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Axonal accumulation of $p75^{NTR}$ and TrkA in the septum following lesion of septo-hippocampal pathways

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Abstract. Septal cholinergic neurones depend on trophic support by nerve growth factor (NGF) which can rescue them from injury-induced degeneration. Since NGF exerts its effects via $p75^{NTR}$ and TrkA receptors coexpressed in vast majority of these neurones and down-regulated without NGF treatment after injury, in this study we aimed to examine how does the lesion to the cholinergic tracts affect distribution of both types of receptor proteins in damaged fibres. Early changes (two and seven days) were examined immunocytochemically within the septum and supracallosal stria after unilateral lesion to the supracallosal area and cingulum transecting some septal cholinergic efferents. We found accumulation of $p75^{NTR}$ and TrkA immunoreactive material (so-called "pile-up") within axonal segments of distended appearance proximal to the transection at two days postlesion and its translocation towards cell bodies seven days postsurgery. We observed $p75^{NTR}$ pile-up to be more intense than TrkA, which may indicate different cellular concentrations of both receptors. Receptor pile-up resembled acetylcholinesterase pile-up reported previously, suggesting a common response mechanism involving axonal transport disturbances.

Key words: axonal pile-up, cholinergic neurones, NGF receptor, LNGFR, $p75^{NTR}$, septum, supracallosal area lesion, TrkA

INTRODUCTION

Since the study by Daitz and Powell (1954) that provided the first description of morphological changes in the septal neurones caused by section of their hippocampal afferents, multiple studies have brought about complete characterization of the morphological and biochemical responses of these cells to injury (Lewis and Shute 1963, Lewis et al. 1967, Shute and Lewis 1967) and of the cholinergic neurones in particular (for review and references see Tuszynski et al. 1990, Fischer and Björklund 1991). While most of these data concerned the lesion effects on the cell bodies and nerve terminals, the changes occurring along the transected fibres were the subject of a few reports only (Lewis and Shute 1963, Lewis et al. 1967, Gage et al. 1986).

Much attention has recently been given to the presence of receptors for nerve growth factor (NGF) of low (neurotrophin receptor $p75^{NTR}$, previously known as the low-affinity NGF receptor, LNGFR) and high (TrkA) affinity, on the cholinergic neurones of the basal forebrain (Gibbs et al. 1989, 1991, Gibbs and Pfaff 1994, Sobreviela et al. 1994, and references therein). These neurones have been shown previously to be NGF dependent, and to be rescued from lesion-caused degeneration by NGF administration (Gnahn et al. 1983, Hefti 1986, Montero and Hefti 1988, Junard et al. 1990). However, the mechanisms underlying NGF activity in the brain are not entirely clear.

Radioautographic studies with [^{125}I]NGF have allowed visualization of the high affinity NGF receptors over neuronal somata in basal forebrain (Richardson et al. 1986, Raivich and Kreutzberg 1987b), although at that time resolution has been limited as neither single fibres nor low affinity binding sites could be demonstrated. Production of antibodies that recognize $p75^{NTR}$ (Chandler et al. 1984, Taniuchi and Johnson, Jr. 1985, Taniuchi et al. 1986), and, much later, Trk A (Steininger et al. 1993, Sobreviela et al. 1994) has allowed numerous laboratories to visualize the presence of these receptors both on the cell bodies and along their axons and nerve terminals. Recent studies have shown a very strong correlation of $p75^{NTR}$ and Trk A distribution within basal forebrain, which colocalize in over 95% in the septum and reveal 90% overlapping with ChAT immunopositive cells (Verge et al. 1992, Sobreviela et al. 1994), proving that almost the entire population of septal cholinergic neurones bears both NGF receptors. It has been documented that lesion to the cholinergic fibres causes loss of neurotransmitter enzymes, i.e., ChAT and AChE in neuronal perikarya and nerve terminals (Sofroniew et al. 1983, Dravid and Van Deusen 1984, Hefti et al. 1984, Oderfeld-Nowak et al. 1984, Stephens et al. 1985, Cuello et al. 1986, Grądkowska et al. 1986), and that these changes are accompanied by parallel decrease of NGF receptors expression (Venero et al. 1994, Figueiredo et al. 1995b) and NGF receptor protein immunoreactivity (Armstrong et al. 1987, Gage et al. 1988, Hagg et al. 1988, 1989), suggesting impairment in their trophic responsiveness. In this regard nothing is known about lesion-induced axonal changes in NGF receptors. To add to our knowledge about mechanisms of neuronal response to injury we characterized, using immunocytochemical tools, axonal changes in NGF receptors in injured neurones within first postlesion week, i.e., the time period proved to be optimal for initiation of the most effective NGF experimental therapy (Hagg et al. 1988, 1989).

