining even 24 h after the sodium selenite injections (Figs. 7a, b, 8a, b). FG single-labeled pyramidal cells were seen subjacent to the mossy fiber zone. Deep in the pyramidal cell layer, many cells double-labeled with FG and silver precipitate were seen, as well as some single-labeled cells of either type (Figs. 7b, c, 8b, c). In the proximal CA3, close to the area dentata, a similar picture was seen, however, with fewer retrogradely FG-labeled pyramidal cells in the deep part of the pyramidal cell layer. *Contralateral* to

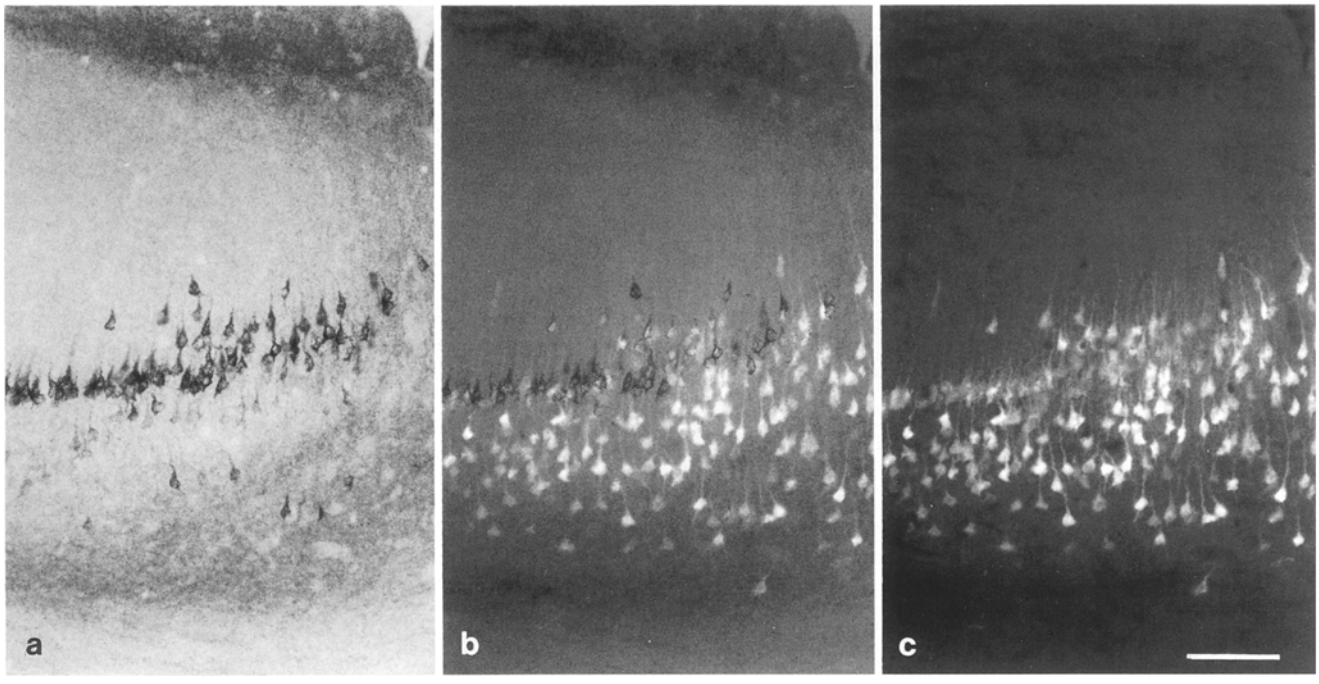

Fig. 5a–c. Zinc selenide and FG labeling in the distal CA1. **a** Zinc selenide-containing neurons of the superficial distal CA1 pyramidal cell layer viewed in brightfield illumination. **b** Zinc selenide- and/or FG-containing neurons in brightfield and UV illumination. Neurons double-labeled with zinc selenide and FG are located in the superficial pyramidal cell layer, whereas FG single-labeled neurons are located in the profound layer. **c** FG-labeled neurons seen in UV illumination. Bar 80 μm

