

BRE 20303

***Phaseolus vulgaris* leuco-agglutinin immunohistochemistry. A comparison between autoradiographic and lectin tracing of neuronal efferents**

G. J. TER HORST¹, H. J. GROENEWEGEN², H. KARST¹ and P. G. M. LUITEN¹

¹Department of Animal Physiology, State University of Groningen, P.O. Box 14, 9750 AA Haren and ²Department of Anatomy, Free University, Van der Boeorchstraat 9, 1081 BT Amsterdam (The Netherlands)

(Accepted March 27th, 1984)

Key words: *Phaseolus vulgaris* leuco-agglutinin — autoradiography — anterograde transport — immunohistochemistry — neuroanatomical tracing

The autoradiographic pattern of anterograde labeling as a result from injections with tritiated amino acids is compared to the labeling of efferents with *Phaseolus vulgaris* leuco-agglutinin after lectin injections in the same nucleus visualized by immunohistochemical methods. This comparison is made for efferents from the ventromedial hypothalamic nucleus to the amygdaloid body.

In recent years the study of neural connections underwent a tremendous development by the introduction of methods that employ exogenous compounds which can be transported by the living neuron. For the study of efferent connections, the autoradiographic technique that is based on the anterograde transport of tritiated amino acids has generally been accepted as the most reliable method¹. A disadvantage of the widely applied autoradiographic technique for neuronal tracing, however, is the indirect identification of labeling in the overlying emulsion that does not allow the positive identification of the actual structures. Secondly, the spread of tracer at the injection site obscures the determination of the effective tracer uptake area. Moreover, the study of very small projections is limited by the inevitable background activity in the emulsion. Apart from major projections, it is also impossible to discriminate between labeling of fibers and terminals in autoradiographic material. Besides, a time-consuming procedure and radioactive waste materials offer additional problems to this method.

Although several other exogenous compounds like horseradish peroxidase (HRP), HRP conjugated with wheat germ agglutinin or free lectins⁵ have been

shown to be transported in the anterograde direction, most of these substances also appear to be carried in the opposite direction. Recently, Gerfen and Sawchenko² have indicated that the plant lectin *Phaseolus vulgaris* leuco-agglutinin (PHA-L) may be used as a selective anterograde tracer for light microscopy. Before this technique can be accepted as a routine method it is essential to demonstrate the anterograde transport properties of PHA-L by comparison with such transport of tritiated amino acids studied in the same system. In this paper we report our observations on anterograde transport after iontophoretically delivered PHA-L and tritiated leucine (³H]Leu) in the ventromedial hypothalamic nucleus (VMH). [³H]Leu was injected and processed according to the procedures of Ricardo⁴. The brain sections were coated with Kodak NTB-2 nuclear track emulsion and exposed for 12–16 weeks at 4 °C. The slides were developed in Kodak D-19b at 16 °C, counterstained, coverslipped and examined with dark-field illumination. For the PHA-L experiments basically the procedure as described by Gerfen and Sawchenko² was followed. During the course of the experiments a number of parameters such as survival times, fixation and immunohistochemical reactions

Correspondence: G. J. ter Horst, Department of Animal Physiology, State University of Groningen, P.O. Box 14, 9750 AA Haren, The Netherlands.

were varied in order to obtain optimal results. The experiments were performed in male albino Wistar rats weighing approximately 300 g. The following procedure proved to give the best results in our hands.