METHODS

Animals and surgery

Sixteen adult, male, Wistar rats (200-250 g body weight) obtained from the facility of the Nencki Institute, were used in the study. Ten animals were subjected under Nembutal anaesthesia (50 mg/kg, i.p.) to unilateral lesions of supracallosal area and cingulum by electrocoagulation (2.5 mA, 2 x 15s), performed essentially as described earlier (Skup et al. 1987). The coordinates were from bregma (Paxinos and Watson 1986): for the first electrode placement AP=0.0; L =0.4; V=2.9; for the second electrode insertion: AP=0.0; L=1.0; V=2.4. The lesion destroyed frontal 1 and 2 cortical regions including

supracallosal stria and encroached to various extent corpus callosum, leaving in most cases cingulate cortex, induseum griseum and genu of the corpus callosum intact (see Figs. 1-4, schemes). Six normal, unoperated rats, serving as controls, were included in the study for comparison.

Immunocytochemistry

Two experimental groups (comprising five operated and three control rats each) were investigated. The survival times were 2 and 7 days after surgery. At these time points animals were sacrificed under deep Nembutal anaesthesia by perfusion through the ascending aorta. Rats were perfused with 250 ml of phosphate-buffered saline (0.01M PBS, pH 7.4) containing heparin (6250 U/l), followed by 500 ml of ice-cold 2% paraformaldehyde with 0.2% parabenzoquinone in PBS. After 20 min of perfusion brains were removed from the skulls, fixed in the same fixative for further 1.5-2 h at room temperature and afterwards cryoprotected in 30% sucrose in 0.1M PBS for 48 h at 4°C. Coronal 25 µm sections were cut on the freezing microtome. Serial sections were collected (levels + 1.2 mm to - 0.8 mm from bregma, see Figs. 1-4) in 0.1 M PBS, pH 7.4, and every third section was taken for low- affinity p75^{NTR} or TrkA receptor immunocytochemistry. Monoclonal antibody IgG 192 against p75^{NTR}, kindly provided by Prof. L. Aloe, Institute of Neurobiology, Rome, and anti-Trk A polyclonal antibody, kindly provided by Prof. L. Reichardt, Howard

