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Multiple nitric oxide synthase systems in adult rat thymus revealed using NADPH diaphorase histochemistry

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SUMMARY

Nitric oxide (NO) has become recognized as a multifunctional mediator, with roles in vascular physiology, neurotransmission and non-specific immune defence. The histochemical marker associated with the neural and endothelial form of NO synthase (NOS), reduced nicotinamide adenine dinucleotide diaphorase (NADPHd), has enabled the indirect localization of potential sites of NO production. Innervation of the thymus and its immunological functions made this tissue a candidate for utilization of various NO systems. In the present study on adult rat thymus, multiple cellular sites expressing NADPHd activity, thereby implicated as sites of NOS activity, have been identified using morphological criteria alone: blood vessel endothelium, dendritic cells, deep cortical or medullary stromal cells, intrinsic neuron-like profiles, granulocytes (possibly neutrophils) and fat cells. In addition, the availability to the thymic microenvironment of another form of NOS in macrophages, which is not stained by the diaphorase technique, was supported by the observation of these cells at corticomedullary and cortical locations. These results indicate that a wide variety of possible immunomodulatory roles can be expected for NO in the thymus including the induction of tolerance, major histocompatibility complex (MHC) restriction, lymphocyte trafficking and regulation of thymic endocrine output.

INTRODUCTION

Evidence from different areas of study suggest that nitric oxide (NO) might be utilized within the thymus. Firstly, the trafficking of lymphoid progenitors between the blood vascular supply and the thymic microenvironment is likely to be dependent upon factors that influence vasodilation and adhesion of cells to the endothelial lining of vessels. A role for NO as endothelium-derived relaxing factor (EDRF) is now well established.[1,2] NO is also known to inhibit platelet aggregation and adhesion.[3] Hence, NO may influence rates of blood flow, cellular adhesion and therefore migration of blood-borne cells across vasculature within the thymus.

Secondly, thymus innervation by autonomic adrenergic and peptidergic fibres is well documented.[4] Cholinergic inputs are however disputed. It is also not yet clear whether some fibres could be classified as non-cholinergic/non-adrenergic (NANC). Because NO has been implicated as a transmitter of other autonomic NANC neurons,[5] a role for NO as mediator of signals between intrinsic or projection neurons and cells of the thymus would therefore be plausible should such cells be shown to be capable of synthesizing NO.

Thirdly, NO released at high levels from activated macrophages (Mϕ) and neutrophils is often the major cytotoxic effector molecule in the defence against tumour cells and many types of infectious organism.[6] Cytotoxic activity may also occur within the thymus during the induction of tolerance. It is believed that bone marrow-derived populations of thymic stromal cells are responsible for the deletion of self-reactive T-cell clones (negative selection).[7–10] Whether NO is utilized in the tolerizing of T-cell populations, perhaps by stimulating apoptosis, is not known. However, in addition to direct cytotoxic effects, Mϕ-derived NO does appear to be capable of mediating more subtle intercellular signalling, such as the suppression of concanavalin A-induced proliferation of rat splenic lymphocytes.[11,12] The possibility exists therefore that the proliferative development of thymocytes might be regulated by NO released from Mϕ or other cells of the thymic microenvironment, were they shown to be capable of its synthesis.

Finally, endocrine output of the thymus appears to be under the influence of NO as dietary supplementation of L-arginine can reverse the age-related decline in plasma thymulin level in mouse and human.[13] L-arginine metabolism may regulate thymic hormone secretion through NO production because this amino acid acts as substrate for nitric oxide synthase (NOS).[14] However, either thymic NOS systems or central neuroendocrine circuits driven by NO may be involved as L-arginine is known to have a secretagogue action on pituitary growth hormone,[15,16] which itself may modulate serum thymulin levels.[17]

The possibility that NO might be synthesized and used by cells within the thymus was investigated by a histochemical procedure for localizing NADPH diaphorase (NADPHd) activity.[17] This method has become recognized as a convenient, although indirect, way of identifying the neural and endothelial

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forms of NOS following the discovery that NADPHd and brain NOS activities co-purify, that these activities are co-localized within discrete populations of neurons, and that cells transfected with NOS cDNA assume diaphorase activity.[14]