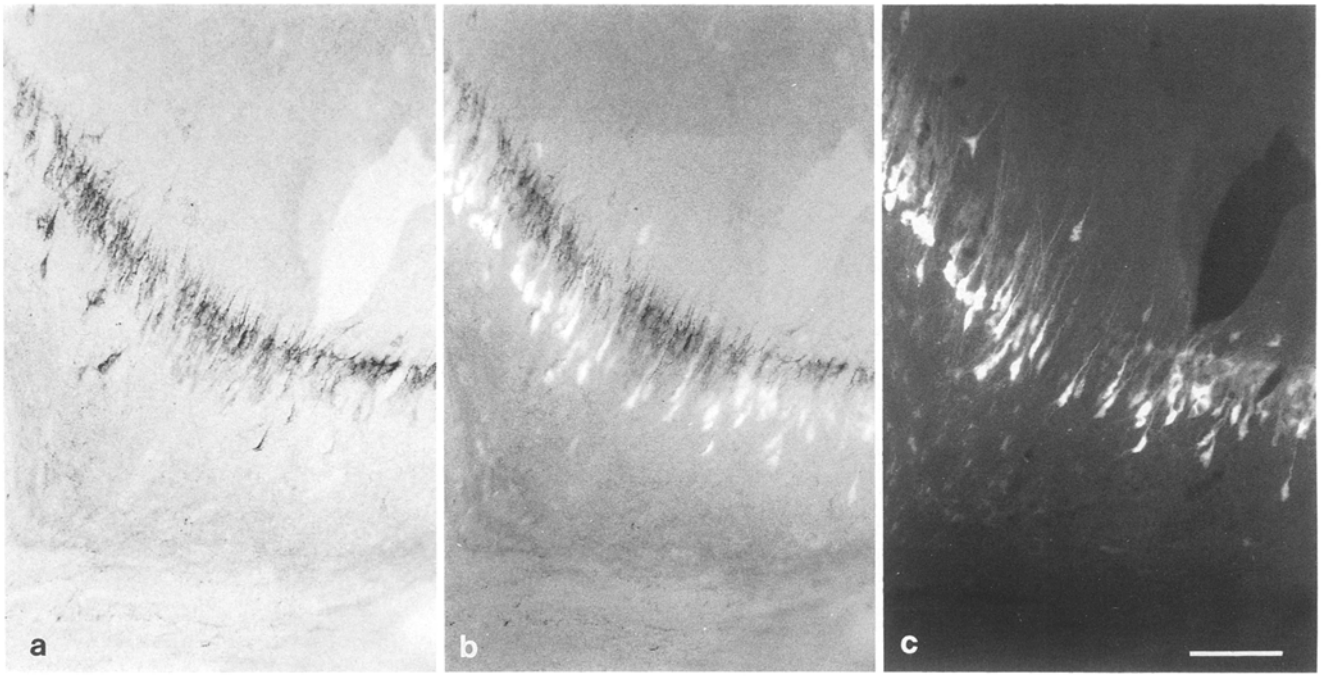

Fig. 6a–c. Zinc selenide and FG labeling in the proximal CA1. **a** Zinc selenide-containing neurons viewed in brightfield illumination. Zinc-containing neurons are seen predominantly in the superficial pyramidal cell layer. **b** Zinc selenide- or FG-containing neurons viewed in brightfield and UV illumination. Neurons labeled with zinc selenide are located in the superficial pyramidal cell layer, whereas FG single-labeled neurons are located in the profound layer. **c** FG-labeled neurons seen in UV illumination. Bar 80 μm