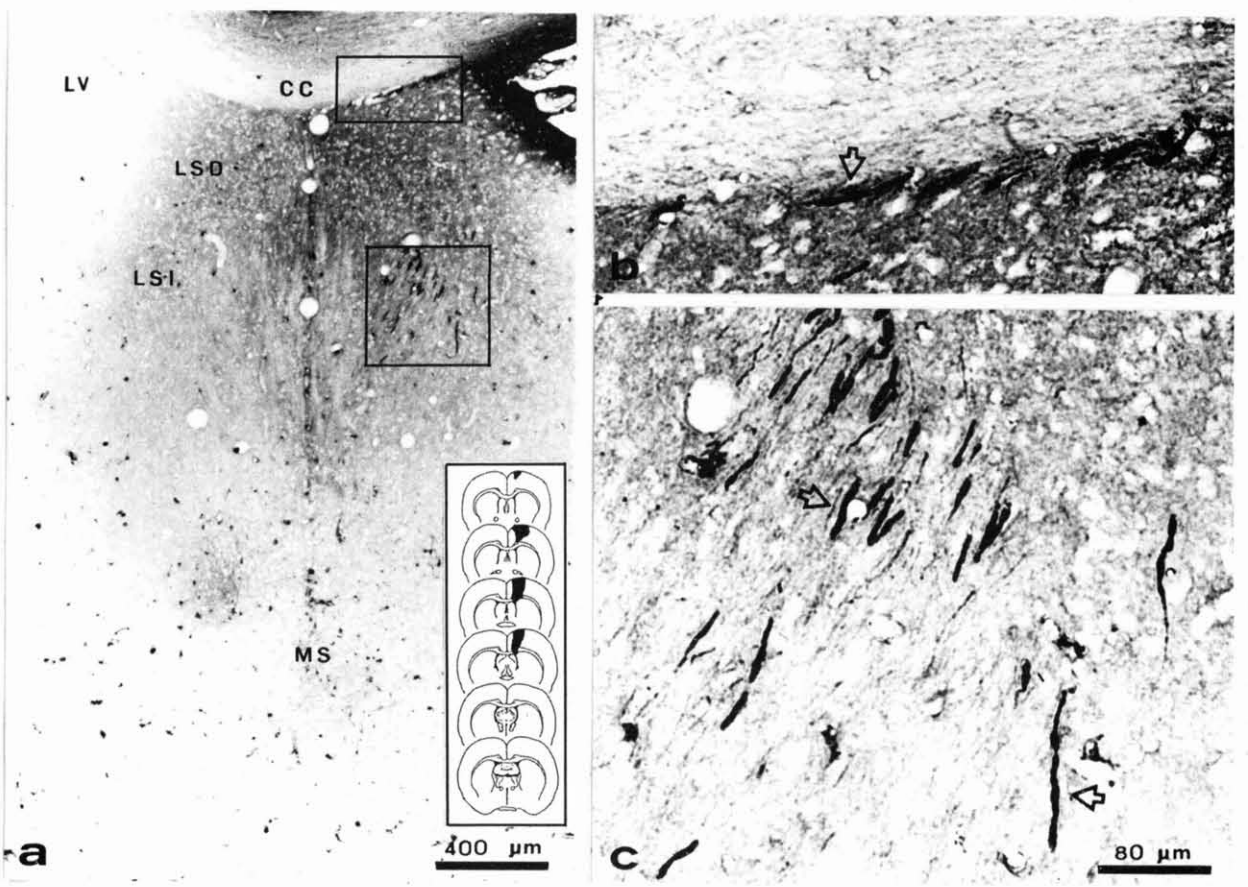

Fig. 1. Photomicrographs of brain section showing the distribution of the low-affinity neurotrophin receptor p75^{NTR}- immunoreactive (p75^{NTR}-IR) material in the septum of the operated rat two days after lesion. (a) unilateral accumulation of p75^{NTR}-IR in neuritic profiles of the lateral septum (framed areas) at the level of the most extensive damage to the cortex and supracallosal stria (see scheme). High-power magnifications of the framed subcallosal area (b) and LSI area (c) show arrangement of dilated axonal fragments densely packed with p75^{NTR}-IR material (arrows). Scale bar on (c) refers also to (b).

Hughes Institute, San Francisco, were used. Sections were processed free-floating. After washes in 0.1 M PBS plus 0.3% Triton X-100 (PBS+T), sections were treated with hydrogen peroxide solution (0.3% H_2O_2, in methanol, 20 min) to reduce endogenous peroxidase activity, then washed in water followed by washes in PBS+T. Nonspecific staining was blocked by subsequent incubation of sections with 5% normal horse serum ($p75^{NTR}$, 1 h) or 5% normal goat serum (TrkA, 0.5 h). Sections were incubated with primary antibodies (anti-$p75^{NTR}$ undiluted; anti-Trk A 1:5,000) overnight at 4°C. All the following steps were carried out at room temperature using PBS+T for washes and reagent dilutions. After incubation with biotinylated secondary antibodies (1:200, Vector Laboratories, Burlingame, CA, 1 h) sections were washed and incubated for 1.5 h in the solution of avidin and biotinylated peroxidase complex (1:100, Vector Laboratories, Burlingame, CA). To visualize the immunocytochemical reaction the sections finally were reacted with 0.1% 3,3'-diaminobenzidine (Sigma) and 0.01% H_2O_2 mixture for 10 min. The reaction was stopped by removal of solution and rinsing. Sections were mounted on chrom alum subbed slides, dried, dehydrated in ascending concentrations of ethanol, cleared in xylene and coverslipped with DPX.

RESULTS

Controls

In the control, unoperated rats, a dense network of $p75^{NTR}$ immunoreactive ($p75^{NTR}$ - IR) fibres in

