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Transient projections from rat occipital cortex are able to respond to a spinal target derived diffusible factor in vitro

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Layer V pyramidal neurons in the occipital part of the rat cerebral cortex project to both the cervical spinal cord and the tectum early in postnatal development. The occipito-spinal projection is transient and is subsequently withdrawn, while a permanent connection is maintained with the tectum. The withdrawal of the transient occipital corticospinal axons may be due to their inability to respond to target-derived influences. In the current study we co-cultured explants of the occipital cortex and cervical spinal gray matter or tectum in 3-D collagen gels. Directional growth of the cortical axons towards either the cervical spinal gray or tectal explant was observed. This indicates that the failure of neurons located in the occipital cortex to maintain collaterals within the spinal cord in vivo is not due to their inability to respond to a target-derived factor, but must be regulated by other extrinsic factors.

The corticospinal tract (CST) in rat is characterized by its postnatal outgrowth throughout the spinal cord [13]. Early in development the cells of origin of the corticospinal projection are more widespread than they are in the mature animal. During the first postnatal week, corticospinal (CS) neurons are located throughout the tangential extent of the neocortex, including occipital areas [10]. During its early postnatal development, the rat CST can be subdivided in three components [7]: two permanent sets of fibers, the first originating from a group of CS neurons situated more anteriorly in the cortex and projecting to the cervical spinal gray matter, and the second with its parent CS neurons in the intermediate part of the cortex and projecting to lumbar spinal gray. The CS axons originating in anterior parts of the cerebral cortex enter spinal gray matter shortly after their arrival in spinal white matter at postnatal day 4 (P4) [7]. In addition a transient component is present, emanating from neurons in the occipital [7, 16] and medial prefrontal [9] parts of the cortex. The transient CS projection disappears from spinal cord levels by collateral elimination [10, 16]. Occipital (visual) cortical neurons with transient spinal axons maintain an axonal projection to one of the other subcortical targets, such as the superior colliculus [10].

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In vitro and in vivo experiments indicate the operation of specific neurotrophic influences between cortical and subcortical visual structures during embryonic development [2, 4]. In vitro, axons of (putative) CS neurons originating in anterior cortical areas respond to a diffusible 'tropic' substance located in the cervical spinal gray matter [8]. Although transient occipito-spinal axons project to the cervical cord levels, their spinal outgrowth is mainly restricted to the white matter. Hence, the transient occipital CS axons may be unable to respond to the diffusible tropic signal released by the cervical spinal gray matter. We have used the collagen co-culture technique [5] to determine if occipital cortical neurons are able to respond to a target-derived diffusible signal in vitro.

Explants of occipital cerebral cortex of 1-day-old rat pups (P1) were co-cultured with cervical spinal gray matter or superior colliculus (tectum) explants from 4-day-old rat pups (P4) (see Fig. 1A). A total of 31 1-day-old (P1) and 4-day-old (P4) rat pups were used in this study. The day of birth is considered as postnatal day 0 (P0). Under sterile conditions the whole brain (P1), the cervical enlargement, or optic tectum (P4) was placed in a drop of Leibovitz (L15 GIBCO) medium. Coronal sections, with a thickness of about 500 μm, were hand cut. White matter was removed from spinal cord gray matter