MATERIALS AND METHODS

Adult male and virgin female Sprague Dawley rats (250–350 g) were anaesthetized with pentobarbitone (i.p.), decapitated, and perfused through the heart with cold saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Vibratome sections (300 μm thick) were cut and stored in cold PBS prior to incubation for 3–6 hr at 37° in a solution consisting of: 30 mM malic acid, 1 mM manganese chloride, 0·2 mM nitroblue tetrazolium (NBT), 1 mM βNADP, 0·5% Triton X-100, in 0·1 M Tris–HCl buffer at pH 7·4. Sections were washed in PBS and mounted in glycerol, coverslipped and viewed without counter staining. These histochemical methods were adapted from that of Sagar.[18] Incubations without βNADP were carried out as controls. Paired control and experimental sections were taken from individual thymi, processed together in adjacent chambers of multiwell plates, and interrupted simultaneously by wash solution. Analysis of thick-sectioned material was chosen because stained cells were relatively infrequent.

RESULTS

No staining was observed in control sections from which βNADP was omitted. NADPHd histochemistry failed to stain either the majority of cortical or central medullary stromal cells or thymocytes. Precipitation of the blue, reduced form of NBT was however observed in several structures, many of them centred about the corticomedullary junction (CMJ).

Blood vessels, radially orientated between the septae and CMJ, were observed to stain positive for the diaphorase throughout the cortex (Fig. 1A, Bv). Groups of darkly stained, neutrophil-like profiles were occasionally observed in the cortex (Fig. 1A, arrows) and distributed about the CMJ (not shown). An aggregation of NADPHd$^+$ neutrophils from within the cortex is shown at higher magnification in Fig. 1B.

In older animals, clumps of fat cells were observed to be positively stained by the diaphorase technique (Fig. 1C, arrow). These cells were found on the capsule of thymic lobules or alongside blood vessels passing deep within the septa.

Diaphorase staining at the CMJ and into the medulla (Fig. 1, star) could be subdivided into several discrete cellular profiles, as follows: a plexus of epithelial-like stromal cells ringing the medulla (Fig. 2A, star); a region of these reactive cells is shown at higher magnification in Fig. 2B. Greater detail of this cellular network is shown alongside other profiles positive for the diaphorase histochemistry in Fig. 3 (SC). Central medullary regions, bordered by the NADPHd$^+$ plexus, were relatively unstained (Fig. 2, Med). Among this network the profiles of mono- and bipolar NADPHd$^+$ somata were also found (Fig. 3, Nu). These neuron-like cells were observed to be associated with what appeared to be blood vessels (Fig. 3A).

One class of cell demonstrated a particularly strong NADPHd$^+$ reaction. These cells had dendritic morphologies and ringed the medulla (Fig. 4, DC). These presumptive interdigitating reticular cells were generally separated from one another, although they were also observed in pairs or clumps.