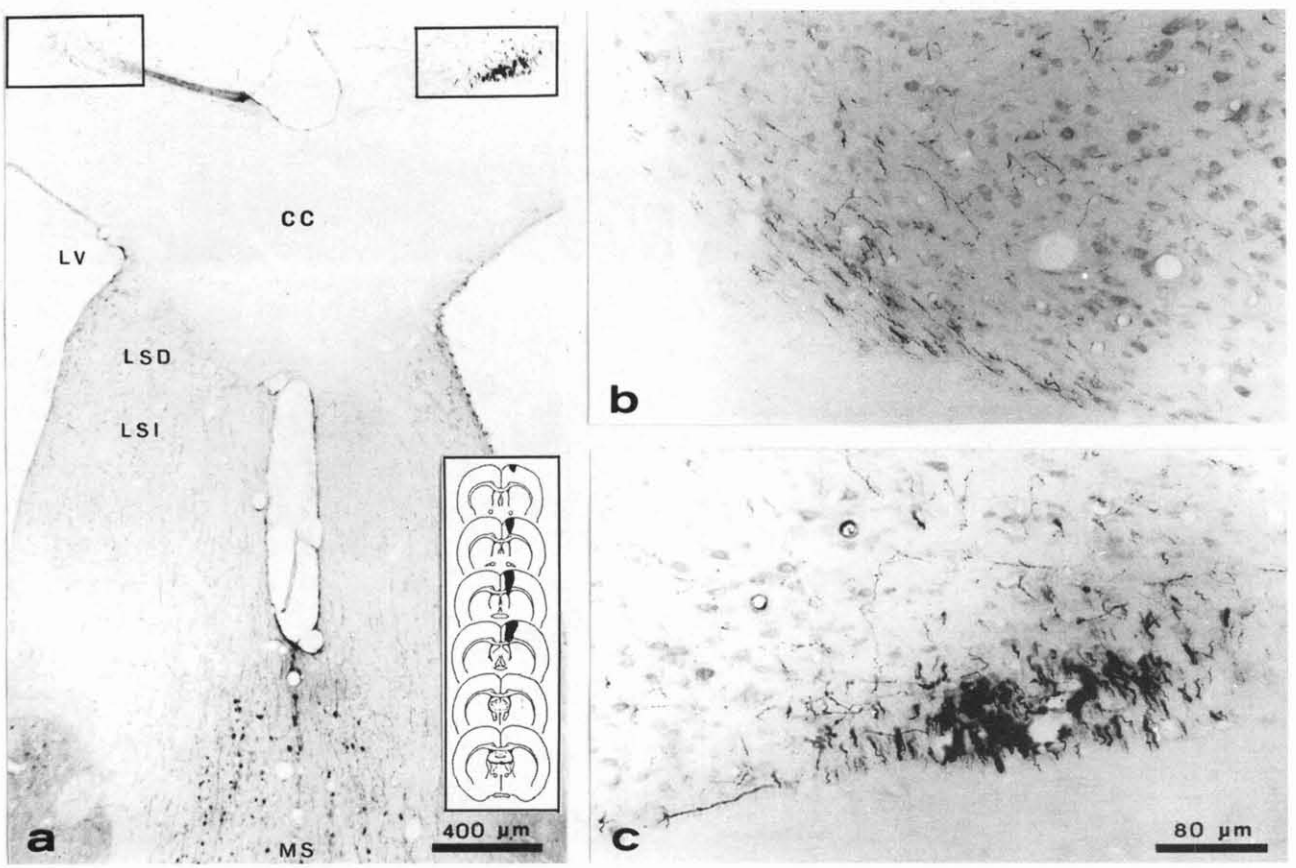

Fig. 2. Photomicrographs of brain section showing the distribution of the high-affinity NGF receptor TrkA -immunoreactive (TrkA-IR) material in/above the septum of the operated rat two days after lesion. (a) TrkA-IR neuronal perikarya of the medial septum (MS) and unilateral accumulation of TrkA-IR in the axonal fragments above the corpus callosum (right framed area) at the level placed anteriorly to the lesion of the supracallosal stria (see scheme). Compare TrkA-IR in the supracallosal stria fibres on the unoperated (b) and operated (c) side. Scale bar on (c) refers also to (b).

the medial septum (MS) and only thin, short, hardly detectable p75^{NTR} -IR fibres of the supracallosal stria were observed (not shown). No p75^{NTR} -IR fibre profiles were detected either in the dorsal (LSD) or intermediate (LSI) parts of the lateral septal nucleus. There were no p75^{NTR} -IR fibres in the posterior septum detected. TrkA immunoreactivity (TrkA-IR) in the control animals revealed a dense network of fibres within the medial septum, scarce TrkA-IR profiles in the LSD and LSI, and a clearly visible bundle of immunoreactive, short fibres penetrating the antero-cingulate cortex between indusem griseum and cingulum, identified as the supracallosal stria projection (Figs. 2b and 3a).