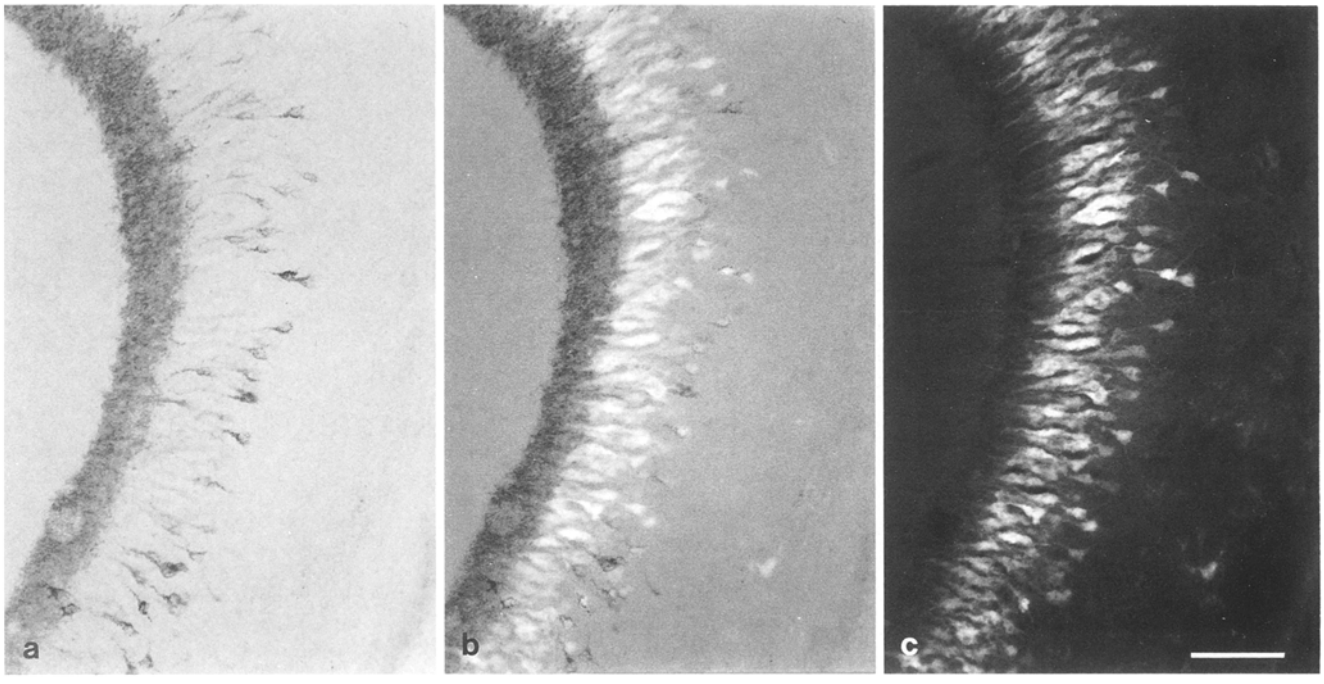

Fig. 7a–c. Zinc selenide and FG labeling in the ipsilateral CA3. **a** Mossy fibers and zinc selenide-containing neurons viewed in brightfield illumination. The zinc-containing neurons are seen in the pyramidal cell layer subjacent to the mossy fiber zone. **b** Zinc selenide- and/or FG-containing neurons viewed in brightfield and UV illumination. Many neurons double-labeled with zinc selenide and FG are located in the profound pyramidal cell layer of the CA3. **c** FG-labeled neurons seen in UV illumination. Bar 80 μm

the injection site, a similar tri-laminar pattern was observed (Fig. 8b), but only extremely few (one or two per section) of the zinc-containing pyramidal cells were labeled with FG (Fig. 8). Throughout CA1 and CA3, many FG single-labeled neurons were observed in the deep part of stratum oriens. Whereas the intensity of the FG labeling in CA3 pyramidal cells decreased towards the dentate area, the FG labeling of the deep stratum oriens neurons remained unchanged along the transverse axis of CA3.