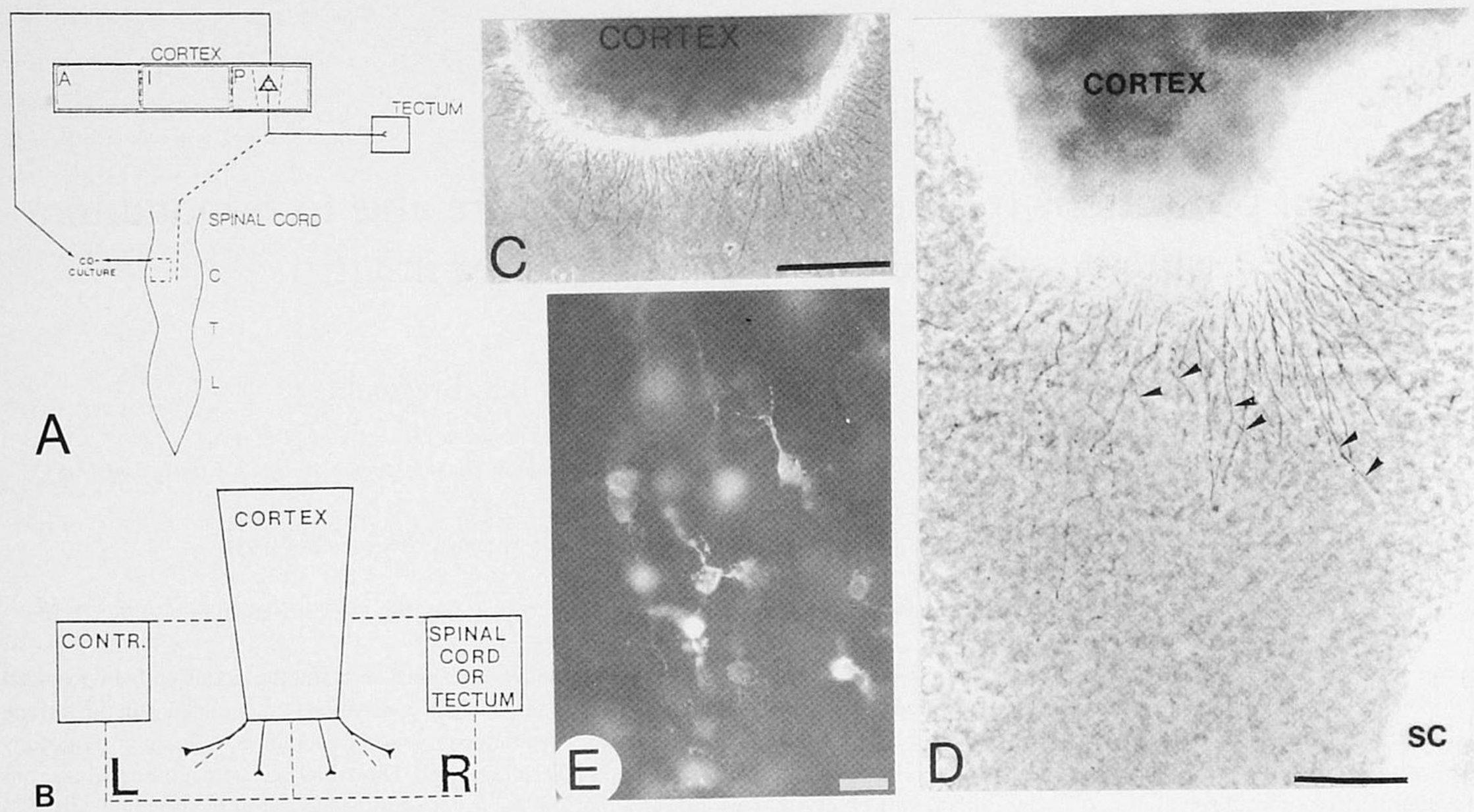

Fig. 1. Panel A: the rat corticospinal tract component originating in the occipital or posterior parts of the cerebral cortex projects to the cervical spinal white matter during the first postnatal days. These occipito-spinal projections are transient. Pyramidal shaped neurons in layer V of the occipital cortex project to the superior colliculus at the same time and subsequently stabilize these connections. Explants from occipital cortex from P1 rats were co-cultured with for instance cervical spinal white matter explants from P4 animals. A: anterior cortex; I: intermediate cortex; P: posterior cortex; C: cervical spinal cord; T: thoracic spinal cord; L: lumbar spinal cord. Panel B: schematic representation of the co-culture experiments and quantification areas. Quantification of the outgrowing axons is carried out in the defined areas (marked by dotted lines: L, left; R, right). Panel C: cortical explant cultured alone: axon outgrowth on the ventricular side of the explant. Axons grow in an inferior direction and no clear preference of outgrowth to the left or the right is noted. Bar = 100 μm. Panel D: cortex explant co-cultured with a cervical spinal gray matter explant. Note the preferential growth of axons towards the spinal cord explant. Axons preferentially turning towards the spinal cord explant are indicated by arrowheads. Bar = 100 μm. Panel E: retrogradely labeled neurons in cortex explant after DiI application in the axonal outgrowth area facing the spinal cord explant. The labeled neurons are confined to one particular layer (presumably layer V) and characterized by their relatively large size and prominent apical dendrite. Bar = 10 μm.

