Synaptic Patterns of Thalamocortical Afferents in Mouse Barrels at Postnatal Day 11

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ABSTRACT

This study focuses on the synaptic output patterns of thalamocortical axons in mouse barrel cortex at postnatal day (P) 11. Axons were labeled by biotinylated dextran amine transported anterogradely following injection in vivo into the ventrobasal thalamus. Labeled axons in the posteromedial barrel subfield were examined by light and electron microscopy and then reconstructed in three dimensions to assess the spatial distribution of their synapses. Thalamocortical axons form asymmetrical synapses, both at varicosities and along cylindrical portions of the axons; usually, only one synapse occurs per site, contrasting with the case in the adult, in which multiple synapses are typical. At P11, varicosities without synapses are common. As in adult barrels, approximately 80% of synapses formed by thalamocortical axons are with dendritic spines; 20% are with dendritic shafts. The similarity in the distribution of thalamocortical synapses onto spines vs. dendrites in developing and mature barrels indicates that adult synaptic patterns already are specified at a very early stage of thalamocortical synaptogenesis. J. Comp. Neurol. 442:63–77, 2002.

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Indexing terms: somatosensory cortex; thalamus; three-dimensional reconstruction; development; synapse

Input to sensory areas of the cerebral cortex from the specific thalamic nuclei has long been recognized as a potent force for determining developmental events in the cerebral cortex (see, e.g., Shatz and Luskin, 1986; Ghosh and Shatz, 1992; Miller et al., 1993; Schlaggar and O'Leary, 1994; Auladell et al., 2000) and for shaping the responses of cortical neurons in the adult brain (see, e.g., White, 1979). Thalamocortical synapses have been implicated in the earliest stages leading to the formation of normal synaptic connectivity in the adult (Herrmann et al., 1994; Pinto et al., 2000). Patterns of adult thalamocortical synaptic connections have been described quantitatively in different cortices in different species (see, e.g., White, 1989; Keller, 1995), but less is known of the developmental events that lead to the adult patterns. In recent years, thalamocortical synaptogenesis has been the focus of intense efforts (see, e.g., Vaughn, 1989; Miller et al., 1993; Catalano et al., 1996; Micheva and Beaulieu, 1996; Castro-Alamancos and Connors, 1997; Molnár et al. 1998; Keller and Carlson, 1999; Auladell et al., 2000; Yamamoto et al., 2000). However, precise quantitative data concerning the development of thalamocortical synaptic patterns are lacking. The need for this information becomes increasingly apparent as the focus of much modern experimentation shifts to the exploration of the mechanisms that underlie cortical development (see Rubenstein and Rakic, 1999). We aim to rectify this deficiency by performing a series of investigations focusing on various characteristics of thalamocortical axons, such as the number of terminals per length, the number of synapses per terminal, and the identification of postsynaptic elements, at different stages of postnatal development. Such studies of normal material will form the bases for comparisons with experimental preparations in which normal, peripheral stimulation has been compromised.

These studies focus on the posteromedial barrel subfield (PMBSF) of mouse primary somatosensory (SmI) cortex (Woolsey and Van der Loos, 1970; see also Rice, 1995), in

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DOI 10.1002/cne.1422
part because of the large body of data available concerning thalamocortical synaptic connectivity in the adult mouse (see, e.g., White, 1989) and the ease and precision with which experimental manipulation can be effected in the whisker-barrel system (see, e.g., Armstrong-James and Callahan, 1991; Armstrong-James et al., 1991; Fox, 1992, 1994; Land et al., 1995). In the mouse barrel cortex, thalamocortical axons first display a somatotopic pattern on P3 (Senft and Woolsey, 1991). Our preliminary results on synaptogenesis in mouse barrels (see White et al., 1997) showed that the number of asymmetrical synapses increases until P20, showing a sharp increase at P13/P14 that coincides with the onset of patterned whisker movements (Weller, 1972) and with eye opening. The source of the asymmetrical synapses was not established but presumably included thalamocortical afferents, which make up 20% of the asymmetrical synapses in adult barrel holows. In the present study, we use injections into the thalamus of biotinylated dextran amine (BDA) to provide unequivocally identified thalamocortical afferents whose synaptic patterns are then examined in three-dimensional (3D) reconstructions made from serial thin sections. The focus of this study is thalamocortical afferents at postnatal day (P) 11, just prior to the period of explosive growth in asymmetrical synapses that was noted in our previous study (White et al., 1997). These findings are expected to serve as a baseline for comparison of thalamocortical synaptic connectivity at different postnatal stages of development and in the adult.

