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Regional Distribution of Calcitonin Gene-Related Peptide and Its Specific Binding Sites in Rats with Particular Reference to the Nervous System

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Abstract. The concentration of calcitonin gene-related peptide (CGRP) in rat tissue extracts was determined by a specific and sensitive radio-immunoassay, and the distribution of its specific binding sites was assessed by radioligand binding studies. A high concentration of immunoreactive CGRP was found at all levels of the spinal cord, in the trigeminal nucleus, in trigeminal and dorsal root ganglia, in the thyroid gland, in blood vessels and in nerves. The highest density of specific binding sites was detected in the cerebellum, where the CGRP content was minimal. The dorsal portion of the spinal cord contained a high concentration of CGRP and its specific binding sites. Specific binding of ^{125}I-CGRP was also demonstrated in a number of other areas of the brain and in certain peripheral tissues. Among the other tissues examined, the spleen, adrenal gland, penis, lungs, bladder, heart and blood vessels all contained a large number of CGRP binding sites, whereas only a negligible number of sites were found in ganglia, nerves, muscle, kidney and liver. The distribution of CGRP and its specific binding sites demonstrated here suggests that CGRP is a neuropeptide with multiple physiological roles.

In addition to calcitonin (CT) and the carboxyterminus-adjacent peptide (CAP) in the rat or katacalcin in man, the CT gene encodes a 37-amino acid peptide named CT gene-related peptide (CGRP) [3, 29]. CT is expressed mainly in the C cells of the thyroid, whereas CGRP is expressed mainly in the nervous system [29], although the presence of CGRP in thyroid gland has been reported in both rats [15, 25, 31, 42] and in man [40]. Preliminary results of the distribution and quantitation of all three products of the CT gene in rats have been reported using specific and sensitive radio-immunoassays (RIA) [42].

Immunocytochemistry and RIA quantitation studies have shown the presence of immunoreactive CGRP (i-CGRP) in the nervous system [29, 33, 40, 42], especially in the spinal cord [11, 25, 42], in the cardiovascular system [24, 27, 42], in the perivascular nerves often associated with the smooth muscles of blood vessels [29] and in a variety of other tissues [10, 24, 25, 40, 42]. Binding sites for CGRP have been demonstrated both in the rat [14] and human [39] central nervous system, in guinea-pig pancreas [30] and in the rat cardiovascular system [32].

The first evidence for the existence of CGRP in human was reported by isolation and characterisation of h-CGRP (α-hCGRP) from medullary thyroid carcinoma [26]. Subsequent investigations have revealed the presence of another gene which encodes for a second (β) CGRP in rats [2, 28] and in man [1, 35]. This peptide differs from the α-CGRP by only 1 amino acid in the rat [2] and by 3 amino acids in the human [35]. The effects of CGRP on vasodilation of rabbit skin (bio-assay) has shown that the three different forms of synthetically available CGRP (rat α-CGRP, human α-CGRP and human β-CGRP) are equipotent [5]. However, in an assay based on stimulation of cyclic AMP in aortic smooth muscle preparations, β-CGRP has been shown to be 5 times more potent than the α-CGRP [McEwan et al., unpubl. data]. Our present RIA and the ligand binding assay are unable to differentiate between α- and β-CGRP, which are equipotent in these assays (data not shown). Here, we report detailed regional distribution of i-CGRP in the rat and its binding sites, studied by RIA and radio ligand binding studies, respectively.

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Materials and Methods

Synthetic rat CGRP (rCGRP), the tyrosinated C-terminal decapeptide (Tyr^0-$rCGRP_{28-37}$) and human β-CGRP (β-hCGRP) were purchased from Peninsula Laboratories, Belmont, Calif., USA. α-hCGRP was supplied by Dr J. Pless (Sandoz, Basel, Switzerland). Cellulose-coated sheep anti-rabbit gamma globulin was obtained from IRE, Fleurus, Belgium. Male Wistar rats weighing 180–210 g were obtained from A. Tuck & Son Ltd. (Laboratory Animal Breeding Station, Essex, UK). ^{125}I-CGRP (spec. act. 2,000 Ci/mmol) was obtained from Amersham International, UK. Aprotinin (Trasylol) was obtained from Bayer (FRG) and Bacitracin was from Sigma (Poole, UK).