Five mg *Phaseolus vulgaris* leuco-agglutinin obtained from Vector Laboratories, Burlingame, U.S.A., was dissolved in 0.05 M Tris buffered saline (pH = 7.4) to a 2.5% solution. This solution was divided into 10 μ l portions and stored at -20°C . Prior to an experiment one portion was stored at 4°C and could be used for 2 weeks without appreciable loss of reactivity, provided it is kept refrigerated between experiments. Small PHA-L deposits into the rat hypothalamus and several other structures like periaqueductal gray, reticular formation, ventral pallidum and amygdala were obtained by means of iontophoresis. Bevelled glass micropipettes (10–20 μ m in diameter) were filled with the PHA-L solution and positioned in the brain stereotactically. A positive pulsed DC current of 4–7 μ A was applied to the pipette using a Midgard CS-3 constant current source for 30 min. Following iontophoresis the pipette was left in situ for 10 min to avoid loss of tracer in the pipette track. Animals were allowed to survive for 7–10 days. Satisfactory fixation was obtained with a transcardial preinse of 50 ml phosphate buffered (0.05 M; pH = 7.4) saline at 37°C containing 10 I.U. heparin/ml, which was followed by 500 ml of a solution of 4% paraformaldehyde in 0.05 M phosphate buffer (pH = 7.4) or a preinse of 50 ml of a phosphate buffered solution containing 0.8% NaCl, 0.8% sucrose and 0.4% D-glucose (37°C), followed by 500 ml fixative made up of 0.5% paraformaldehyde, 2.5% glutaraldehyde and 4% sucrose in phosphate buffer. The advantage of the latter fixative is a lower background precipitate in the subsequent immunohistochemical staining procedure.

Following 2–4 h post-fixation brains were stored overnight at 4°C in 20–30% phosphate buffered sucrose and cut in 40 μ m transverse sections on a freezing microtome. Sections were collected in chilled Tris buffered saline (TBS) and rinsed overnight prior to incubation.

Immunohistochemical procedure

Every third section was transferred to a solution made up of 0.05 M Tris buffer (pH = 8.6), 0.5 M

NaCl and 0.5% Triton X-100 (TBS-T). This solution contained the primary antibody goat-anti-Phaseolus (Vector) diluted to a concentration of 1:2000. The tissue container placed on a rocker was stored for 36 h at 4°C or 18 h at room temperature with the same effect on the reaction. Then the sections were rinsed 3×15 min TBS-T and incubated with rabbit-anti-goat whole serum (Sigma) in TBS-T (1:400) for 2 h at 20°C . Sections were washed thoroughly again in TBS-T and transferred to a goat peroxidase-antiperoxidase (PAP) (Nordic, Immunochemicals) in TBS-T solution (1:400) for at least 4 h. The prolonged PAP incubation strongly intensified the density of the reaction product. After rinsing in several changes of TBS (pH = 7.4) the sections were incubated with 40 mg diaminobenzidine (DAB) in 100 ml Tris buffer (pH = 7.4) to which 0.9 ml H_2O_2 1.5% was added. The DAB incubation should be extended to at least 1 h for optimal density of the precipitate. Then the sections were washed thoroughly, mounted, counterstained and coverslipped. The PHA injection sites appeared as clusters of labeled cell bodies (Fig. 2C). The amount of cells and cluster size can be correlated to the pipette diameter and current density. There also appeared to be a relation between the amount of labeled somata and the number of marked fibers, which suggests that the effective injection site can be defined by the amount of labeled cell bodies. In the case of VMH injections as exemplified in Fig. 1 efferent pathways ascend ventrally and dorsally from this hypothalamic nucleus. The ventral bundle traverses the lateral hypothalamic area (LH), courses dorsal to the tractus opticus (TO) (Fig. 2E, F) and enters the amygdala. Within this nuclear complex the bundle divides in lateral and medial directed fibers. In experiments in which [^3H]Leu was injected into the VMH local increases of silver grains can be observed in several amygdaloid nuclei, which suggest termination areas. In the PHA-L material these supposed termination fields coincide with fine branching of fibers concomitant with the appearance of large numbers of varicosities (Fig. 2A, B). These morphological criteria we used for the identification of terminal fields in PHA-L material (see also ref. 2).

The lateral coursing fibers in the amygdala terminate within the lateral nucleus (AL) after travelling dorsal to the central nucleus (AC). The fibers coursing medially terminate within the medial nucleus