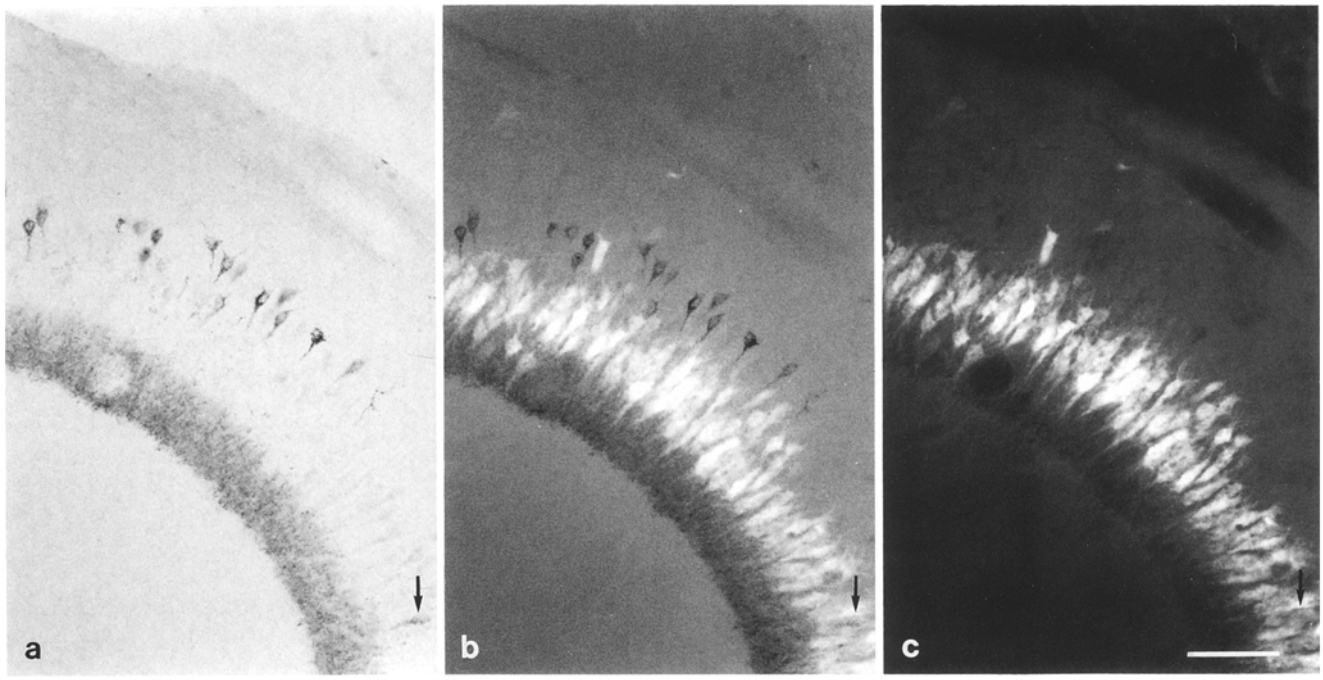

Fig. 8a–c. Zinc selenide and FG labeling in the contralateral CA3. **a** Mossy fibers and zinc selenide-containing neurons viewed in brightfield illumination. The zinc-containing neurons are seen in the pyramidal cell layer subjacent to the mossy fiber zone. **b** Zinc selenide or FG-containing neurons viewed in brightfield and UV illumination. A rare example of a neuron double-labeled with zinc selenide and FG is seen in the lower right part of the micrograph (*arrow*). **c** FG-labeled neurons seen in UV illumination. Bar 80 μm

Area dentata

Several FG-labeled hilar cells were observed, but no double-labeled cells could be identified due to the high level of neuropil staining in the hilus, even 24 h after the sodium selenite injections.

Discussion

Hippocampal zinc-containing projections to the septum

In the present study we have demonstrated that zinc-positive afferents in the septal nuclei of the rat originate from subpopulations of zinc-containing neurons in the hippocampal formation. While the ipsilateral projections from CA1 and CA3 were mixed zinc-positive and zinc-negative, the contralateral projection from CA3 was predominantly zinc-negative.

The zinc-positive connections from the hippocampal region to the septum in the rat were examined by combining the generalized chemospecific retrograde labeling of zinc-containing neurons in the hippocampal formation after intraperitoneal injection of sodium selenite (Slomianka et al. 1992) with the retrograde fluorescent tracer Fluoro-Gold (Garrett et al. 1992; Schmued and Fallon 1986). The distribution of retrogradely labeled zinc-containing neurons in the hippocampal region agreed with that previously described (Slomianka et al. 1992). The size of the FG injection sites (Fig. 2) and the FG-labeling pattern ruled out the possibility of tracer spread by the blood or via the cerebrospinal fluid. The observed retrograde hippocampal FG labeling, after FG injection into the septum, was in accordance with other studies on the efferent connections of the hippocampal region (Chronister and DeFrance 1979; Gaykema et al. 1991; Schwerdtfeger and Buhl 1986; Siegel et al. 1974; Swanson and Cowan 1977, 1979; Swanson et al. 1980). Also, using intraperitoneal injections of sodium selenite with shorter survival times (1 h) that produced intense neuropil labeling (Danscher 1982; Slomianka 1992), we could demonstrate zinc-positive hippocampo-septal afferents by observing axonal degeneration after electrolytic hippocampal or knifecut fimbria-fornix lesions in the mouse (Fig. 1; Slomianka and Sørensen, unpublished observations). The degeneration of the zinc-positive hippocampo-septal terminals led to a bleaching of the lateral septal nucleus in sections stained for vesicular zinc, and the degenerating axon terminals could be visualized by the Fink-Heimer stain (Fig. 1i). These results are consistent with the known hippocampal efferent connections to the septum, and the present results.