Tissue Extraction

To minimise blood contamination of tissues, animals were exsanguinated through the dorsal aorta following ether anaesthesia. Brain dissections were carried out as previously described [13, 19] and tissues collected on dry ice. Wet weights were recorded and tissues homogenised in an acid mixture [4] (1 *M* HCl, 15% TFA, 5% formic acid (v/v) and 1 *M* NaCl) on ice. The homogenates were centrifuged at 7,000 *g* for 15 min at 4 °C and the pellets rehomogenised in fresh acid mixture and recentrifuged. Pooled supernatants were applied to C-18 Sep-Pak disposable cartridges (Waters Associates) primed with methanol and eluted with amixture of methanol, water and trifluroacetic acid (TFA), (90:9:1, v/v). The extracts were vacuum-dried and stored at –20 °C. The recoveries of synthetic rCGRP and ^{125}I-rCGRP added to the control tissues (n = 8) were 85 ± 0.6 and 87 ± 0.8% (mean ± SEM), respectively.

Membrane Preparation

Membranes were prepared by a modification of a previously described method [34]. Fresh rat tissues were dissected and placed in 15 vol of ice-cold 50 m*M* Tris/HCl buffer (pH 7.5) containing 0.32 *M* sucrose, 1 m*M* dithiothreitol and 5 m*M* EDTA. They were homogenised with a Dounce homogeniser at 4 °C and the homogenate was centrifuged at 1,000 *g* for 10 min. The supernatant was then centrifuged for 30 min at 30,000 *g* and the pellet resuspended in 10 vol of 50 m*M* Tris/HCl buffer. Following further centrifugation at 30,000 *g* for 30 min, the pellet was finally resuspended in fresh 50 m*M* Tris/HCl buffer and the protein concentrations were determined by the method of Lowry et al. [23]. The final protein concentration was adjusted to 2 mg protein/ml with assay buffer (50 m*M* Tris/HCl, 10 m*M* KCl, 5 m*M* $MgSO_4$, bacitracin 0.1%, aprotinin 100 KIU/ml and 30 m*M* sodium azide). Aliquots (1–2 ml) were immediately frozen on dry ice and stored at –70 °C and used within 6 weeks of preparation.

Receptor Binding Studies

The labelled peptide (2.5 fmol of [^{125}I]His-hCGRP) was incubated with rat tissue membranes (400 µg of protein) in the presence of 1% (w/v) heat-inactivated bovine serum albumin, in a total volume of 300 µl for 50 min at 23 °C (equilibrium conditions) in polypropylene micro-centrifuge tubes (Sarstedt). After incubation, 700 µl of assay buffer containing 1% BSA was added to each tube and immediately centrifuged at 11,000 *g* for 2 min. The supernatant was discarded and the pellet layered with another 750 µl of the same buffer, recentrifuged and the supernatant discarded. The radioactivity present in the pellets was measured using an NE-1600 gamma counter (Nuclear Enterprises) with a counting efficiency of 60%. Specific binding was calculated from the total amount of ^{125}I-CGRP bound minus the amount bound in the presence of 0.5 µ*M* unlabelled peptide and expressed as ^{125}I-CGRP bound (fmol/g membrane protein). For rat cerebellar tissues, IC50 for ^{125}I-CGRP was 64 fmol/tube and the K_d 0.24 n*M* (data not shown).

Radio-Immunoassay

Radio-iodination of the C-terminal decapeptide of rCGRP was carried out by a modification of the chloramine T method [16] and the products were purified by high-performance liquid chromatography (HPLC). Antiserum (CG-39) was raised in rabbit against the same decapaptide coupled to ovalbumin [26]. Prior to RIA, samples were dissolved in 20 µl of 0.01 *M* formic acid, neutralised with 500 µl of the assay buffer and centrifuged. RIA was performed as previously described [43] using a non-equilibrium assay with addition of label after 3 days. The antiserum-bound peptide was precipitated by sheep anti-rabbit gamma globulin after a further 2 days of incubation. Extracts were assayed in at least two dilutions in duplicate and results were expressed as picomoles per gram wet weight of tissues. 50% displacement of the tracer required 20 fmol of rCGRP, with a detection limit of 0.5 fmol/assay tube. Intra- and inter-assay variations were 5 and 9%, respectively.