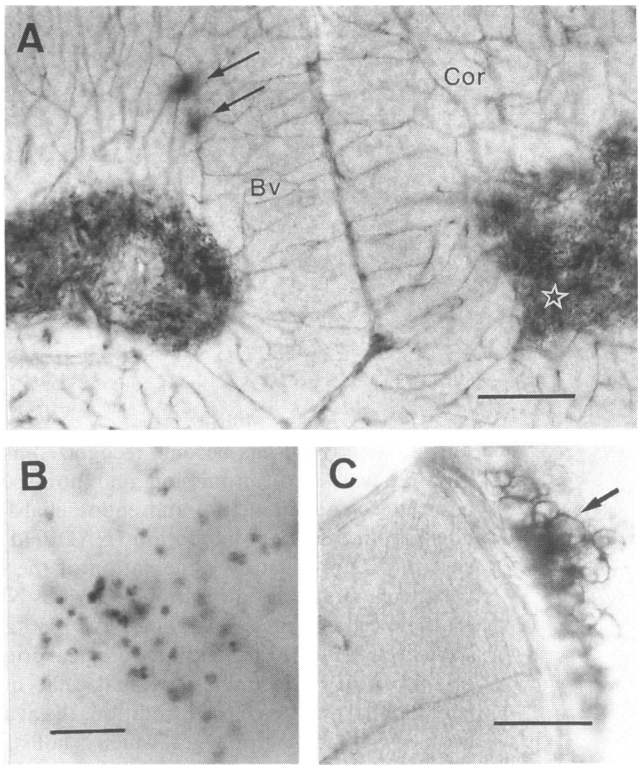

Figure 1. Bright field light micrographs of thick (300–400 μm) thymic sections stained for diaphorase activity. (A) Low magnification view of NADPHd staining patterns within adjacent adult rat thymic lobules. Bv, blood vessel endothelium found throughout the cortex, radially orientated between corticomedullary junction and capsule; Cor, cortex; star, NADPHd$^+$ plexus including various types of histological profile that have been identified in subsequent micrographs; Arrows, out of focus image showing location of two clumps of NADPHd$^+$ neutrophils residing within cortex. (B) Higher magnification detail of an aggregation of stained neutrophils. (C). Arrow, NADPHd$^+$ adipocytes clumped together and attached to the outer surface of the thymic lobule, sometimes also found within septal regions. Scale bars: (A) 200 μm; (B) 50 μm; (C) 100 μm.

Finally, a diffuse reaction sometimes accompanied other NADPHd$^+$ reaction profiles (Fig. 4, Cor). This stain was particularly noticeable throughout the cortex, however, central medullar regions appeared clear (Fig. 4, Med).

Mϕ were observed to be marked by an endogenous red/brown pigmentation in some preparations, especially in older animals. These cells did not stain for diaphorase activity. They were typically positioned at the CMJ, outside the ring of NADPHd$^+$ reaction (Fig. 5, arrows), but were also occasionally observed within the cortex (Fig. 5, uppermost arrow).

Variability was observed in the combination of profiles stained, numbers of NADPH$^+$ cells, and the area covered by stained profiles (compare Figs 2, 4 and 5). Blood vascular staining was on occasion insufficient to identify the radial orientation of vessels in the cortex (Fig. 4). In extreme cases, the stromal cell plexus was unstained (Fig. 4) or heavily stained (Figs 1 and 2). In some preparations visible reaction was restricted to dendritic cells. The endogenous pigmentation of Mϕ was also variable, being strongest in older animals.

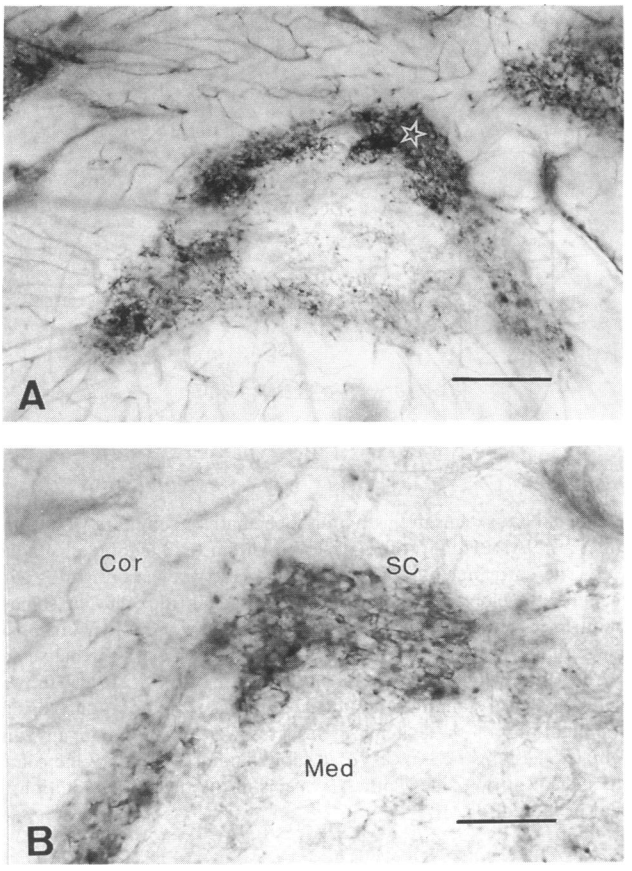

Figure 2. NADPHd$^+$ plexus of a stromal cell-like network surrounding the central medulla. (A) At lower magnifications a meshwork of NADPHd$^+$ reaction is shown that begins at the corticomedullary junction and extends approximately one-third of the way into the medulla. The area of SC plexus marked in (A) with a star is enlarged in (B). In some sections (see Fig. 1) less of the unstained central medullary area was seen. (B) At higher magnification an interconnected network of cellular profiles is revealed that is typical of epithelial cells. More detail of this cell type is also presented in Fig. 3. Cor, cortex; Med, medulla; SC, NADPHd$^+$ stromal cell-like plexus. Scale bars: (A) 100 μm; (B) 200 μm.