Two days postlesion

In three of five rats representing a two days survival group, the lesion caused the most extensive damage to the supracallosal area and cingulum at the level of columnae fornici as shown on the schemes in Figs. 1 and 3. In one rat the injury was smaller but continued towards anterior septum (Fig. 2, scheme), and one rat received extensive lesion at the

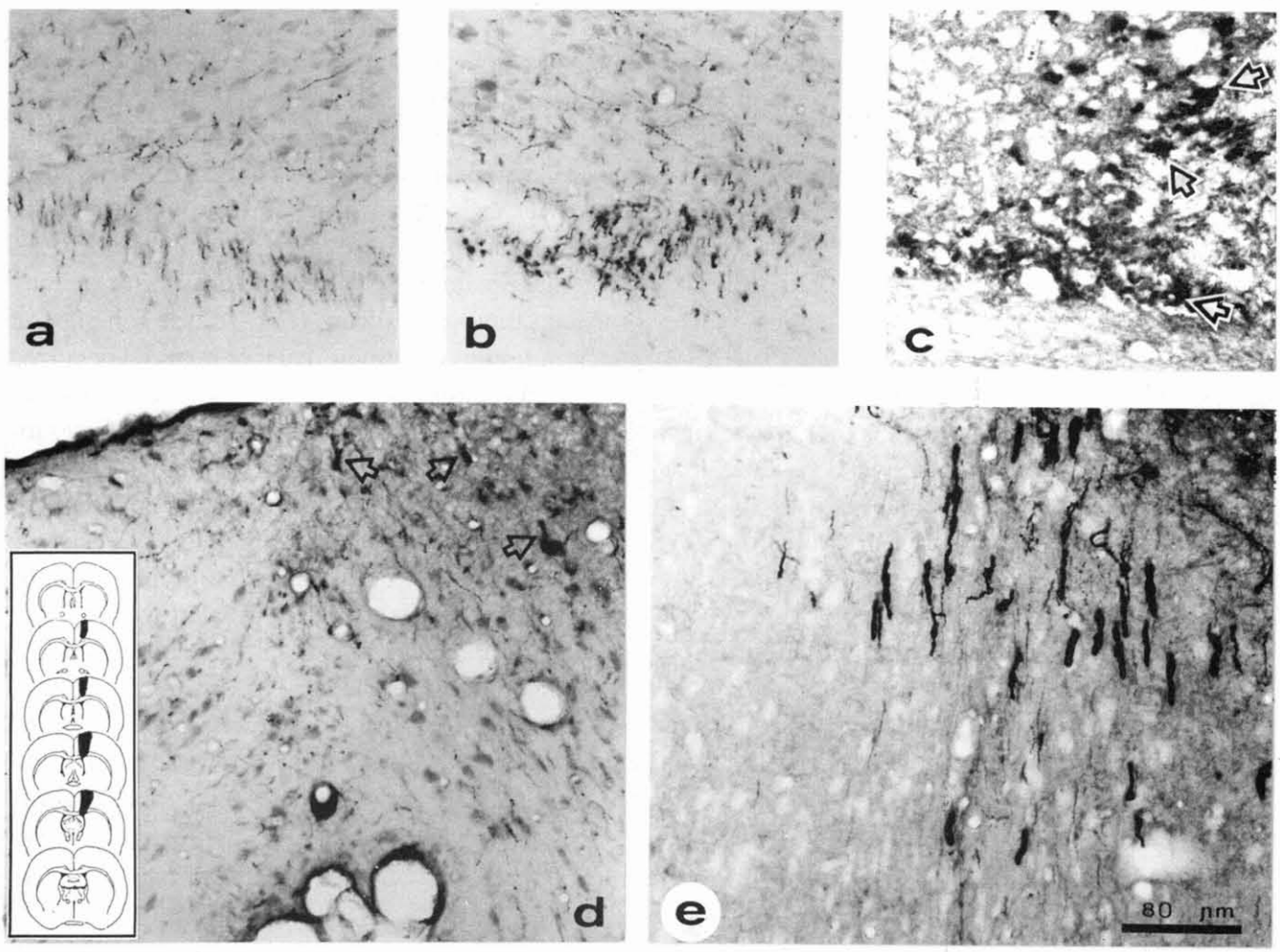

Fig. 3. Photomicrographs showing at the consecutive brain sections lateral septum (d,e), and supracallosal area (b,c), the pile-up of TrkA (b,d) and p75^{NTR} (c,e) in the operated rat two days after lesion. Note differences in the degree of accumulation of TrkA-IR and p75^{NTR}-IR on the operated side (compare b-c and d-e). See also the differences in TrkA-IR pattern between control (a) and operated (b) side in the supracallosal area, comparable to that occurring more anteriorly (Fig. 2). Scale bar refers to all photomicrographs.