Specificity

None of the following peptides at a concentration of 1 nmol/tube resulted in displacement of tracer in either RIA or radioligand binding assay: corticotrophin, $ACTH_{1-39}$, LHRH, Pro-opio-melanocortin (National Institute of Health, Bethesda, Md., USA), somatostatin, glucagon, substance P, bombesin, human and porcine insulin, corticotrophin-releasing factor, growth hormone, oxytocin, vasopressin, prolactin, leu- and met-enkephalins, secretin, gastric inhibitory peptide (Sigma Ltd., Poole, UK) katacalcin and CAP (Peninsula). Salmon CT (Sandoz) with 30% homology to CGRP and human CT (Ciba-Geigy) with 19% homology [25] were both able to displace receptor-bound CGRP, but only at 10^4 and 10^5 molar excess, respectively.

Results

Regional Distribution of CGRP and Its Binding Sites in the Rat Nervous Tissues

Immunoreactive CGRP. The highest concentrations of i-CGRP were found in the trigeminal nucleus (635 pmol/g) followed by the dorsal root ganglia, dorsal half of the spinal cord and trigeminal ganglia (table I). Levels were intermediate in the trigeminal and sciatic nerves, medulla and pons, pituitary and substantia nigra. However, concentrations were found to be low in the hypothalamus, midbrain and striatum, and minimal in the hippocampus, cerebral cortex and cerebellum. The i-CGRP content decreased steadily between the cervical and the lumbar regions of the spinal cord, but in contrast a markedly high level was found in the sacral region.

Table I. The tissue concentration of immunoreactive CGRP and its specific binding sites in the nervous system of the rat

Tissue	^{125}I-CGRP bound fmol/g protein (n = 4)	i-CGRP pmol/g wet weight (n = 8)
Cerebellum	2,385 ± 67	1.0 ± 0.1
Grey matter	2,968 ± 51	1.6 ± 0.2
White matter	1,381 ± 33	0.6 ± 0.1
Spinal cord (cervical)	1,603 ± 61	265.0 ± 6.9
Dorsal portion	2,011 ± 55	370.0 ± 10.2
Ventral portion	1,235 ± 73	35.5 ± 3.1
Upper cervical	1,625 ± 52	311.0 ± 11.1
Lower cervical	1,732 ± 60	237.0 ± 9.3
Thoracic	1,130 ± 29	172.0 ± 8.1
Lumbar	1,566 ± 41	86.8 ± 3.5
Sacral	1,711 ± 51	270.5 ± 7.8
Substantia nigra	690 ± 31	20.1 ± 1.0
Medulla and pons	635 ± 34	72.6 ± 2.6
Olfactory tubercle	512 ± 25	9.7 ± 1.0
Striatum	458 ± 27	5.2 ± 0.4
Hypothalamus	415 ± 32	10.0 ± 0.9
Retina	370 ± 31	10.3 ± 0.8
Olfactory bulb	355 ± 22	6.1 ± 0.3
Midbrain	341 ± 24	10.5 ± 0.9
Hippocampus	252 ± 22	12.0 ± 0.3
Cerebral cortex	195 ± 19	1.0 ± 0.1
Pituitary	174 ± 13	31.5 ± 1.6
Trigeminal nucleus	76 ± 5	635.0 ± 17.5
Trigeminal ganglion	14 ± 2	182.0 ± 9.6
Dorsal root ganglion	11 ± 2	411.0 ± 13.1
Trigeminal nerve	5 ± 1	91.6 ± 5.0
Sciatic nerve	5 ± 1	85.0 ± 2.9

Values are presented as means ± SEM.