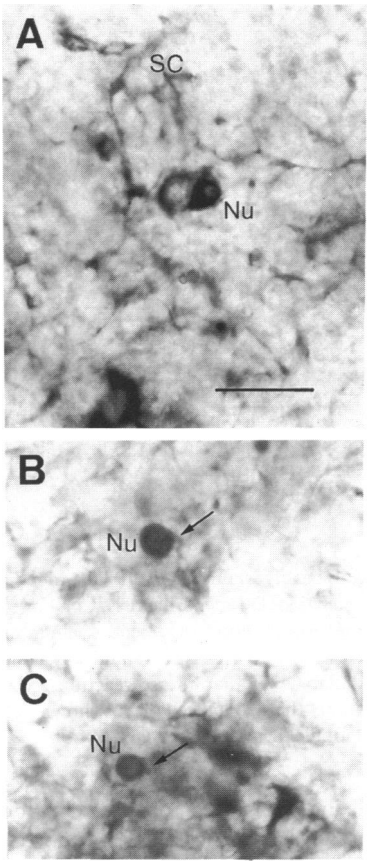

Figure 3. High-power magnification of corticomedullary junction zone. (A) NADPHd$^+$ profiles include stromal cell network (SC), and a neuronal-like somata associated with blood vessel (Nu). (B, C) Other NADPHd$^+$ neuron-like objects observed within this zone. Arrows point to primary processes. Scale bar: 40 μm for all three micrographs.

DISCUSSION

NADPHd reactivity of several populations of cells, identified morphologically, within the adult rat thymus is summarized in Fig. 6, and provides the first histological evidence to support diverse roles for NO within this primary lymphoid organ.

NADPHd as a histochemical marker for NOS

Several independent lines of evidence support the view that NADPHd activity can be a useful marker for certain forms of NOS. First, many histological studies have co-localized the cytochemical reactivity for NADPHd with immunocytochemical reactivity for neural NOS (reviewed in ref. 19). Although some exceptions to the co-localization of these two activities have been reported, there is general agreement on the usefulness of this marker for both central and peripheral neurons having NOS.[20,21] Secondly, biochemical evidence states that both brain NOS and NADPHd activities co-purify to homogeneneity, that these activities are also both immunoprecipitated by antibody to neuronal NADPHd, and that NOS activity is completely inhibited by the NADPHd substrate, NBT.[22] Finally, transfection of human kidney 293 cells with cDNA for NOS engenders these cells with NADPHd reactivity *de novo*.[14,23]

Another class of NOS, having different cofactor requirements, which consequently is not labelled by the diaphorase histochemical reaction, is however known to be present within Mϕ.[14] Also, a family of structurally related enzymes has been recognized, including cytochrome P450 reductase.[24]

NADPHd$^+$ reaction of blood vasculature

EDRF has now unequivocally been identified as NO.[1,2,25] Production of NO in the vicinity of blood vasculature may regulate not only blood flow but also leucocyte adhesion.[26] Both these actions could influence the trafficking of immunocytes into and out of the thymus, which would have important consequences for the extent to which cells would be exposed to thymus-dependent inductive influences. NADPHd$^+$ staining of thymic cortical blood vasculature is consistent with this view.

Innervation and intrinsic neurons

Innervation of lymphoid tissues has been clearly demonstrated (reviewed in refs 27 and 28). For the thymus, both noradrenergic