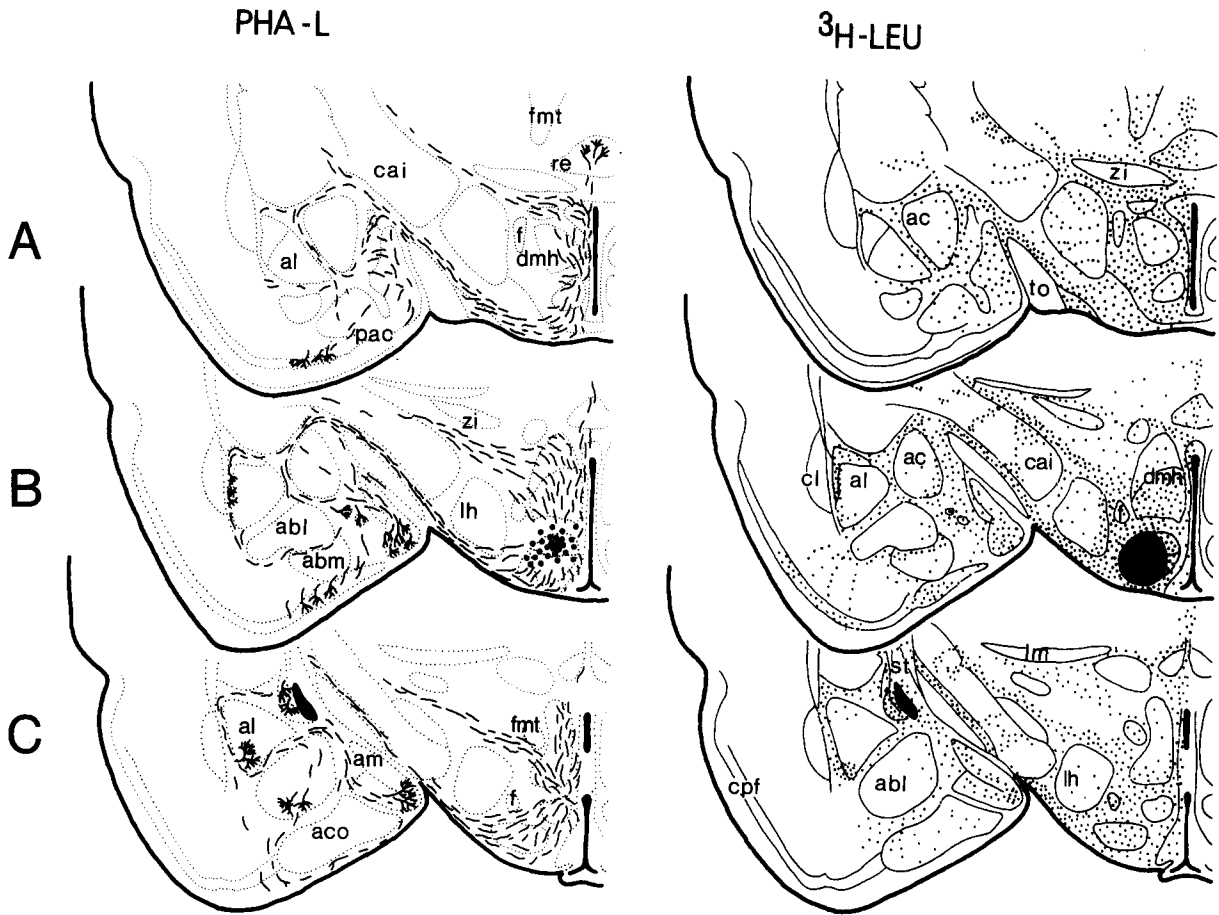


Fig. 1. Two series of identical transverse sections from anterior (A) to posterior (C) representing the efferent projections to the amygdala after iontophoretic *Phaseolus vulgaris* leuco-agglutinin (PHA-L) and [^3H]leucine (^3H -LEU) injections into the ventromedial hypothalamic nucleus (VMH). Abbreviations: abl, basolateral amygdaloid nucleus; abm, basomedial amygdaloid nucleus; ac, central amygdaloid nucleus; aco, cortical amygdaloid nucleus; al, lateral amygdaloid nucleus; am, medial amygdaloid nucleus; cai, capsula interna; cl, claustrum; cpf, piriform cortex; dmh, dorsomedial hypothalamic nucleus; f, fornix; fmt, fasciculus mammillothalamicus; lh, lateral hypothalamic area; lm, lemniscus medialis; pac, peri-amygdaloid cortex; re, nucleus reuniens; st, stria terminalis; to, tractus opticus; zi, zona incerta.