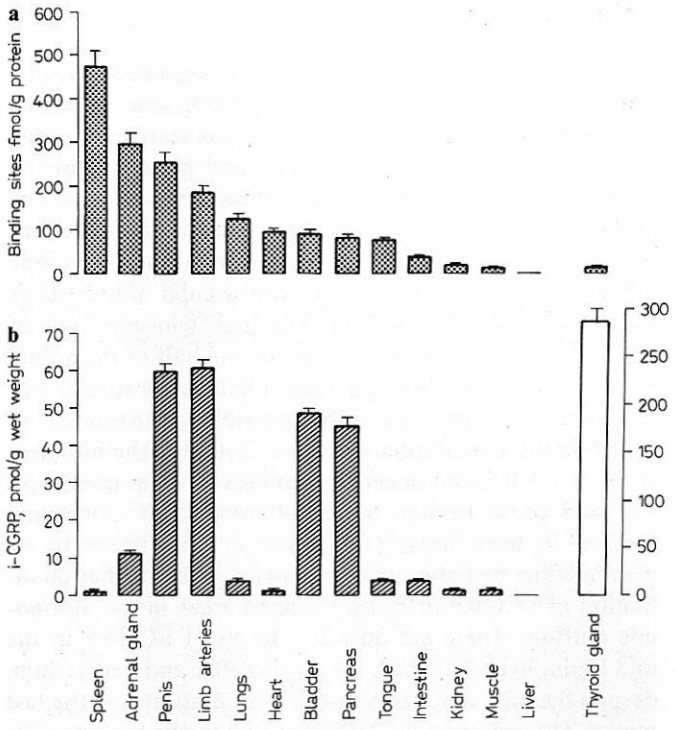

Fig. 1. Specific binding sites and i-CGRP in non-neural tissues (mean ± SEM). ^{125}I-CGRP bound (n = 4) (**a**) and i-CGRP concentration (n = 8) (**b**).

Specific Binding Sites. The highest concentration of specific binding was found in the cerebellum (2,385 ± 67 fmol/g protein) where the content of i-CGRP was minimal (1 pmol/g) (table I). High concentrations of specific binding sites were also found in the spinal cord (1,603 ± 61 fmol/g protein) where i-CGRP levels were also high (265 pmol/g). Markedly different numbers of specific binding sites were found in the grey and white matter of the cerebellum, and in the dorsal and ventral halfs of the spinal cord. Higher numbers of specific binding sites were also found in the hypothalamus, substantia nigra, medulla and pons, olfactory tract, striatum, mid brain and hippocampus in comparison to the cerebral cortex, pituitary and the trigeminal nucleus. Minimal or no specific binding was detected in the sensory ganglia and on nerve trunks. Non-specific binding was relatively constant at 4–6% of the total radioactivity added to the membranes.

Other Tissues

The highest concentration of i-CGRP outside the nervous system was found to be in the the thyroid gland (287 ± 18.8 pmol/g) (fig. 1). No significant binding could however be demonstrated in this tissue. A high concentration of i-CGRP and also a high number of specific binding sites were found in the adrenal gland. However, the spleen contained the highest concentration of specific binding sites (475 ± 41 fmol/g protein) outside the nervous system, despite the presence of minimal amounts of CGRP (0.7 pmol/g). In addition, i-CGRP was measured in the pancreas, penis, bladder and intestine. Specific binding was also found in several other tissues including penis, lungs, bladder and pancreas, but was negligible in muscles, kidney and liver. The heart contained only a small quantity (1 pmol/g) of CGRP, but large amounts of its binding sites (95 ± 6 fmol/g protein), particularly in the right atrium (not shown).

Discussion

The main purpose of this study was to determine the distribution of CGRP and its specific binding sites in rat tissues with special reference ot the nervous system. The distribution of CGRP-producing cells and pathways in the central and peripheral nervous system and in endocrine and other systems suggests potential functions in nociception, ingestive behaviour and modulation of the autonomic and endocrine systems [29], cardiovascular homeostasis and mineral metabolism [28]. The high concentration of i-CGRP in the neural ganglia and dorsal half of the spinal cord with lower levels in the ventral half of the spinal cord described here reinforces the possible involvement of CGRP in the modulation of sensory activity. The high levels of i-CGRP found in cervical and sacral areas of the spinal cord could be due to the abundance of autonomic outflow in these areas [18]. However, this needs to be proven either by immunocytochemistry or by further quantitation of i-CGRP in microdissected areas in the autonomic outflow. There are only low levels of i-CGRP in the mid brain, hypothalamus, cerebral cortex and cerebellum, despite the high number of specific binding sites in the last region. The presence of CGRP and its specific binding sites in the olfactory area of the brain is consistent with the possible role of CGRP as a neuromodulator/neurotransmitter in this sensory pathway.