MATERIAL AND METHODS

Neurosurgery and labeling

Five CD/1 mice (Charles River Breeding Laboratories) were used in this study. At P10 (the first 24 hours after birth were termed P0), the animals were anesthetized intraperitoneally with sodium pentobarbitone (20 mg/kg) and placed into an ice-filled box for 5–10 minutes before being transferred to a stereotaxic apparatus especially designed for the neurosurgical manipulation of mice at early postnatal ages. The animals were kept chilled by ice during the surgery. The stereotaxic coordinates of ventrobasal nucleus (VB) thalamus (VBm nucleus of Van der Loos, 1976; VPM nucleus of Zantua et al., 1996; see also Franklin and Paxinos, 1997) were based on previous work (see, e.g., White and DeAmicis, 1977; White, 1978), with corrections obtained from analysis of injections of Sudan black B made into P10 mouse brain. Briefly, a craniotomy was made over the region of tectum and adjacent cerebellum. The exposed blood vessels were used as landmarks for the micropipette injection of 4% lysin-fixable BDA (molecular weight 3,000; D-7135; Molecular Probes, Eugene, Oregon). Four percent BDA in 0.01 M phosphate buffer (PB) at pH 7.25 was delivered iontophretically through a 20–30 μm glass micropipette into the left VB thalamus. The tracer was injected at three different depths at intervals of 500 μm using a high-voltage precision current source Microguard CS-3 (Transkinetics) set for 5 μA positive current pulses (7 seconds on, 7 seconds off) for a total period of 6–10 minutes. The edges of the scalp wound were gently cleaned of blood and glued together with Histocryl surgical glue (Braun) prior to returning the treated animal to the cage. After a survival period of 24 hours, the animals were anesthetized deeply with 4% chloral hydrate and perfused transcardially with 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M PB, pH 7.4, heated to 37°C. Shortly after perfusion, the animals were decapitated, and the heads were placed in the same fixative solution at 4°C overnight. On the next day, the brains were removed, and hemispheres ipsilateral to the injection site were cut in the coronal plane at 60 μm on a vibratome (Electron Microscopy Science OTS-3000-03). All sections through the PMBSF cortex were collected in sequence and processed for BDA histochemistry according to the protocol of Veenman et al. (1992) but using cobalt intensification (Adams, 1981). All surgical procedures conformed to current NIH guidelines and were approved by the university animal care and use committee.

Light microscopy

The 60-μm-thick sections were examined with the light microscope (LM) to identify those sections that contained anterogradely labeled thalamocortical axons. An axon was considered to be labeled by anterograde transport when it could be followed as a single fiber from the lower part of layer VI or below and branched upon reaching layer IV. Axons were also considered to be labeled anterogradely when they were obtained from preparations containing no more than three lightly labeled (ghost-like) somata on any single 60-μm-thick section, with no labeled cell body occurring closer than 0.75 mm, in any direction, to any part of the selected axon. Employing strict criteria for the identification of labeled axons severely constrained the number of specimens available for examination but greatly increased the reliability of our identification of selected axons as thalamic in origin. Sections containing selected axons were placed in 1.5% aqueous OsO4 for 30 minutes, stained for 1 hour with 1.0% uranyl acetate, dehydrated, embedded in epon/araldite, and then mounted onto plastic cylinders. Blocks were trimmed so that they contained only a single labeled axon and its branches, with the longest labeled axonal segment oriented as parallel as possible to the block face. The blocks were cut into long consecutive series of thin sections and mounted on formvar-coated slotted grids with the long axis of the longest axonal segment orthogonal to the long axis of the slot. Standardization of section thickness was effected by performing all thin sectioning with a single micromtome (Porter-Blum MT2-B) under identical conditions of temperature, humidity, lighting, and viewing angle. Sections of constant silver/gold interference color estimated to be 80 nm in thickness (Williams and Meek, 1966) were obtained. An additional approach for estimation of section thickness involved dividing the diameter of longitudinally sectioned mitochondria as viewed in thin sections by the number of sections in which the mitochondria appeared (Chicurel and Harris, 1992). The thicknesses obtained with this approach also averaged 0.08 μm. Thin sections were rinsed with 70% ethanol (http://medic.bgu.ac.il/ homes/Ed/dirt_EM.html), stained with lead citrate, and examined with a JEOL 1200 EXII electron microscope (EM).