level of posterior septum damaging paracentrally lateral septal nucleus, dorsal part (LSD) and its part bordering lateral ventricle (not shown). In all the animals irrespectively to the differences in the lesion placement, unilateral accumulation of $p75^{NTR}$-IR material in the axonal segments of dilated appearance was observed (Figs.1 and 3c,e). These distended segments of various length were localized in restricted areas of the dorso-lateral quadrant of the medial septum (Fig. 1a,c, arrows), in the posterior septum (Fig. 3e) and in the supracallosal area (Fig. 3c, arrows) proximal to the lesion. In one rat, additionally to the dispersed pattern of altered fibres within intermediate part of the lateral septum (LSI), there was a group of short, parallel fibres with condensed $p75^{NTR}$ -IR material just below the corpus callosum at this level (Fig. 1b). In the rat with a small, anterior lesion (Fig. 2, scheme) no change in the posterior septum and, more anteriorly, weak $p75^{NTR}$ immunoreactivity accumulation in the supracallosal area was found (not shown). The intensity of $p75^{NTR}$ immunoreactivity varied in different fibres and along the same fibres where fragments with less accumulation of $p75^{NTR}$ -IR material had varicose-like appearance (Fig.1c, arrows). In a rat with a posterior lesion encroaching dorsal septum (not shown) there was a varicose-like accumulation of $p75^{NTR}$ -IR material in a proximity to the lesion boundaries, within the fibres running parallel to the sectioning surface from the LSD towards the midline of the septum. In all operated rats no $p75^{NTR}$ immunoreactivity accumulation in the fibres in the medial septum (MS) and the diagonal band of Broca was detected and no reduction of the number of $p75^{NTR}$ immunopositive perikarya was observed at this time point.

TrkA-IR accumulation within injured fibres had been also detected. Its appearance and intensity were different from that of $p75^{NTR}$ (Fig. 2a,c, compare also Fig. 3b and c). It was particularly evident in the supracallosal neuritic bundle where the network of TrkA-IR fibres of altered morphology was dense but individual fibres seemed fine, thread-like. At the lateral septum Trk A-IR material accumulated much less than $p75^{NTR}$ and its condensation was detectable in the isolated fibres only (Fig. 3d, arrows). No change of TrkA-IR in the septal cell bodies had been found at this postlesion time.

Seven days postlesion

Seven days after surgery in most cases there was no accumulation of $p75^{NTR}$ immunoreactivity in the lateral part of the posterior septum while an altered distribution was detected more distally to the lesion placement (Fig. 4). In the case where the lesion caused damage to the supracallosal stria including fornix (one animal, Fig. 4a,b, see the scheme with the lesion reconstruction) there was an accumulation of $p75^{NTR}$ -IR material in axons located mostly in the medial septum. This phenomenon was expressed with different intensity in various fibres (see Fig. 4a, framed areas shown on b); some accumulation was of varicose-like appearance, but in a great majority of processes it was intense, filling uniformly fragments of fibres of an enlarged diameter. In rats with the lesion that presumably spared the projection running through the dorsal fornix (Fig. 4c, see scheme) an accumulation of immunoreactive material occurred in a smaller number of fibres at the level of the medial septum (Fig. 4c, frame e) and in a few fibres located above, in the septohippocampal nucleus (frame 4d). Strikingly distended appearance of some of them evidenced that strong axonal response persisted (Fig. 4d,e, head arrows). In these rats the cell bodies neighbouring the dilated axonal fragments revealed unchanged morphology and $p75^{NTR}$ immunostaining intensity (Fig. 4e, black arrows). At this time point in an animal with more extensive lesion damaging dorsal fornix there was a mild loss of $p75^{NTR}$ -IR cells in the medial septum (not shown).

DISCUSSION

This report presents an immunocytochemical study of the changes in the accumulation of the $p75^{NTR}$ and TrkA receptor molecules in the axotomized cholinergic neurones projecting from the nucleus of diagonal band throughout supracallosal stria, and in some cases in the pathways running