The present study has shown the presence of a high concentration of i-CGRP as well as a high number of specific binding sites in the dorsal spinal cord when compared with the ventral spinal cord. No significant specific binding was found in the trigeminal or dorsal root ganglia and on nerve trunks. The relative concentrations of specific binding sites demonstrated in the present study are not in agreement with the ratios previously reported [14, 39]. The highest concentration of specific binding sites were detected in the cerebellum followed by the spinal cord. Goltzman and Mitchell [14], found specifig binding of CGRP in rat pituitary membranes comparable to that in spinal cord and several fold greater than in the cerebellum. Tschopp et al. [39], however, could not find any CGRP binding sites on human pituitary membranes. The present study shows that the number of specific binding sites for CGRP on the pituitary membranes was some 14-fold less than that of cerebellum. Similarly, binding in the brain stem was found to be 4-fold less than in the cerebellum. Marked differences in CGRP-specific binding sites exist in different areas of the cerebellum and spinal cord, and this is consistent with the autoradiographic localization of CGRP binding sites [17]. This distribution of specific binding sites and i-CGRP suggests that CGRP may play a part in sensory neurotransmission and in cerebellar function.

CGRP has been shown to inhibit basal and stimulated gastric acid and pepsin secretion [22] together with a lowering of plasma gastrin, enteroglucagon and neurotensin [22]. Furthermore, CGRP has been shown to cause a dose-related contraction of intestine in rats [7] and contraction of the guinea-pig ileum which could be blocked by either antihistamine or anticholinergic drugs [38]. The interaction of CGRP with its receptors has been shown to release amylase from exocrine pancreas in a dose-dependent manner [30], perhaps to assist the digestion of food. Furthermore, the finding of a high concentration of i-CGRP and its specific binding sites in mesenteric arteries (not shown) supplying the viscera could reflect a role in the diversion of the blood supply following a meal and perhaps enhancing absorption.

CGRP has been shown to have potent cardiovascular effects including positive chronotropic and inotropic actions on the heart [9, 24, 32, 38] and to cause vasodilation in man [6, 12, 36] and in the rabbit [5, 6], hamster [6] and rat [8]. The positive inotropic action of CGRP, however, may be due to the reflex sympathetic stimulation as shown by a rise of plasma noradrenaline levels after administration of CGRP [8, 9, 36]. Nevertheless, direct action on the heart cannot be excluded, in view of the presence of the CGRP-specific binding sites.

The high concentrations of i-CGRP and its specific binding sites in peripheral arteries may be important in increasing blood supply during exercise. It is possible that some of the specific binding sites demonstrated in highly vascular tissues, such as penis and lungs, could be due to the binding sites present on the blood vessels. The spleen contained the highest concentration of specific binding sites outside the nervous system (fig. 1), and this may reflect the known ability of the spleen to pool blood in certain situations. Whether this high specific binding reflects splenic vessels or some other component in the spleen remains to be elucidated. The finding of a high concentration of CGRP and its specific binding sites in the peripheral vasculature together with the proven potent vasodilatory activity of CGRP [5, 6, 12, 36] makes it likely that the peptide has an important role as a neuromodulator of peripheral vascular tone.

CGRP has been measured in plasma from normal man [12] and in rat [43] plasma. Furthermore, high concentrations of i-CGRP have been found in both the thyroid and plasma [26] of patients with medullary thyroid carcinoma. In the rat thyroid, CGRP has been shown to coexists with CT in the C cells and with substance P in the perivascular nerve fibers [15]. On HPLC of Sep-pak (C-18)-purified thyroid extracts, the predominant i-CGRP peak coeluted with the synthetic $rCGRP_{(1-37)}$ [31]. In addition, gel-permeation studies of rat thyroid extracts revealed that 70% of the total yield of i-CGRP coeluted with the synthetic $rCGRP_{(1-37)}$, suggesting the presence of monomeric CGRP in the thyroid gland [Wimalawansa, unpubl. data]. CGRP receptors associated with smooth muscle cells have been

shown to be linked to adenylate cyclase [20, 32], but no similar interaction has been demonstrated in the CNS [14]. Recently, it has also been reported that CGRP may act on vascular smooth muscle indirectly via an endothelial cell-dependent mechanism [6, 20], but further studies are needed to elucidate the nature and in particular the cellular localisation of specific binding sites in blood vessels by autoradiography or by immunocytochemical techniques.