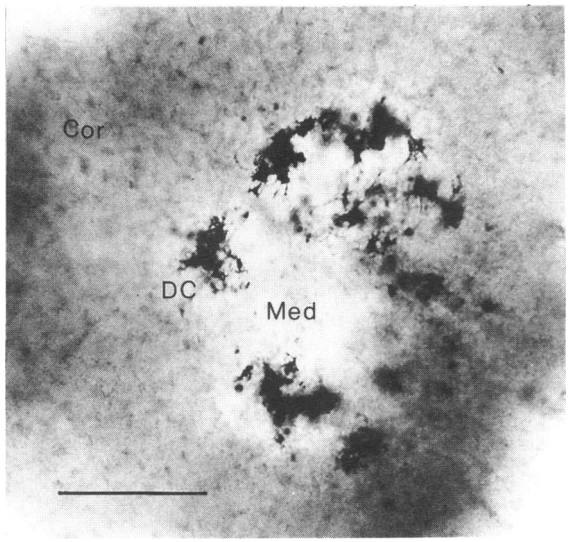

Figure 4. Ring of darkly stained NADPHd$^+$ dendritic cells at the corticomedullary junction. DC, dendritic cells; Cor, cortex; Med, medulla. Cross-sectional profiles of blood vessel endothelium are faintly visible throughout the cortex. The cortex is also stained by a diffuse reaction not seen in the medulla. Other diaphorase reactions seen within former micrographs, notably the stromal cell plexus shown in Figs 2 and 3, are absent from this lobule. Scale bar: 200 μm.

and cholinergic input have been proposed, in addition to other peptidergic fibres. The presence of NANC fibres among thymic innervation has not been established and yet, by analogy with other peripheral targets, this possibility still exists. Recent evidence implicates NO as an alternative inhibitory neurotransmitter of other NANC neurons.[5] Neuronal input to the thymus via this novel neuronal mediator was therefore eminently feasible. However, the results do not point to the presence of diaphorase labelling within projection neurons along the known routes of entry of autonomic input (septa). In addition, although a plexus of staining was observed at one possible site of autonomic terminal arborization, the CMJ, staining by other cell types seems to account for this pattern (Fig. 2, SC).

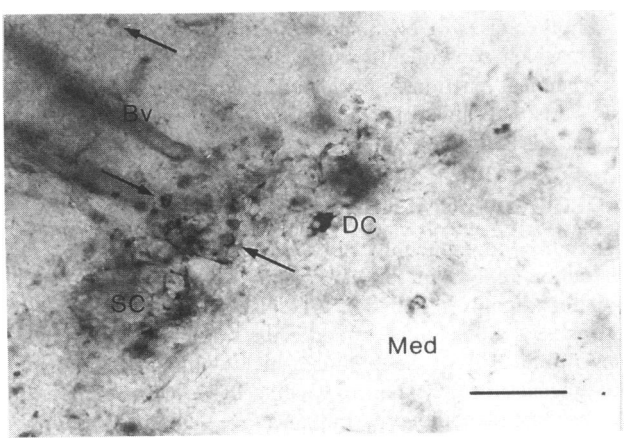

Figure 5. Corticomedullary junction zone showing locations of macrophages (arrows). These cells had endogenous red/brown pigmentation but did not stain for diaphorase. Med, medulla; NADPHd$^+$ profiles: Bv, blood vessel endothelium; SC, stromal cell network; DC, dendritic cell. Scale bar: 100 μm.

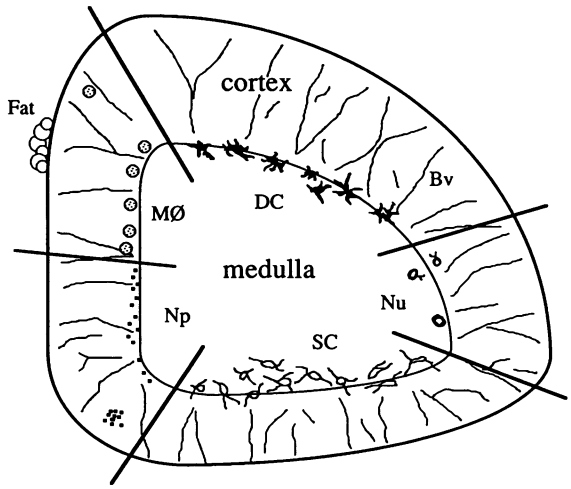

Figure 6. NO systems in the thymus. Schematized summary view of the different NADPHd$^+$ profiles observed within thymic lobules of adult rats. The corticomedullary junctional zone is drawn contiguous with medulla. Bv, blood vessel endothelium; DC, dendritic cells; Nu, somata of intrinsic neuron-like cells; SC, stromal cell network; Np, neutrophils; Fat, adipocytes. The location of macrophages (Mϕ), unreactive for NADPHd but known to contain another form of NOS, are also drawn. Diffuse cortical staining is not included in this representation. Various combinations of these itemized features were observed in any one lobule. These histochemical results for diaphorase activity indicate a number of nitric oxide synthase systems in the thymus in addition to that proposed for Mϕ.