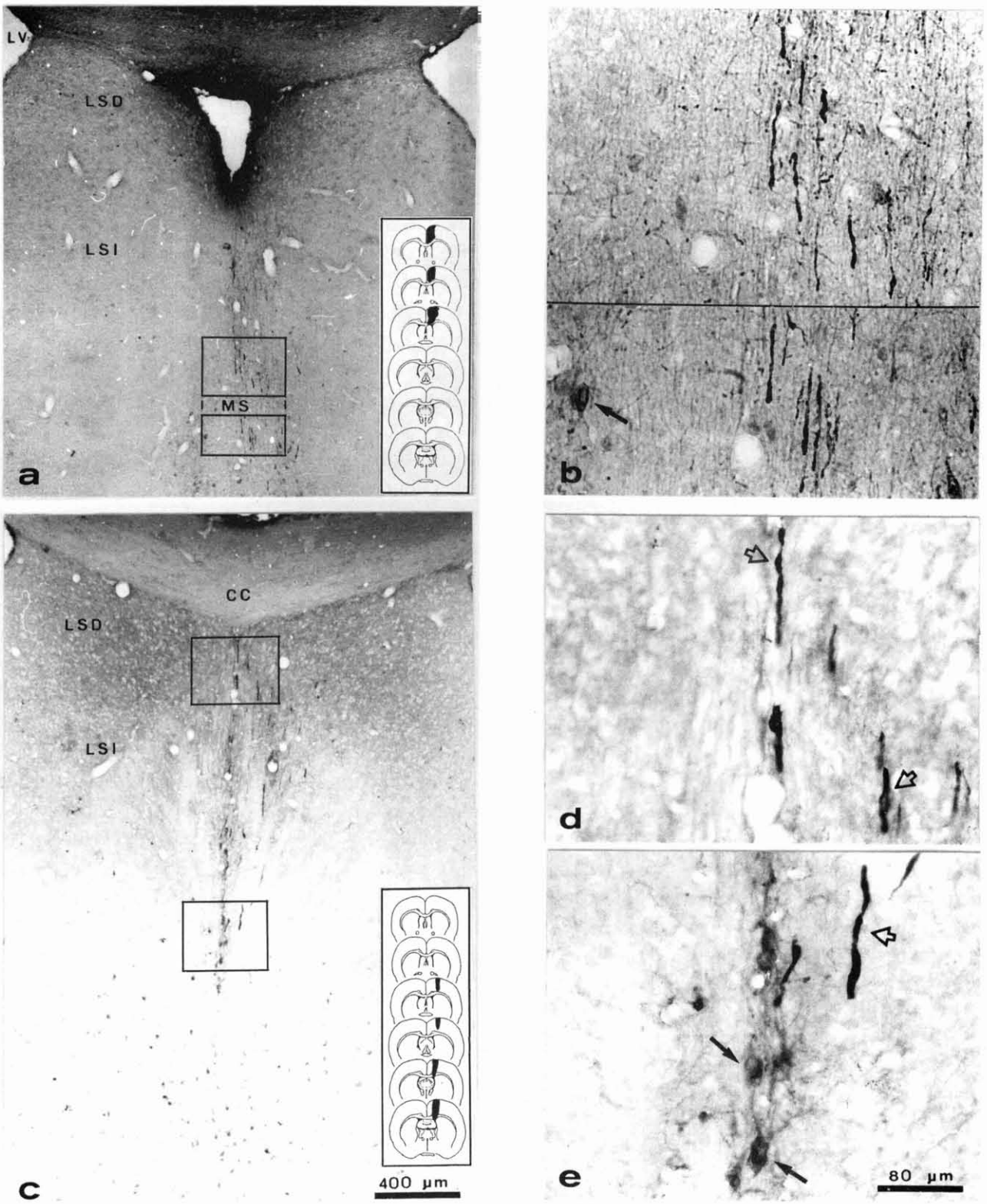

Fig. 4. Photomicrographs of brain sections showing the distribution of the p75^{NTR}-IR material at various levels of the medial septum of two rats (a,c) differing in lesion location, seven days after surgery. Note the differences in the distribution pattern of axonal accumulation of p75^{NTR}-IR and its intensity in single axonal fragments (higher magnification, b, d, e, head arrows). Black arrows point to p75^{NTR}-IR cell bodies of unchanged appearance. Scale bar on (c) refers also to (a); scale bar on (e) refers also to (b) and (d).

