Ecdysteroid receptors in the neuroendocrine-endocrine axis of a moth

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Abstract. With a combination of thaw-mount autoradiography using a tritiated 20-hydroxyecdysone agonist, ponasterone A, and immunocytochemistry with a monoclonal antibody to 29 K-prothoracicotropic hormone, high affinity binding sites for ecdysteroids were identified in the tissues of the neuroendocrine-endocrine axis in *Manduca sexta* larvae. At specific times during larval-pupal development in fifth stadium larvae, nuclear ecdysteroid binding sites were present in the cerebral prothoracicotropes, the corpora allata and prothoracic glands, the main axis for the regulation and production of ecdysteroids. A stage-specific appearance of ecdysteroid receptors also occurred in cells of fat body, midgut and Malpighian tubules, tissues which convert ecdysone into 20-hydroxyecdysone. Our data identify new target tissues for ecdysteroids and suggest that ecdysteroids could affect their own production at the genomic level via long and short feedback loops.

Key words. Ecdysteroid receptor; prothoracicotropic hormone; neuroendocrine-endocrine axis; feedback regulation; autoradiography; immunocytochemistry; *Manduca sexta.*

The steroid hormones of eukaryotic organisms are phylogenetically old[1,2] regulatory molecules which act via receptor proteins in target cells to control an array of physiological processes. In vertebrates, steroids like estrogens, progestins and androgens control development and reproduction, while others like glucocorticoids, mineralocorticoids and vitamin D control stress responses and/or ion homeostasis[3]. These hormones influence their own production via feedback regulation of the cerebral neuroendocrine axes that control their synthesis. Steroids are also key endocrine effectors in invertebrates, and the ecdysteroids are thought to be responsible for the control of development, growth and reproduction. Correspondingly, the ecdysteroids regulate their target tissues via nuclear receptors, and the regulation of ecdysteroidogenesis is also under neuroendocrine control via the peptidergic prothoracicotropic hormone (PTTH), which is synthesized by four lateral neurosecretory cells (LNSC-III)[4]. PTTH is released at precise times into the hemolymph from neurohemal organs, the corpora allata (CA), to stimulate the prothoracic glands (PG) to synthesize and secrete ecdysteroids[5]. Ecdysone (E) is then hydroxylated by numerous tissues to the principal active ecdysteroid, 20-hydroxyecdysone (20 E). While ecdysteroids are generally viewed to exert precise temporal control over most of the organs and tissues of the insect and may control their synthesis by feedback regulation of the PTTH axis[5,6], direct evidence for these actions of ecdysteroids at the cellular level, particularly in terms of receptor presence and turnover, has been lacking. This study presents evidence that in all tissues involved in the synthesis of the endogenously active 20 E, nuclear ecdysteroid receptors are present at distinct times during development.