explants, since oligodendrocytes at P4 might already contain inhibitory proteins [1]. Postnatal day-4 olfactory bulb or cerebellum (tissues which do not receive any direct cortical input) were used as control tissues.

The cortical explants were either cultured alone (n = 20) or co-cultured (n = 107) in collagen gels. The explants were positioned above the floor of a 35-mm plastic culture dish (Costar) within a hemisphere matrix of collagen (collagen type I, Boehringer Chemicals). Spinal cord, tectum and/or control explants were positioned on the flanks of the cortical explant at a distance varying between 200 and 400 μm. The cultures were incubated in a 50 : 50 mixture of HAM F12 and MEM (Gibco) with 5% bovine serum (Hyclone) for 48 h. Quantification of outgrowing axons was carried out as described previously [8]. Briefly, outgrowing cortical axons were counted only if their growth cones were visible in areas defined by dotted lines (areas L,R). The spinal cord outgrowth coefficient was calculated by dividing the number of axons growing in the area facing the spinal cord explant by the total number of outgrowing axons from the ventricular side of the cortical explant. 'Turning' axons (in areas L and R) are those that have an initial trajectory when exiting from the cortical explant that would miss the spinal cord, tectal or control tissue explant, but subsequently turn by > 30° in the direction of either the target or control explant (see Fig. 1B).

In order to label retrogradely the cortical neurons whose axons project preferentially to the cervical spinal cord explant, small crystals of the neuronal tracer 1,1-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) were applied to the area of axonal outgrowth on the ventricular side of the cortex explant [3, 8] in fixed cultures. After 6–8 weeks, the cultures were examined by fluorescence microscopy.

When the occipital cortex is cultured by itself in the collagen matrix, axon outgrowth is predominantly from the ventricular surface (Fig. 1C). Axons grow uniformly from the ventricular surface of the explant; no preferen-