(AM), basolateral nucleus (ABL) and periamygdaloid cortex (PAC). Fibers directed dorsally from the VMH injection site traverse the dorsomedial nucleus (DMH) and divide in lateral and dorsal coursing bundles. This dorsal bundle terminates partly within the nucleus reuniens (RE) and the rest ascends to the habenular complex. Lateral coursing fibers travel under zona incerta (ZI) towards the capsula interna (CAI). Some fibers cross this CAI, others bend dorsally towards stria terminalis (ST).

The PHA-L results show a remarkable similarity with this and previous autoradiographic studies on VMH projections^{3,6}. The [^3H]Leu injections, how-

ever, appear as rather diffuse deposits, which do not allow the precise delineation of the tracer uptake area. Moreover, the tracer diffusion prohibits the identification of short connections. DMH injections of PHA-L (not illustrated) show a strong short projection to the VMH which could not be identified after similar injections with [^3H]Leu. Furthermore, after the same DMH injections of PHA-L long descending projections of individual fibers could be observed to the lower medulla, previously demonstrated with retrograde transport methods⁷, whereas these minor projections can hardly be discerned in the autoradiographic material. Another advantage

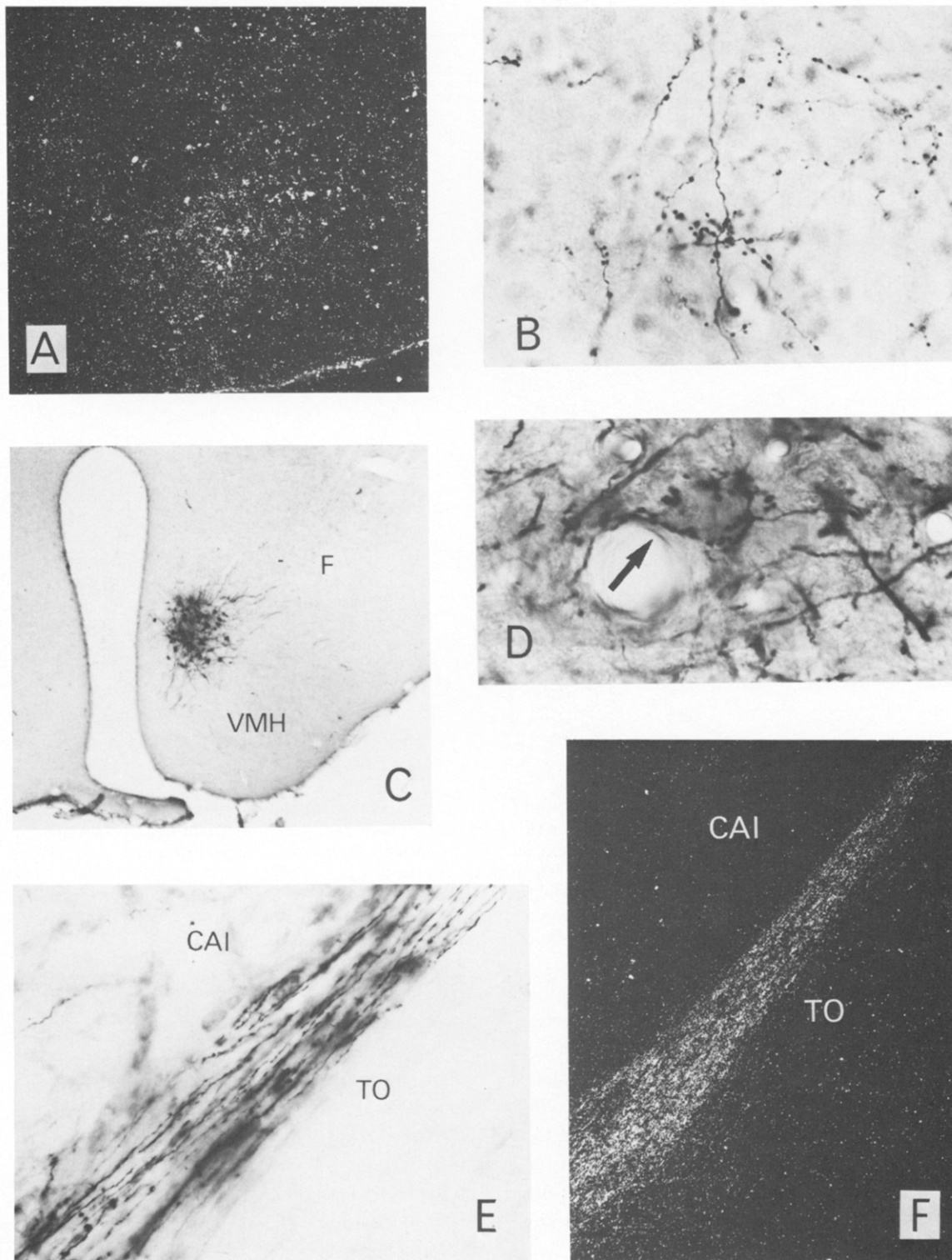


Fig. 2. A-F: photomicrographs illustrating labeling obtained after autoradiographic and PAP immunohistochemical processing of [^3H]leucine and *Phaseolus vulgaris* leuco-agglutinin injections into the ventromedial hypothalamic nucleus (VMH). A: dark-field photomicrograph showing tritiated leucine terminal labeling within the periamygdaloid cortex (see also Fig. 1A, B). B: bright-field photomicrograph of PHA-L terminal labeling within the periamygdaloid cortex in the area illustrated in A. C: photomicrograph of a PHA-L iontophoretic injection into the ventromedial hypothalamic nucleus (VMH). D: bright-field photomicrograph illustrating PHA-L terminal labeling embracing a receiving neuron (arrow). E, F: photomicrographs showing the labeling in the amygdalofugal pathway after PHA-L (E) and [^3H]Leu (F) injections into VMH, respectively.