Materials and methods

Manduca sexta were raised and staged according to Granger et al.[7]. Only gate II larvae were used. Tissues such as brain, CA, PG, fat body, Malpighian tubule, gut and others were dissected from fifth instars at days 0, 1, 2, 3; 3.5, 4.0, 4.8, 6.0, 6.8, 7.8 and P_0, and maintained for 2 h in a large volume of Grace's insect culture medium (Gibco, Grand Island, NY) at 27 °C under 95% O_2/5% CO_2, to reduce endogenous ecdysteroids[8]. Individual tissues were then incubated in 40-µl drops of Grace's medium containing 4 nM ^{3}H-PNA (spec. act. 178 Ci/mmol; a gift from Dr P. Maroy, Dept. of Genetics, University of Szeged, Hungary) for 1 h under the same conditions, followed by a 2 × 10 min post-incubation in a large volume of Grace's medium, to eliminate non-specifically attached tracer[9]. The specificity of binding was verified by competition experiments, in which tissues were incubated in ^{3}H-PNA in the presence of 400 nM unlabeled PNA (a gift from Dr D. H. S. Horn, CSIRO, Melbourne, Australia) or 500 nM 20 E (Calbiochem, San Diego, CA) according to the same incubation schedule[9,10]. After in vitro labeling, all tissues were dried on filter paper and frozen in liquid nitrogen. 4-µm sections of the frozen tissues were cut and prepared for thaw-mount autoradiography. The thaw-mount autoradiograms and the exposure of sections and development of the autoradiograms were done according to Stumpf and Duncan[11], and Stumpf et al.[12]. Autoradiograms for colocalization of PTTH were developed and pretreated for immunostaining as described by Bidmon et al.[13]. Sections were then incubated for 14 h at 6 °C with a monoclonal antibody to 29 K-PTTH (A_2H_5) (gift of Dr W. E. Bollenbacher, Dept. of Biology, University of North Carolina, Chapel Hill, NC)[4], diluted 1:500 in 0.1 M phosphate buffered saline (PBS). The sections were rinsed twice in PBS and incubated for 2 h at room temperature in peroxidase-coupled antimouse immunoglobulins (Organon Teknika Corp., West Chester, PA). After two rinses in 0.05 M Tris-HCl buffer (pH 7.6), antibody binding in the autoradiograms was made visible by an 8-min incubation in a solution of 75 mg 3'3'-diaminoben-

zidine hydrochloride (Sigma, St. Louis) and 2.3 µl H_2O_2 in 100 ml Tris-HCl buffer. Control sections were incubated in normal mouse serum instead of primary antibody and processed as described above.

For each developmental stage and tissue, the number of silver grains per defined nuclear volume was counted in autoradiograms prepared according to Stumpf et al.[12] from six larvae, except for a few stages where some of the tissues were difficult to preserve (see fig. 2). The number of bound PNA molecules per volume was calculated according to Stumpf et al.[12]. For the quantitative evaluation of the LNSC-III, sections adjacent to those immunostained were developed for quantitative autoradiography.

Results and discussion

High affinity nuclear binding sites for ^{3}H-PNA fulfill most of the qualifications described for ecdysteroid receptors (ERec)[9,14], and thus the term ERec has been used to denote high affinity ecdysteroid binding sites in autoradiographic studies[15]. In the present investigation binding sites or ERec were found in all organs involved in 20 E production during fifth stadium development (figs 1 and 3), and binding of the 20 E agonist ^{3}H-PNA was not observed in the tissues incubated in the presence of non-labeled PNA or 20 E (not shown, see also Bidmon et al.[9]). These results are thus similar to those described for the central nervous system of *Calliphora vicina* and *Manduca sexta*[8–10,15]. However, a more detailed examination of the last larval stadium revealed differences among the various tissues of the ecdysteroid-endocrine axis in the time of the appearance of nuclear ERec.

During the first three days of fifth stadium development, when the 20 E titer is low, no ERec were observed in the prothoracicotropes (LNSC-III) (fig. 2A). Weak nuclear and cytoplasmic binding first appeared on day 3.5, during the scotophase when PTTH is released to induce the commitment peak[5] in the hemolymph ecdysteroid titer. Clear nuclear ERec were evident by day 4.0 in these cells (figs 1a and 2A). Cytoplasmic binding of ^{3}H-PNA might be evidence of rapid induction of receptor synthesis by an extranuclear action of 20 E or by a fast nuclear action, as has been described for the fast action of progesterone in the mammalian hypothalamus[16]. The rapid induction of ecdysteroid receptors has been observed in another insect system, where measurable amounts of ecdysteroid receptors were found between 1 and 2 h after 20 E administration to *Drosophila* embryos and larvae[17].