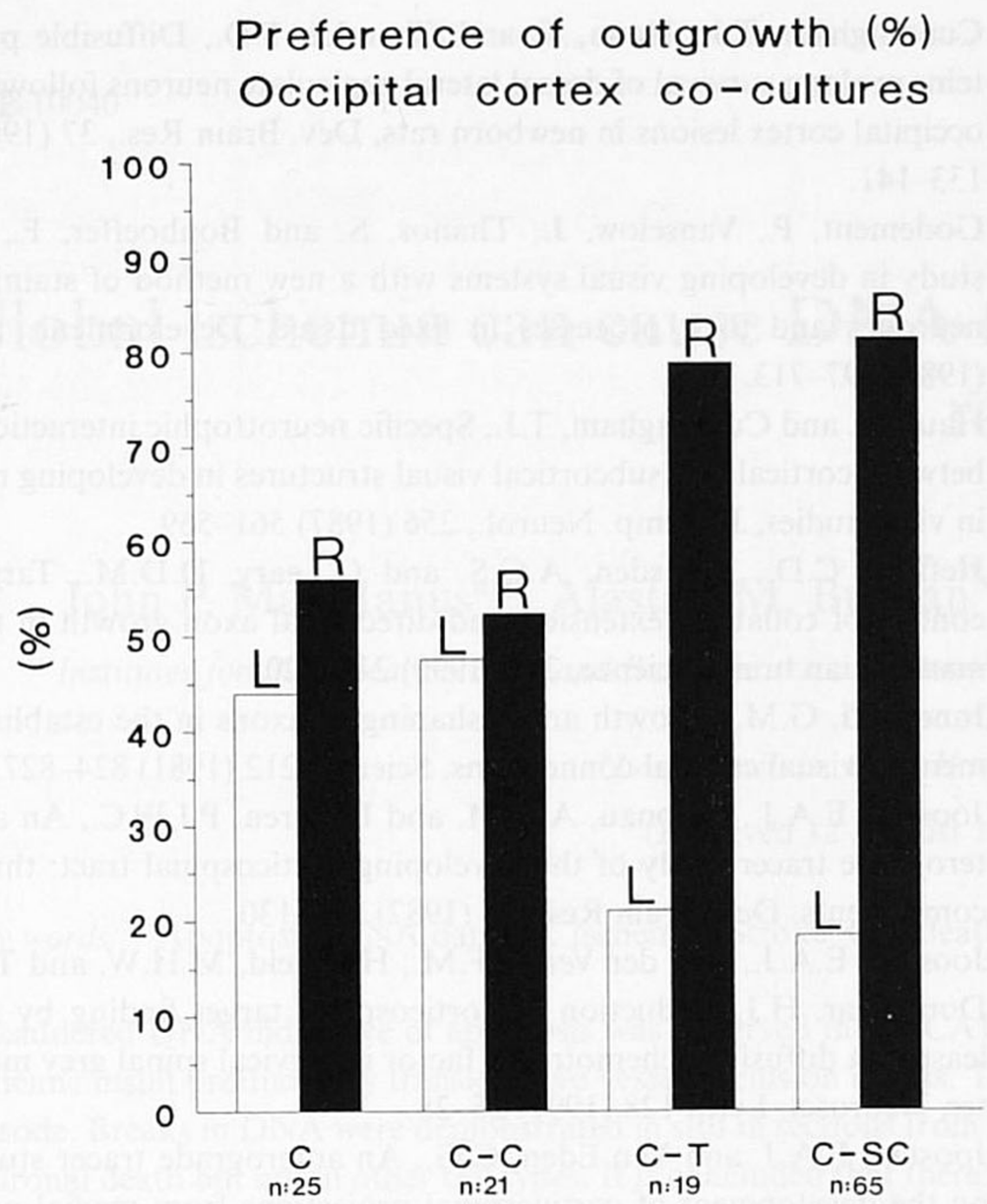

Fig. 2. Diagrammatic representation of the preference of cortical axon outgrowth. C, cortex; CON, controls (olfactory bulb or cerebellum); SC, spinal cord; T, tectum. Bars indicate the percentage of cortical outgrowth to the left or right area.

tial outgrowth from either side of the explants can be observed in an inferior direction; no preference to the left or the right can be noted (Fig. 1C). When cortical explants are co-cultured with either cervical spinal cord or tectal explants, however, axon outgrowth is enhanced from the side of the cortical explant facing the spinal cord or tectal explant (Fig. 1D).

A preference of cortical axon growth towards the cervical spinal cord or the tectal explant can be deduced from Fig. 2. Quantitative measurements of axon outgrowth support this conclusion: in 65 co-cultures (cortex-spinal cord or control-cortex-spinal cord) 3042 axons were scored at the ventricular side of the cortex explant and 1921 axons were located in the area facing the spinal cord and are growing toward the target explant (a spinal cord outgrowth coefficient of 0.63); in 19 co-cultures (cortex-tectum or control-cortex-tectum) 1536 were scored and 883 were located in the area facing the tectal explant (a tectal outgrowth coefficient of 0.57). Furthermore, cortical axons that would have missed the cervical spinal gray explant if they had maintained their initial trajectory preferentially turn toward it (Fig. 1D). For 269 axons scored there is a spinal cord turning coefficient of 0.88. The tectal turning coefficient is 0.91. In the control experiments (control-cortex co-cultures or cortex alone) virtually no turning axons could be observed.