Electron microscopy

Each section was examined through microscope-mounted binoculars at a screen magnification of 8,000× to identify synapses made by the labeled axons. Serial electron micrographs were taken at 10,000–15,000× of each thalamocortical synapse; the location of the synapse was
recorded with reference to section number and grid. These electron micrographs were used subsequently to identify the type of synapse (asymmetrical vs. symmetrical) and postsynaptic element (dendritic shaft, spine, or cell body). Video images of overlapping fields containing the labeled axons were captured at 8,400× using a Gatan 673 wide-angle video camera (Gatan Inc., Warrendale, PA). These images were assembled to produce 3D reconstructions of the labeled thalamocortical axons.

**Three-dimensional reconstruction**

Three-dimensional computer graphic reconstructions of labeled axonal segments were made using a reconstruction system running software designed specifically for this research (Noesis, Velizy, France). Briefly, video images of every portion of the labeled axon as it appeared in each thin section of the series were digitized using a Series 151 image processor (Imaging Technology Inc., Woburn, MA). The images were then viewed in sequence on a high-resolution monitor and “classified” in a procedure that enabled the reconstruction software to treat the images as single or as part of a montage and to place each in the final reconstruction at its appropriate level (z-axis) within the series of thin sections. The “horizontal” registration of montage images and the “vertical” registration of images from adjacent sections were effected by simultaneously displaying overlapping or sequential images on the monitor with one image rendered transparent. The transparent image was then rotated and translated with respect to the other image to obtain a best-fit alignment, using all the elements visible in the two images. Once registered, the images were recalled, and the contours of processes to be reconstructed were demarcated. At this stage, reference was made to the electron micrographs taken previously of thalamocortical synapses, to assist in plotting the synapses onto the contours in their appropriate locations. The locations were rendered on the final reconstruction as colored circles.

Reconstructed axons were then displayed, and the lengths of their segments and the distances between their synapses determined using appropriate software functions. An additional function allowed the display of thalamocortical axons as straight lines representing the long axes of reconstructed elements, on which the precise spatial distribution of their synapses was plotted (see Fig. 6). The reconstructions were correlated with LM measurements of axonal length, but this was not feasible in all instances, for instance, when branches of the reconstructed axon were not clearly visible at the LM level prior to thin sectioning. The long, complex trajectories of thalamocortical axons precluded LM or EM examination of entire axons.

Making reconstructions of long axonal segments sectioned along their long axis often required making montages of a length that exceeded the ability of the reconstruction software to display them. In these instances, the images of the axonal segment were divided into groups that were reconstructed as separate axonal segments that were joined later in correct register by matching structures at the abutting ends of adjacent segments.

**RESULTS**

**Light microscopy**

**Injection site.** Examination of 60-μm-thick coronal sections through the thalamus confirmed that the injection was contained within the VB (Van der Loos, 1976; Zantua et al., 1996; Fig. 1). No BDA-filled neuronal cell bodies were visualized in this region because of the high concentration of reaction product. Although the diameter of the central (opaque) part of the injection site was approximately 150–250 μm, the BDA diffused over an area of about 350 μm. BDA-labeled fibers were observed in the reticular nucleus of the thalamus (cf. Keller, 1995) and in the internal capsule on their way to the ipsilateral SmI cortex.

**Identification of thalamocortical axons.** Coronal sections obtained from five mice with injection sites confined to VB thalamus were examined with the LM. Clusters of BDA-labeled axons were observed in layer IV in the 60-μm-thick coronal sections through ipsilateral PMBSF cortex (Fig. 1). Because of their location, size, and shape, these clusters were considered to be barrels of the PMBSF of SmI cortex (Woolsey and Van der Loos, 1970).