Evidence for intrinsic neurons within the thymus has occasionally been obtained by immunocytochemical studies (M. D. Kendall, personal communication). NADPHd$^+$ intrinsic neurons have been identified in association with blood vasculature supplying central and peripheral nervous tissues.[29] The results with NADPHd lend support to the notion that NO-synthesizing, intrinsic neurons of the thymus may likewise be involved in the regulation of vascular circulation within this organ.

Identity of NADPHd$^+$ thymic stromal cells: DC and SC plexus

Two populations of NADPHd$^+$ stromal cells were observed encircling the medulla at the CMJ. A heavily stained population of mostly separated cells grouped as a necklace around the medulla (Fig. 4, DC). From their stellate morphological appearance and location within the lobule these cells were identified as bone marrow-derived dendritic cells.[30] A second class of labelled cellular profiles appeared to form an interconnected plexus throughout the deep cortex or superficial medulla (Figs 1–3, SC). Clarification of the identities of both cell types awaits further studies combining immunolabelling of cryosections firstly with anti-stromal cell antibodies[31] and subsequently by NADPHd histochemistry.[5]

Immunomodulatory roles for NADPHd$^+$ thymic stroma

Signals derived from the thymic microenvironment appear to regulate the major histocompatibility complex (MHC) restriction

of T-cell response to antigen and confer tolerance to self-antigen. A commonly held view is that thymic cortical epithelial cells[10,32] along with mesenchymal cells[33] are required in order to establish the specificity of MHC restriction, while bone marrow-derived cells (Mϕ and/or DC) may impart tolerance to self-antigen.[7–9] This role for bone marrow-derived cells in the induction of tolerance is inferred from the failure of T cells to develop tolerance to graft-type H-2 determinants following the selective experimental depletion of bone marrow-derived components of thymic stroma, by pretreatment with deoxyguanosine.[8]

NADPHd staining of putative DC suggests that NO might be utilized as a 'negative selection' signal delivered to self-reactive T cells, conveying either a tolerizing message, or perhaps mediating clonal deletion through lethal doses of this reactive radical. On the other hand, the NADPHd$^+$ staining of a thymic stromal cell plexus, which may represent a deep cortical epithelial cell type, is consistent with an additional or alternative role for NO in the 'positive selection' of T cells in the process of MHC restriction. However, further experimentation is required in order to verify the role of intrathymic NO in the regulation of T-cell development.

Macrophage and neutrophil NOS

The best-known function of Mϕ NOS is in the mediation of cytotoxic effects. During the process of clonal deletion of self-reactive T cells, thymic Mϕ NOS activity could conceivably be invoked in order to achieve tolerance, either as a cytotoxic dose of reactive radical interrupting respiratory enzyme function or as a more subtle intercellular message transduced through the cGMP pathway.[6,14]

A neutrophil NOS has been reported[34,35] and is known to be different in form to either the Mϕ or neural/endothelial NOS. However, diaphorase activity was found in these cells. Their presence within the thymus may indicate a response to infection. The patterning of these cells at the CMJ and in clumps within the cortex may prove to have functional significance.

Involvement of NO in control of thymulin release

The synthesis of NO is dependent upon the provision of L-arginine: Mϕ are unable to mount cytolytic challenges when stimulated in the absence of this amino acid.[14] Dietary arginine has also been implicated in the restoration of normal levels of serum thymulin in both mouse and man.[13] The possibility arises therefore that NO production in the thymus by the endothelial-type NOS labelled by NADPHd histochemistry, or by Mϕ-type NOS, may be involved in the regulation of thymulin release. Medullary cells capable of synthesizing thymulin would appear to be in the vicinity of NO released from either DC or the SC plexus. Double labelling of thymulin-secreting medullary epithelial cells, along with NADPHd histochemistry, maybe helpful in evaluating the proximity of these cells to those thymic cells synthesizing NO. Alternatively, central neuronal circuits governing endocrine functions may be involved, as NO is known to act centrally to enhance the release of pituitary peptides (e.g. growth hormone)[17,36] which themselves influence the release of thymic hormones.[37]

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