No preference of outgrowth can be noticed using cortex explants only (Fig. 2). To exclude the possibility that an inhibitory influence from the control explants might affect our observations we co-cultured cortex and control explants (olfactory bulb or cerebellum) only. The results indicate that control explants do not inhibit axonal outgrowth: in 21 co-cultures (control-cortex) 863 axons were scored and 427 (or 49.5%) were located in the area facing the control explant.

Retrograde DiI labeling was used to determine the origin and cellular morphology of the cortical neurons whose axons preferentially project to the cervical spinal cord explant in the collagen co-cultures. DiI implanted into the axonal outgrowth area on the proximal side of the cortical explant retrogradely labels neurons which are mainly located in one particular layer. This is presumably layer V because in in vivo retrograde labeling of corticospinal neurons, the same layer of a cortex explant was labeled (not shown). Furthermore, the labeled cell bodies are characterized by their pyramidal-like cell shape (Fig. 1E). These retrograde DiI-labeling experiments indicate that axons from pyramidal cell shaped neurons are mainly located in one particular layer (presumably layer V) of the occipital part of the cortex, and grow preferentially toward the cervical spinal cord in vitro.

In vivo, the occipital CS projection is transient, and is characterized by the virtual absence of spinal gray ingrowth at any spinal cord level and at any age [7, 10]. The transiency of the occipital CS projection can be explained by collateral axonal loss without cell death, a phenomenon called collateral elimination and first described during the development of cortical callosal projections [6]. With the use of various long-lasting retrograde tracers, the change in the distribution of CS neurons due to collateral elimination has now been demonstrated in various mammals, including rat [10, 15, 16]. Many of the occipital cortical neurons in layer V with transient CS axons maintain and stabilize permanent connections to the superior colliculus and pons [10, 15]. During embryonic development, specific trophic influences act between cortical and subcortical visual structures [2, 4]. Our in vitro experiments indicate that a diffusible trop(h)ic factor is also involved during the postnatal formation of (permanent) cortico-tectal connections. The question arises, for future research, whether this postnatal diffusible factor is identical to the one which acts during embryonic development of the cortico-tectal connections.

The cellular processes and mechanisms underlying collateral elimination and stabilization are still uncertain. Since transient exuberant projections may play an important role in developmental brain plasticity, it is essential to understand the key events involved in processes regulating the maintenance or elimination of particular

axonal projections. Transplantation experiments have demonstrated that differences among cortical regions, with respect to the eventual targets of layer V neurons, are not due to inherent differences in the layer V subcortical projection neurons in the various cortical areas [12]. Rather, all layer V neurons appear to have similar projectional capacities [11, 12, 14]. The question remains which 'extrinsic' key events are involved in collateral elimination. Our data indicate that axons of layer V neurons in the occipital cerebral cortex are able to respond to a diffusible factor located in the cervical spinal gray matter. The enhanced neurite outgrowth from the proximal face of the occipital explant towards the spinal cord tissue as well as the observation that axons which would have missed the spinal cord explant turn preferentially toward it indicate that the diffusible factor is probably tropic. To exclude the possiblity that the diffusible factor might increase the survival of layer V neurons (i.e. a 'trophic' effect), a detailed quantitative morphometrical study (beyond the scope of the present investigation) would be required.

The signal that causes the cortical axons to enter the spinal gray matter in vivo might occur at the level of the parent cell bodies (the layer V pyramidal cells in the sensorimotor cortex). Thalamocortical fibers appear to form synapses as soon as they enter the sensorimotor cortex during the first postnatal week [17]. Their synapse formation on a certain population of layer V pyramidal cells might be the trigger for the maintenance or withdrawal of collaterals within particular targets. The inability of the transient occipitospinal projections to grow into the cervical spinal gray matter in vivo, might therefore reflect the absence of somatosensory thalamocortical input on the pyramidal cells of origin.

The extrinsic influences which regulate the elimination of exuberant collateral projections in vivo are unknown. In any event, our in vitro data indicate that the elimination of transient corticospinal axons emanating from the occipital cortex is not due to an inherent inability of these neurons to respond to target derived factors.

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