**Electron microscopy**

The appearance of neuropil surrounding the labeled axons was consistent with that observed in layer IV barrels of normal mice at P11 (White et al., 1997): Basically, the neuropil consisted of a feltwork of fine processes, containing relatively few synapses compared with later ages and no myelinated axons. Eight BDA-labeled axonal segments of various lengths were exam-
ined with the EM and reconstructed from series of thin sections. Comparison with drawings made with the LM, with which the general outlines of barrels can be observed (Fig. 1), indicates that the selected axons occurred within barrel hollows. Labeled axons were identified by the content of electron-dense reaction product that filled their cytoplasm (Fig. 2); usually, mitochondria were more lightly stained than the background axoplasm (see also Fig. 7).

Commonly accepted morphological criteria were used to identify thalamocortical synapses: the presence of distinct, parallel pre- and postsynaptic membranes, separated by a synaptic cleft wider than the normal extracellular space, and a band of postsynaptic dense material, typical of asymmetrical synapses, adherent to the cytoplasmic surface of the postsynaptic membrane (see, e.g., Peters et al., 1976). Usually, the presence of a cluster of synaptic vesicles at the presynaptic membrane was difficult to discern because of the electron-dense staining of the background axoplasm. Labeled thalamocortical axons formed synapses with dendritic spines and shafts (Figs. 4, 5, 7). No synapses between labeled axons and cell bodies were observed.

Quantitative analysis of reconstructed axonal segments

Three-dimensional reconstructions. Reconstructed axonal segments had many varicosities along their lengths; some segments displayed a greater concentration of varicosities than others (Figs. 3, 6). Rotation of the images of the reconstructed axons around their long axes confirmed that the varicosities were essentially spheroid, sometimes oblate spheroids, but were never so flattened that they were not visible from all angles of view. Varicosities ranged from about 1 μm to 1.5 μm in diameter; roughly cylindrical segments of axon connecting the varicosities ranged from 0.3 μm to 0.5 μm in diameter. A large portion of the volume of axonal varicosities was occupied by mitochondria (Fig. 7), an observation made previously in adults (Anderson et al., 1992; Kharazia and Weinberg, 1993, 1994; see also review by Castro-Alamancos and Connors, 1997); the presence of mitochondria has been suggested to indicate a high level of tonic activity at the site of the varicosity (Wong-Riley, 1989).

Synapses were distributed irregularly along the reconstructed segments, and the concentration of synapses was conspicuously different for different axons. Future studies of thalamocortical synaptic patterns at later ages will
clarify whether this variability is a developmental phenomenon that reflects the presence at P11 of axons at different developmental stages or whether the synaptic patterns of different thalamocortical axons are indeed markedly different.

**Synaptology of thalamocortical axons.** All labeled axons formed exclusively asymmetrical synapses (Figs. 4, 5, 7). Many thalamocortical synapses were formed along cylindrical lengths of thalamocortical afferents; others were formed at regions that were difficult to define as cylindrical or varicose, whereas still others were formed at axonal varicosities (Figs. 3, 6). The latter nearly always contained only one synapse and rarely two. Axonal varicosities having no synapses were also observed. Overall, the ratio of axospinous to axodendritic thalamocortical synapses was 4:1. No association was observed between the type of postsynaptic element contacted (i.e., spine vs. dendrite) and whether the synapse was made at an axonal varicosity. Quantitative data on the distribution of thalamocortical synapses made by the reconstructed axonal segments are presented in Table 1; the average number of thalamocortical synapses per micrometer was 0.12 ± 0.04.