By day 4.8, between the commitment and prepupal peak in the ecdysteroid titer, nearly all ERec in the LNSC-III were nuclear. This was seen in autoradiograms developed for quantitative receptor autoradiography (not shown) and after subtraction of background. However, the number of ERec per LNSC-III cell had slightly decreased by day 4.8 (figs 1b and 2A). The number of ERec per prothoracicotrope increased again during the prepupal peak (day 6.0) (figs 1c,d; 2A). These same changes in the

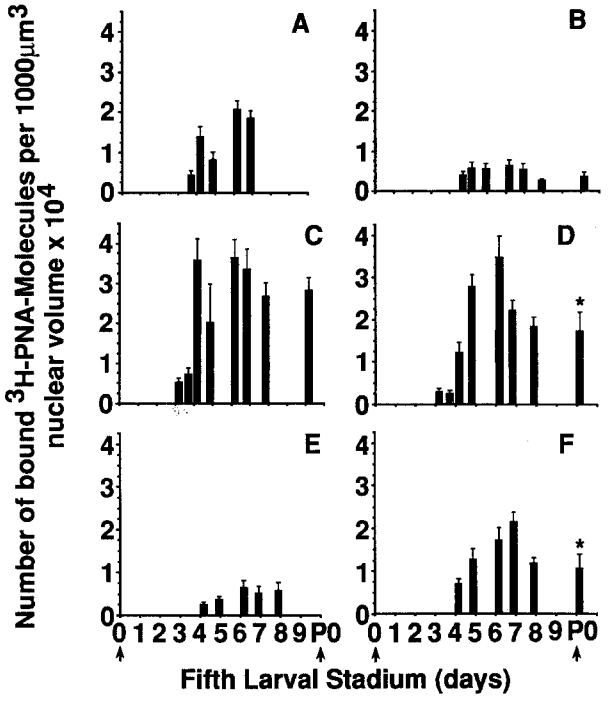
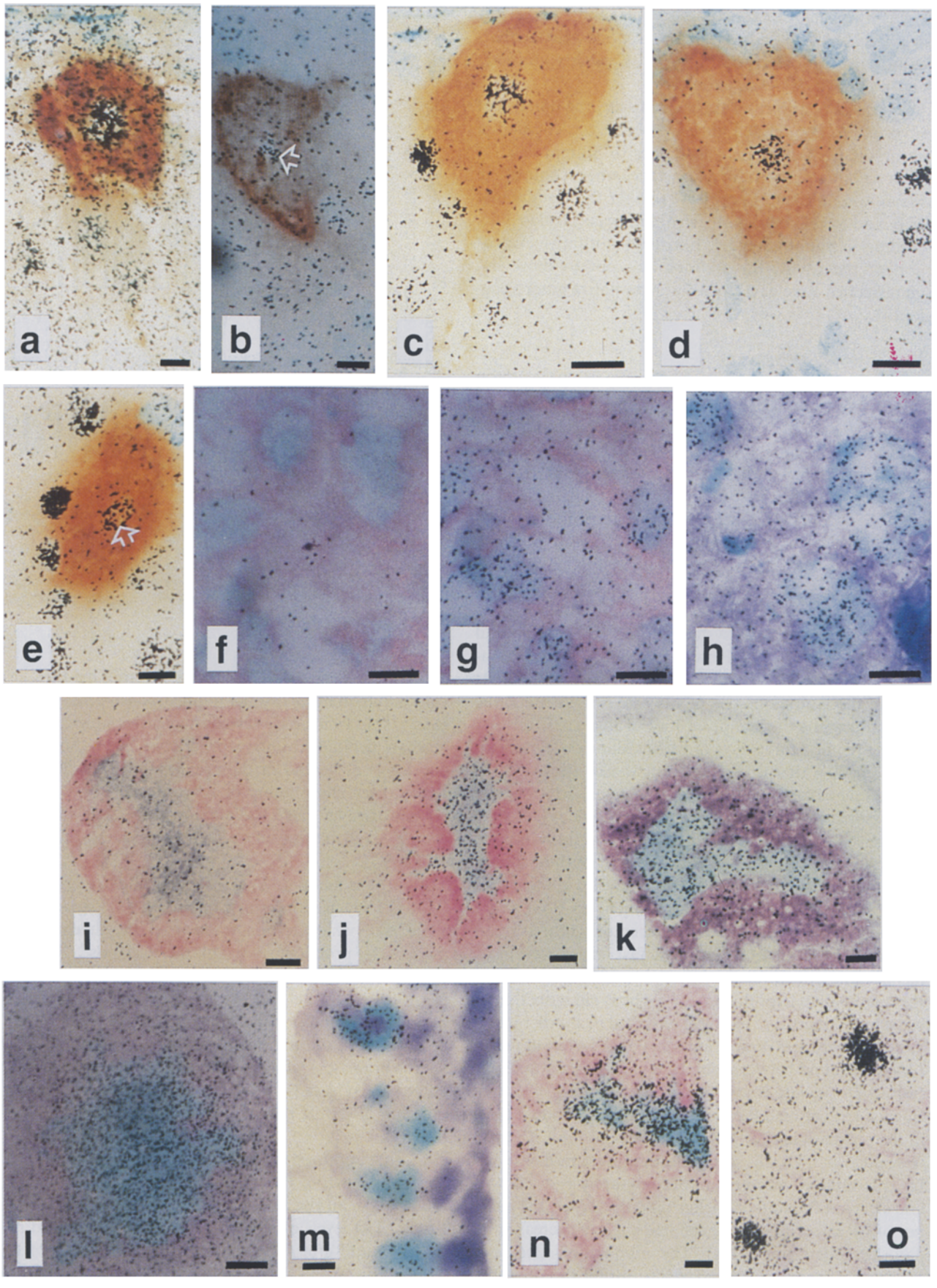