Although CGRP has been shown to be present in plasma [12, 43], no definitive humoral role has been established. However, the distribution of i-CGRP and its specific binding sites strengthen the postulated actions of CGRP as a neuromodulator involving more than one function. No conclusions can be drawn about the differential expression of the two calcitonin genes or the possibility of subclasses of receptors. It has been shown that CGRP coexists at a higher concentration with other neuropeptides such as substance P in the trigeminal and dorsal root ganglia [21, 41] and also in sensory nerve fibres [15, 24]. It has also been shown to coexist with acetylcholine in neurones in the hypoglossal, facial and ambiguous nuclei [37] of rat brain.

The widespread distribution of CGRP and its specific binding sites suggests that CGRP (whether α, β or both) may have a physiological role. In view of the close structural homology between the α- and β-CGRP, the differentiation of the α- and β-gene expression may be difficult using conventional immunological methods, and more easily approached using gene-specific probes.

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Original Papers

Neuropeptide Y Stimulates the Release of Luteinizing Hormone-Releasing Hormone from Medial Basal Hypothalamus in vitro: Modulation by Ovarian Hormones
Crowley, W.R.; Kalra, S.P. (Memphis, Tenn./Gainesville, Fla.) 97

Growth Hormone Secretion after Hypothalamic Periventricular Lesions in the Rat
Praputpittaya, C.; Arita, J.; Kimura, F. (Yokohama) 104

In vivo Release of Dopamine, Luteinizing Hormone-Releasing Hormone and Thyrotropin-Releasing Hormone in Male Rats Bearing a Prolactin-Secreting Tumor
Voogt, J.L.; de Greef, W.J.; Visser, T.J.; de Koning, J.; Vreeburg, J.T.M.; Weber, R.F.A. (Rotterdam/Leiden) . . . 110

Electrical Stimulation of Ventral versus Dorsal Mesencephalic Tegmental Areas in the Conscious Rat: Effects on Luteinizing Hormone Release
Bergen, H.; Leung, P.C.K. (Vancouver) 117

A Pharmacological Dose of Melatonin Increases PRL Levels in Males without Altering Those of GH, LH, FSH, TSH, Testosterone or Cortisol
Waldhauser, F.; Lieberman, H.R.; Lynch, H.J.; Waldhauser, M.; Herkner, K.; Frisch, H.; Vierhapper, H.; Waldhäusl, W.; Schemper, M.; Wurtman, R.J.; Crowley, W.F. (Cambridge, Mass./Boston, Mass./Vienna) 125

Regional Distribution of Calcitonin Gene-Related Peptide and Its Specific Binding Sites in Rats with Particular Reference to the Nervous System
Wimalawansa, S.J.; Emson, P.C.; MacIntyre, I. (London/Cambridge) 131

Growth Hormone Secretory Patterns in Young, Middle-Aged and Old Female Rats
Takahashi, S.; Gottschall, P.E.; Quigley, K.L.; Goya, R.G.; Meites, J. (East Lansing, Mich.) 137

The Corticotropin-Releasing Factor Release in Rat Hypophysial Portal Blood Is Mediated by Brain Catecholamines
Guillaume, V.; Conte-Devolx, B.; Szafarczyk, A.; Malaval, F.; Pares-Herbute, N.; Grino, M.; Alonso, G.; Assenmacher, I.; Oliver, C. (Marseille/Montpellier) 143

Assessment of Corticotropin-Releasing Factor, Vasopressin and Somatostatin Secretion by Fetal Hypothalamic Neurons in Culture
Clarke, M.J.O.; Lowry, P.; Gillies, G. (London/Reading) 147

Immunoreactive Prolactin in the Rat Hypothalamus: In vitro Release and Subcellular Localization
DeVito, W.J.; Connors, J.M.; Hedge, G.A. (Morgantown, W.Va.) 155

Growth Hormone Responsiveness in vivo and in vitro to Growth Hormone Releasing Factor in the Spontaneously Diabetic BB Wistar Rat
Serri, O.; Brazeau, P. (Montréal) 162

Rapid Naloxone-Induced Alterations of Androgen Variables in the Growing Male Rat
Marić, D.; Stojilković, S.; Krsmanović, L.; Simonović, I.; Kovačević, R.; Andjus, R.K. (Novi Sad/Belgrade) . . . 167

Reevaluation of the Effects of Castration on Naloxone-Sensitive Opiate Receptors in the Male Rat Brain
Cicero, T.J.; O'Connor, L.H.; Bell, R.D. (St. Louis, Mo.) 176

Announcement 184

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