through the dorsal fornix to the hippocampal formation. Analysis of the serial brain sections allowed us to reconstruct spatial alterations of NGF receptors accumulation within injured fibres. It revealed at early postlesion time (two days) progressing hypertrophy of axonal segments in the lateral septal area proximal to the transection and slightly more distally above the corpus callosum, which moved towards neuronal perikarya of the medial septum one week after the lesion. In any of the time points studied there was no immunopositive material in the superior part of the cingulate cortex where intracortical collaterals of supracallosal stria are known to project (Lewis and Shute 1967). In earlier studies of the septal cholinergic neurones in which the cell body and axonal response to damage had been mapped with AChE staining and assessed with ChAT activity, extensive brain injuries causing more complete transection of the septo-hippocampal pathways were produced (Lewis and Shute 1963, Lewis and Shute 1967, Lewis et al. 1967, Gage et al. 1986). By producing that type of injury causing fimbria lesion, Lewis and Shute (1963) were the first to show that if a tract rich in acetylcholinesterase (AChE) is interrupted, fibres on the cell body side of the lesion become distended and stain much more intensely whereas fibres distal to the lesion rapidly lose their capacity to stain. A term "pile-up" was carried to describe the hypertrophic state on the septal side of a fimbria lesion. In this paper we show that NGF receptor molecules are subject to "pile-up" as well. In particular, in one rat with an extensive lesion to the posterior septum $p75^{NTR}$ pile-up revealed striking similarity to the one described by Gage et al. (1986) for AChE after a comparable lesion. This suggests a common response mechanisms of both classes of molecules. The gradual shift of the immunopositive $p75^{NTR}$ particles back to the region of cell body may be explained by progressive dying back of the damaged axon towards the perikaryon (Gage et al. 1986) accompanied by a loss of immunoreactive material and/or immunostaining capacity. It can be speculated that "piling-up" of both NGF receptors reflects the disturbances of bidirectional axonal transport (Lubińska et al. 1964, Lubińska and Niemierko 1971), see also Lubińska (1975). The mechanism of fast axonal flow described in early paper of Lubińska and Niemierko (1971) for AChE, was reported more recently for $p75^{NTR}$ (Raivich and Kreutzberg 1987a), and suggested by the others (Cuello et al. 1990, Pioro and Cuello 1990, Pioro et al. 1990). Such data are missing for TrkA. However, the results of the present study allow to speculate that the general pattern of TrkA axonal flow is similar.

First significant signs of AChE pile-up in the dorso-lateral quadrant of the septum were reported to occur in the immediate proximity to the lesion placement already one day after extensive aspirative lesion causing damage to the supracallosal stria and fimbria-fornix (Gage et al. 1986). We document occurrence of $p75^{NTR}$ and TrkA accumulation of the same pattern both in the lesion proximity and more anteriorly two days after the lesion. Irrespectively to the pile-up distribution pattern however, there was a reproducible difference in the axonal appearance revealed by $p75^{NTR}$ and Trk A immunoreactivity. Fibres immunoreactive to TrkA seemed less dilated, revealed presence of multiple varicosities that were invisible in most of the neurites filled with $p75^{NTR}$ -IR material. These differences may find explanation in the quantitative differences in the amount of both proteins within the cell: if the proportion of the $p75^{NTR}$ and TrkA density at the nerve endings membrane equalling to 10:1 (Hempstead et al. 1989, 1991) reflects the rate of protein synthesis of these two proteins, then one could expect much stronger accumulation of $p75^{NTR}$ following fibre transection.

Despite strong axonal reaction, no detectable shrinkage or loss of immunoreactive cell bodies was found either in the nucleus of diagonal band or in the medial septum two days after injury and only mild loss of immunoreactive cells occurred in the medial septum seven days after injury. It remained in contrast to more severe cell loss reported by Gage et al. (1986) and Tuszynski et al. (1990). This may be explained by the fact that electrocoagulative supracallosal lesion performed stereotactically damages short fragments of fibres less severely than suction does, which causes complete destruction of

all septo-hippocampal connections (fimbria-fornix bundle and supracallosal stria) (Gage et al. 1986).

It is worth mentioning that axonal response of NGF receptors shown in this study was accompanied by bilateral elevation of endogenous NGF content in the septum, related to both neuronal and glial component as revealed with NGF-immunoreactivity (Oderfeld-Nowak et al. 1995). This lends strong support for the evidence that lesion-induced alterations of NGF in the brain tissue are not sufficient to protect cholinergic fibres from gross lesion-induced changes (Gasser et al. 1986, Figueiredo et al. 1995a) including axonal pile-up of proteins, although some effect counteracting neuronal degeneration cannot be excluded. On the other hand multiple data proved effectiveness of exogenously administered NGF in protection of neurones against degeneration (Montero and Hefti 1988, Junard et al. 1990, Fischer and Björklund 1991, Garofalo et al. 1992, Garofalo and Cuello 1994) and showed that the maximal therapeutic effect is achieved after immediate onset of treatment after surgery (Hagg et al. 1988, 1989). These observations suggest that axonal accumulation of NGF receptor molecules occurring at the first postlesion week does not deprive injured neurones of responsiveness to NGF treatment. Whether NGF effects at this stage are mediated via receptors located on perikarya involved in anterograde neurotrophin signalling (Bartheld et al. 1996) or on terminals of uninjured collaterals remains to be established.

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