Figure 2. Changes during fifth stadium development in the number of specifically bound ^{3}H-PNA molecules per 1000 µm^3 nuclear volume (± SD) in cells of organs (n = 6; *n = 3) involved in 20 E synthesis, metabolism and excretion: LNSC-III (*A*), CA (*B*), PG (*C*), fat body (*D*), midgut (*E*) and Malphigian tubule (*F*). Note absence of nuclear PNA binding in LNSC-III before day 3.5 and after day 7.8 (prepupal development) (*A*).

number of ERec were also observed during the fifth larval stadium in other distinct populations of cerebral neurons[9,10], in which ecdysteroids may affect processes other than peptide hormone production[18,19]. Following day 6.8 and the end of the prepupal peak in the ecdysteroid titer, the number of ERec in the LNSC-III decreased (figs 1e and 2A), until by day 7.8, no labeling was observed in these cells with either tritiated or iodinated PNA[20].

The corpora allata (CA) are the neurohemal organs for PTTH. They are innervated in part by the axons of the LNSC-III, which terminate between the juvenile hormone (JH)-producing cells of these glands[4]. Nuclear ERec were present in the CA cells from day 3.5 on (figs 1f–h, 2B), i.e. from just prior to the commitment period. Since the previously described[7,21] 20 E control of CA activity during the commitment period is exerted via the brain, it is not clear what function is served by the ERec in the CA cells. Possible functions include the coordination of PTTH release and the inhibition of JH synthesis[22].