of the PHA-L technique over the autoradiographic method is that in several projection areas it is possible to identify detailed contacts between the efferent terminals and the receiving somata (Fig. 2D). In these cases large numbers of labeled boutons can be seen embracing the target perikarya.

In summary, it may be concluded that the technique of anterogradely transported PHA-L is basically as effective as the autoradiographic method for the study of neuronal efferents, but offers several additional advantages. Its superior sensitivity en-

ables the user to study the fine structure of even small efferent projections. A disadvantage may be that the PHA-L technique does not appear suitable for survey studies of efferents from large brain areas.

The authors wish to express their appreciation to Mrs. Joke Poelstra for typing the manuscript. This work was supported in part by the Foundation for Medical Research FUNGO (Grant 13-46-36) which is subsidized by the Dutch Organization for the Advancement of Pure Research (ZWO).

- 1 Edwards, S. B. and Hendrickson, A., The autoradiographic tracing of axonal connections in the central nervous system. In L. Heimer and M. J. Robards (Eds.), *Neuroanatomical Tract-Tracing Methods*, Plenum Press, New York, 1981, pp. 171–205.
- 2 Gerfen, C. R. and Sawchenko, P. E., An anterograde neuroanatomical tracing method that shows the detailed morphology of neurons, their axons and terminals: immunohistochemical localization of an axonally transported plant lectin, *Phaseolus vulgaris*, leucoagglutinin (PHA-L), *Brain Research*, 290 (1984) 219–238.
- 3 Krieger, M. S., Conrad, L. C. A. and Pfaff, D. W., An autoradiographic study of the efferent connections of the ventromedial nucleus of the hypothalamus, *J. comp. Neurol.*, 183 (1979) 785–816.
- 4 Ricardo, J. A., Efferent connections of the subthalamic region in the rat: I. Zona incerta, *Brain Research*, 214 (1981) 43–60.
- 5 Ruda, M. and Coulter, J. D., Axonal and transneuronal transport of Wheat Germ Agglutinin demonstrated by immunocytochemistry, *Brain Research*, 249 (1982) 237–246.
- 6 Saper, C. B., Swanson, L. W. and Cowan, W. N., The efferent connections of the ventromedial hypothalamus of the rat, *J. comp. Neurol.*, 169 (1976) 409–422.
- 7 Ter Horst, G. J., Luiten, P. G. M. and Kuipers, F., Descending pathways from hypothalamus to dorsal motor vagus and ambiguus nuclei in the rat, *J. auton. nerv. Syst.*, in press.