Parallel and diverging zinc-positive or zinc-negative hippocampal efferents

The new information obtained in the present study is that not all hippocampal zinc-containing neurons project to the septum. The zinc-positive hippocampal efferents to the septum thus originate primarily from pyramidal cells in the distal end (relative to the area dentata) of the superficial and deep parts of the pyramidal cell layer of CA1 and CA3 respectively. More specifically,

the zinc-containing pyramidal cells in the distal CA1, in conjunction with the exclusively zinc-negative pyramidal cells project to the ipsilateral septum. Likewise the zinc-containing pyramidal cells in the CA3 project to the ipsilateral septum, whereas the zinc-negative pyramidal cells terminate in the septum bilaterally. The presubicular efferents to the septum also originate in segregated zinc-containing and zinc-negative layers of cortical neurons. In contrast, even though there is a mixed population of zinc-containing and zinc-negative cells in the parasubiculum, apparently only the zinc-negative cells project to the septum.

The pyramidal cells of the hippocampal subfields are generally regarded as homogeneous populations of neurons. However, recently it has been shown that not all cells in the hippocampal region are zinc-containing (Slomianka 1992). The zinc-containing pyramidal cells of CA1 and CA3 are thus confined to distinct strata of the pyramidal cell layer, and whereas there are numerous zinc-containing cells in the pre- and parasubiculum, no zinc-containing cells are present in the subiculum. Moreover, there is a complex and highly topographical organization of the intrinsic hippocampal connections. The CA3 thus projects in an ordered proximo-distal fashion to corresponding proximo-distal parts of the CA1 (Ishizuka et al. 1990), and it has been demonstrated that the CA1 has a specific topographic organization of efferents to the subiculum, so that the proximal CA1 projects to the distal subiculum, and the distal CA1 in turn projects to the proximal subiculum (Amaral et al. 1991; Tamamaki and Nojyo 1991). Furthermore, the subiculum displays a proximo-distal heterogeneity with respect to the efferent projections to cortical and subcortical areas (Witter et al. 1990), and the pre- and parasubiculum display a rough organization with regard to their projections to the entorhinal cortex (Caballero-Bleda and Witter 1993; Köhler 1985). Accordingly, the reported topography of the subcortical CA1 septal afferents arising from zinc-containing neurons in the distal CA1 and from zinc-negative neurons in the proximal CA1, may follow a general principle regarding the proximo-distal organization of the intrinsic and extrinsic projections of the hippocampal region.

Functional significance

The functional significance of these zinc-positive hippocampo-septal connections lies in the fact that zinc modulates the effects of glutamate and GABA on their receptors. There is firm evidence for an inhibition of the NMDA-receptor response by zinc, through binding to a receptor zinc site (Chadwick and Choi 1990; Nakanishi 1992; Peters et al. 1987; Westbrook and Mayer 1987; Wong and Kemp 1991). In contrast, zinc potentiates quisqualate- or AMPA- induced currents through glutamate ionotropic receptors (Koh and Choi 1988; Mayer and Vyklicky 1989; Rassendren et al. 1990) and counteracts the desensitization of the kainic acid receptor response (Rassendren et al. 1990). The role of zinc in GABA-mediated transmission, however, remains more obscure, as so far no GABAergic boutons or cells have been found to contain zinc (Slomianka 1992; Beaulieu et al. 1992). One function of the hippocampo-septal zinc-positive projection may thus be to modulate the septal system in a way that antagonizes NMDA and favors AMPA receptor-mediated glutamatergic neurotransmission. In order to differentiate between zinc-positive and zinc-negative neuronal pathways that may serve different modulatory functions in forebrain neurotransmission, more hodological studies of the zinc-containing neuronal systems must, however, be carried out.

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