ERec first appeared in the cells of the PG (figs 1i and 2C) about 12 h before PTTH release, at approximately the time of a small rise in the basal hemolymph ecdysteroid titer[23]. The levels of nuclear ERec in these cells did not increase until after day 3.5, when in response to PTTH stimulation, the PG cells showed clear signs of synthetic activity (fig. 1j, day 4.8) including cytoplasmic invagina-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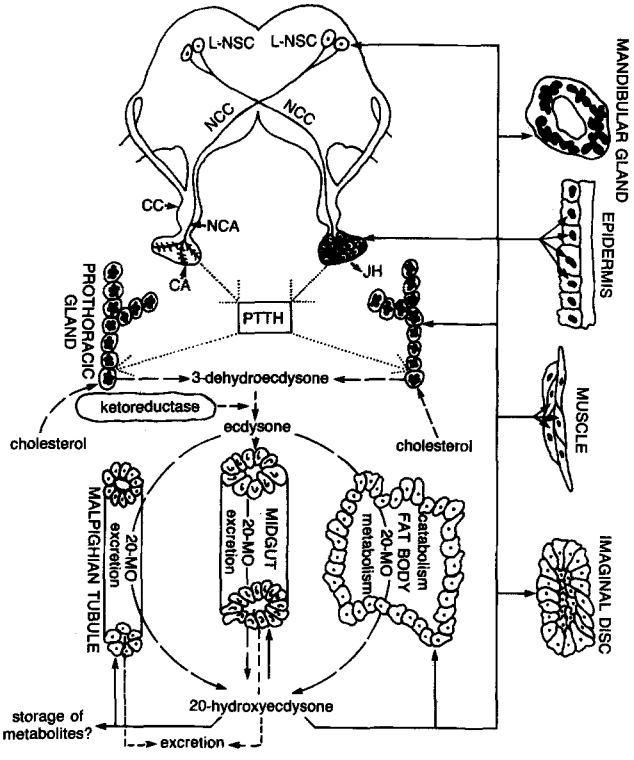

Figure 3. Schematic of identified steps in the pathway of 20 E production and metabolism (dashed lines and arrows), as described in the literature[4-6,27-30,32]. Levels in the ecdysteroid neuroendocrine-endocrine axis and ecdysteroid target tissues with high affinity nuclear binding sites are indicated by solid black dots representing nuclear ERec, and a connecting line of solid arrows. Interrelationships between sites of ecdysteroid synthesis, metabolism, and ecdysteroid targets indicate the possible regulation of the entire system by 20 E. It is further indicated that 20 E may regulate its stage-specific titer via a long feedback loop involving the cerebral lateral neurosecretory cells (LNSC-III) and the retrocerebral complex, and/or via a short feedback loop involving prothoracic glands, fat body, midgut and Malpighian tubules. CA = corpus allatum, CC = corpus cardiacum, JH = juvenile hormone, 20-MO = 20-monooxygenase, NCA = nervi corporis allati, NCC = nervi corporis cardiaci, PTTH = prothoracicotropic hormone.

tions under the basal lamina[24]. The timing of the increase in the number of nuclear ERec in these cells thus paralleled that in the LNSC-III. However, ERec were still present in PG cells on day 7.8, after the prepupal peak in the ecdysteroid titer (fig. 1k), and remained evident until after pupal ecdysis (fig. 1l). It is possible that ERec are involved in a short ecdysteroid feedback loop regulating ecdysteroid production at the PG level, as it is described by Sakurai and Williams[25] for certain developmental time points during the fifth stadium. However, these authors found that late prepupal PG do not respond to 20 E in their assay system. Thus the ERec present during the times of prepupal development may have different functions, such as stimulation of protein synthesis[26] or inhibition of ecdysteroid synthesis.

After production by the PG, 3-dehydroecdysone is reduced by a ketoreductase to ecdysone in the extracellular spaces of the PG and in the hemolymph (fig. 3)[27] and is further hydroxylated at the C-20 position to 20 E (fig. 3) by the action of a 20-monooxygenase from fat body, Malpighian tubules and midgut (fig. 3)[28-30]. In addition to this involvement in ecdysone metabolism, it has been recently reported that the midgut of Lepidoptera synthesizes ecdysteroids in vivo and in vitro in the presence of a trophic factor from the brain[31]. In all three of these organs (figs 1m–o, 2D–F), ERec were present in cell nuclei from day 3.5 on, and a developmental increase in high affinity PNA binding sites occurred in all three at the time of commitment, similar to that observed in the PG cells (fig. 2D). In fat body cells, low amounts of receptor were present even earlier, on day 3.0 (fig. 2). Since the functions of both the fat body, the main tissue in which 20 E metabolism and catabolism takes place, and the Malpighian tubules, which excrete ecdysteroids, appear to be stimulated by ecdysone and 20 E[32] (fig. 3), the receptors in these organs are essential for the regulation of their functions.