**DISCUSSION**

The present study provides quantitative data on synapses made at P11 by identified thalamocortical axons in mouse SmI cortex: All synapses were of the asymmetrical type. Reconstructions made from serial thin sections were used to determine the numbers of synapses per length of thalamocortical axon and whether the synapses were with spines or dendritic shafts. Results showed that the proportion of thalamocortical synapses at P11 onto spines vs. dendritic shafts is identical to the proportion observed for populations of identified thalamocortical synapses in the adult mouse (White, 1989). This proportion, roughly 4:1 axospinous vs. axodendritic synapses, is similar for different species and in different cortical areas (Colonnier, 1981). For example, axospinous synapses constitute 83% of the thalamocortical synapses in visual cortex of adult rat (Peters and Feldman, 1976) and cat (Garey and Powell, 1971).
Fig. 3. Computer-assisted reconstructions made from serial thin sections of two axonal segments belonging to BDA-labeled thalamocortical axons. Blue circles denote the locations of synapses; measurement bars adjacent to the reconstructions indicate the lengths of central, longitudinal axes calculated for each branch. d, Thalamocortical synapse onto a dendritic shaft; all other synapses are made with dendritic spines. Asterisks next to the upper of the two reconstructions indicate synapses depicted in the electron micrographs of Figures 4 and 5.
Fig. 4. A–D: Electron micrographs of serial thin sections showing an asymmetrical, axospinous synapse made by the upper axonal segment shown in Figure 3 (Fig. 3, asterisk at left). s, Spine head. In C, note the spine neck connecting the head (s) with the parent dendritic shaft (d). Scale bar = 0.25 μm.
Labeling of thalamocortical afferents

The ventrobasal nuclear complex (VBm nucleus of Van der Loos, 1976), equivalent to the ventroposterior medial nucleus (Zantua et al., 1996; Franklin and Paxinos, 1997), is reciprocally connected with the somatosensory cortex (Van der Loos, 1976; Saporta and Kruger, 1977; White and Keller, 1987; Brown et al., 1995; Keller, 1995; Zantua et al., 1996). Barrel-like clusters of thalamocortical afferents from VB thalamus are observed in the mouse as early as P3 in the deep regions of the cortical plate (Senft and Woolsey, 1991) and at P4 in a distinct layer IV (Senft and Woolsey, 1991; Agmon et al., 1993). Postnatally, thalamocortical afferents undergo accelerated, intrabarrel terminal arborization (Senft and Woolsey, 1991; Agmon et al., 1993, 1995; Catalano et al., 1996), possibly with modest pruning of inappropriate branches (Senft and Woolsey, 1991) to produce the adult pattern in which afferents from VB thalamus fill barrel hollows of one or more barrels (Arnold et al., 2001) while sparing the septa between them (adult rat: Killackey, 1973; Killackey and Leshin, 1975; Jensen and Killackey, 1987; Lu and Lin, 1993; mouse: White, 1979; Keller et al., 1985; Bernardo and Woolsey, 1987).

In this study, care was taken to label anterogradely thalamocortical afferents to PMBSF cortex in such a way that we avoided or minimized concurrent labeling of local axon collaterals belonging to retrogradely labeled corticothalamic projection cells (see, e.g., Keller, 1995; Hirsch et al., 1998). BDA having a molecular weight of 10,000 or more is thought to effect anterograde labeling better,
Fig. 6. Three-dimensional reconstructions made from serial thin sections through BDA-labeled thalamocortical axonal segments. Blue circles denote synapses made onto spines (unlabeled) or onto dendritic shafts (d). The synapses are plotted in their proper spatial relationship on the scales beneath the reconstruction (scale units = 1.0 μm). The scales were calculated using the central longitudinal axis of the longest segment of each reconstruction. Asterisks in A (right) indicate synapses, at left onto a spine, at right onto a dendritic shaft (d), depicted in the electron micrographs of Figure 7.
whereas BDA of lower weight (3,000 MW) is preferred for combined anterograde/retrograde labeling (Veenman et al., 1992; Reiner et al., 2000; Arnold et al., 2001).

During the early postnatal period, there is intensive growth of thalamocortical afferents (Senft and Woolsey, 1991) and a rapid increase in the number of asymmetrical synapses formed in the barrel neuropil (White et al., 1997); some of these synapses are presumed to be made by thalamocortical axons. To maximize the labeling of thalamocortical afferents at specific postnatal ages, it was
Fig. 7. Electron micrographs of adjacent thin sections showing asymmetrical, axospinous, and axodendritic synapses indicated by the asterisks in Figure 6A. The synapse at left is made with a spine head (s) and the one at the center of A and B with a dendritic shaft (d) that is shaped like a spine head in A, but whose dendritic nature begins to be appreciated in B. At the right of A is an asymmetrical synapse between an unlabeled axon and a spine (s). Inset is an underexposed print of the axodendritic synapse shown to demonstrate better the synaptic cleft. Scale bars = 0.25 μm.
the approaches of the present study is needed to clarify and Gilbert, 1976). Examination of adult material using varicosities (see, e.g., White, 1979, 1989; but see LeVay or their possible incorporation by shrinkage into adjacent most studies of thalamocortical synaptic organization later incorporated into axonal varicosities. Until now, at P11 by cylindrical regions of thalamocortical axons are thalamocortical afferents. It may be that synapses made at P11, a relatively early stage in the development of results of this study, but clearly the results are obtained in the white matter and observed to branch in layer IV or occurred in volumes of tissue where retrogradely labeled cell bodies were entirely absent.