To compare the temporal program for the tissue-specific appearance of ERec in the ecdysteroid pathway with that

Figure 1 a–o. Autoradiograms of organs involved in regulation and production of 20 E, generated with ^{3}H-PNA; (*a–e*) are also immunostained with antibody A_2H_5; (*f–o*) are counterstained with methyl green-pyronin which stains DNA blue-green and RNA red. LNSC-III stained with a monoclonal antibody to PTTH contain nuclear binding sites on days 4.0 (*a*); 4.8 (*b*); 6.0 (*c*, *d*, showing the two cells in one hemisphere) and 6.8 (*e*). Nuclear and cytoplasmic binding is absent in the corpora allata cells on day 3.0 (*f*), but is obvious by day 3.5 (*g*), just prior to commitment, and on day 7.8 (*h*). Nuclear PNA binding is present on day 3.0 in the PG (*i*), increases during commitment, day 4.8 (*j*), and is still high after the premolt peak in the ecdysteroid titer, day 7.8 (*k*), and on pupal day 0 (*l*). Nuclear binding sites are present in cells of midgut on day 6.0 (*m*), Malphigian tubules on day 7.8 (*n*), and fat body on day 6.8 (*o*). Exposure time in days: 84, *b–e*, *g–h*, *m–o*; 60, *i–l*; 126, *a*; 206f. Bar 10 µm.

in traditional ecdysteroid targets, the presence of ERec was examined in salivary glands, muscle cells, imaginal discs, epidermis, and circumtracheal ectodermal cells during the commitment period and prepupal stages (fig. 3). Cells of all these tissues exhibited high levels of ERec predominantly during the prepupal stages. Only salivary gland cells, a few epidermal and endodermal cells, and a small proportion of muscle cells exhibited low levels of nuclear ERec during the commitment phase.

Our data reinforce the idea that ecdysteroids are systemic hormones, since the cells of all tissues examined in this study possessed nuclear ERec. However, these ERec were not present at all times, but were expressed or activated in a temporally specific manner in the different tissues and organs of the ecdysteroid neuroendocrine-endocrine axis, in organs involved in ecdysteroid metabolism, and in certain ecdysteroid target tissues (summarized in figs 2 and 3). Since the sensitivity of cells to steroid hormone signals is determined by the presence of steroid hormone receptors[33,34], developmental changes in the presence and number of ERec in each of these tissues may reflect changes in their sensitivity to ecdysteroids. Changes in receptor numbers seem to be regulated by the ecdysteroid titer itself[10,20], because increases in ERec occur only at times of active ecdysteroid synthesis. However, the initial step in the induction of ERec expression, which occurs in PG and fat body prior to the commitment peak in the ecdysteroid titer, remains unclear. Levels of ERec below the sensitivity of our detection methods may always be present, and these may respond to the first increase in hemolymph ecdysteroids, i.e. the small hemolymph ecdysteroid peak described by Riddiford[23] on late day 2.

Alternatively, membrane receptors for ecdysteroids may be involved in the induction of nuclear ERec. The existence of membrane receptors for ecdysteroids could be concluded from the finding of a fast ecdysteroid action on the nervus corporis cardiaci II[35]. Membrane steroid hormone receptors in mammals usually have a much lower K_D-value than nuclear steroid hormone receptors[36], and they are involved in the modulation of cellular responses to neurotransmitters[37]. Therefore, it is possible that slight, but specific, alterations in the very low baseline level of ecdysteroids may induce the stage-specific expression of nuclear ERec. Another possibility is that the decline in the JH titer during days 1 and 2[38] induces the expression of the first ERec; JH inhibits 20 E production in precommitment PG cells[22].

Our data further suggest that some fine tuning of the ecdysteroid titer could be achieved by short and long ecdysteroid feedback loops at several steps in the ecdysteroid metabolic/catabolic pathways (fig. 3), similar to the situation with steroid synthesis and metabolism in vertebrates[39]. Ecdysteroid receptors belong to the steroid hormone receptor family[40], which controls transcription via binding to specific hormone response elements in the DNA. Thus the response to ecdysteroids by the prothoracicotropes and by cells synthesizing 3-dehydroecdysone, ketoreductase and the 20-monooxygenase could involve transcriptional changes. The transcriptional changes affecting the ecdysteroid titer could also occur rapidly; steroid hormone- or peptide hormone-inducible transcription factors in mammals, such as the protooncogene *c-fos*, are early genes[41] which are expressed and act within 5–10 min[42] and ecdysteroid-inducible early genes have been identified in several insects[43,44]. The timing of the appearance of ERec at some of the levels of the ecdysteroid axis further supports the idea that they represent an overt cytological expression of ecdysteroid feedback regulation. For example, nuclear ERec in the identified prothoracicotropes, which together with the retrocerebral complex represent the long feedback loop, were clearly evident at times of active 20 E production during the fifth stadium, but were fewer in number during the interpeak period. Since these cells innervate the CA, their neurohemal organ, ecdysteroids could either be affecting PTTH release and therefore the activation of the PG, or affecting CA activity via ecdysteroid feedback loops known to be present in the brain at the times of the two ecdysteroid peaks[5,7,21]. The absence of nuclear and cytoplasmic ERec in the ecdysteroid neuroendocrine axis after the active phases of ecdysteroid production, but their presence in tissues responsible for the metabolism/catabolism and excretion of ecdysteroids, suggests that under the influence of ecdysteroids, these tissues control the crucial pre-molt decline in the ecdysteroid titer. This is essential for eclosion hormone release[45] and successful pupal ecdysis.

The results presented provide evidence that ecdysteroids (20 E) are humoral coordinators of multiple functions during larval-pupal development in *Manduca sexta* and that ERec can be visualized and localized at the morphological level by the combination of quantitative receptor-autoradiography and immunocytochemistry.

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Peptide YY enhances NaCl and water absorption in the rat colon in vivo

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Abstract. Peptide YY (PYY) is thought to possess paracrine and endocrine functions. The highest concentrations of this peptide are in the colonic mucosa. The effect of PYY on electrolyte and water transport in the rat colon was studied in vivo. Under urethane anesthesia, rat colonic loops were perfused at a constant rate with physiological buffer solution containing phenol red as a nonabsorbable volume marker, and net movements of water, sodium, chloride and potassium in the perfused colon were determined every 10 min. Intravenous administration of PYY produced a dose-dependent increase in the net absorption of sodium chloride and water, as well as a decrease in the net secretion of potassium. PYY inhibited the reduction in net absorption of sodium chloride and water evoked by vasoactive intestinal peptide (VIP), but did not affect the VIP-evoked increase in net potassium secretion.
These findings suggest that PYY acts as an enhancer of sodium chloride and water absorption and as an antagonist to VIP-induced secretion in the colon.
Key words. Peptide YY; vasoactive intestinal peptide; colon; electrolyte and water transport; rat; in vivo.

Peptide YY (PYY), a 36 amino acid-polypeptide, is localized in the endocrine-like cells in the gastrointestinal mucosa, which are numerous in the distal intestine, particularly in the colon [1–3]. Immunohistochemical studies have revealed that PYY cells sometimes have long cytoplasmic processes extending from the basal portion to the neighboring epithelial cells [1, 3], which suggests that PYY cells may have dual endocrine and paracrine functions.

Although hormonal effects of PYY have been suggested, such as inhibition of gastric or pancreatic secretion [4, 5], inhibition of gastrointestinal motility and constriction of intestinal vasculature [1, 6], little is known about the paracrine roles of PYY. Vasoactive intestinal peptide (VIP), which is a neuropeptide widely distributed in the central and peripheral nervous systems, has been shown to stimulate intestinal secretion [7]. Recently we reported [8]