Electron microscopy

Attempts to quantify synapses of individual axons using traditional light or confocal microscopy suffer from a tendency to equate synapse number with the number of varicosities. Clearly, synapses formed along cylindrical portions of axons would go unrecognized with this approach. In addition, the occurrence of multiple thalamocortical synapses at single varicosities, a common feature of thalamocortical axons in the adult (see, e.g., Freund et al., 1989; Anderson et al., 1992, Jones et al., 1997) suggests that counts relying on LM observations largely may be underestimated. Using molecular approaches to identifying synaptic components (see, e.g., Friedman et al., 2000) would overcome some of these difficulties, but they introduced an added complication to studies of specifically labeled axons.

In the present study, we have relied on the making of 3D reconstructions of thalamocortical axons from serial thin sections for the quantification of thalamocortical synapses. Although this approach suffers from the inability to identify synapses whose synaptic membranes are sectioned parallel to the plane of sectioning, the analysis of serial thin section reconstructions remains the most reliable and efficient approach to obtain precise quantitative and spatial information on the distribution of synapses along individual axons.

Synapse location

The generally accepted notion that synapses occur preferentially at axonal varicosities is not borne out by the results of this study, but clearly the results are obtained at P11, a relatively early stage in the development of thalamocortical afferents. It may be that synapses made at P11 by cylindrical regions of thalamocortical axons are later incorporated into axonal varicosities. Until now, most studies of thalamocortical synaptic organization have involved the examination of degenerating afferents at a stage following the phagocytosis of preterminal axons or their possible incorporation by shrinkage into adjacent varicosities (see, e.g., White, 1979, 1989; but see LeVay and Gilbert, 1976). Examination of adult material using the approaches of the present study is needed to clarify the relationships of thalamocortical synapses with varicos vs. cylindrical regions of the afferents.

Varicosities of thalamocortical axons at P11 that form synapses typically form only one. In contrast, in the adult mouse, varicosities of thalamocortical afferents typically form two or three synapses (multiple synapses; see, e.g., White and Keller, 1987), a common occurrence in adult animals (Freund et al., 1989; Anderson et al., 1992; Jones et al., 1997). Varicosities that do not form synapses have been reported also for kitten visual cortex (Anderson et al., 1992). Again, additional studies are needed to define better an association between the number of synapses per varicosity at different stages of development. It seems natural to assume that the development of thalamocortical axons would progress from few to more synapses per varicosity, but it might also be that varicosities with no synapses displayed synapses at some earlier stage or simply never had them.

Postsynaptic elements

In adult primary sensory cortex, spines are the major postsynaptic target for thalamocortical afferents (see, e.g., Colonnier, 1981; White, 1989) accounting for roughly 80% of thalamocortical synapses. Similarly, at P11 in mouse barrels, the ratio of axospinous to axodendritic synapses is 4:1. The similarity in the distribution of thalamocortical synapses onto spines vs. dendrites in developing and mature barrels indicates that adult synaptic patterns already are specified at a very early stage of thalamocortical synaptogenesis.

The results of the present study represent a first step in the development of a detailed baseline of normal synaptogenesis for thalamocortical afferents. Ultimately, these data are essential for the interpretation of phenotype in knock-out and transgenic mice and the elaboration of realistic computational models of neuronal development.

ACKNOWLEDGMENTS

The authors are indebted to Martin and Rena Blackman, President Avishai Braverman, and Mme. Suzan Zlowtowski for providing the funds and environment that enabled us to embark on a study that would be considered unrealistic under normal circumstances and time constraints. This work was supported in part by grant 52/00 from the Israel Science Foundation to E.L.W.

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