Changes in 17β-estradiol concentration in autonomic regulatory nuclei following MCAO

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ABSTRACT

Stroke-induced lesions in the insular cortex of the brain have been linked to autonomic dysfunction leading to arrhythmiogenesis and sudden cardiac death. In experimental models, systemic exogenous estrogen has been shown to reduce stroke-induced cell death in the insular cortex. However, estrogen's ability to reduce stroke-induced autonomic dysfunctions appears to be due to its effects at other extra-cortical autonomic regulatory nuclei. We therefore set out to test the hypothesis that estrogen concentrations increase in autonomic nuclei in a rodent model of middle cerebral artery occlusion (MCAO) in the absence of system changes in estrogen concentrations. Plasma estrogen concentrations were not altered by MCAO (22.86±0.14 pg/ml vs. 21.24±0.33 pg/ml; $p>0.05$). In contrast, estrogen concentrations in the parabrachial nucleus (PBN) increased significantly by 10 minutes after MCAO (36.43±0.57 pg/ml vs. 83.71±2.33 pg/ml; $p<0.05$) and then gradually declined to below baseline values (11.43±0.43 pg/ml). Estrogen concentrations also increased significantly in the central nucleus of the amygdala (CNA), but the increase was gradual, reaching four times that of baseline values (20.83± 0.54 pg/ml vs. 76.67±1.59 pg/ml; $p<0.05$) by four hours post-MCAO. Local infusion of an aromatase inhibitor (letrozole) into the CNA at the time of MCAO prevented the increase in estrogen concentration, suggesting that estrogen is synthesized locally. Infusion of letrozole into the PBN did not prevent the initial rise in estrogen post-MCAO. This suggests that the change in estrogen concentration in the PBN may be due to release of estrogen, rather than an increase of synthesis. Following immunohistochemical analysis for the aromatase protein, it was determined that there was no significant change in aromatase enzyme expression in the cell bodies of the PBN or CNA following four hours of ischemic insult when compared with the sham treatments. Perhaps a larger sample size could have demonstrated significant changes. However, the presence of the aromatase enzyme demonstrate that estrogen is synthesized in these brain areas. These results also suggest that an increase in endogenous estrogen concentration in some autonomic regulatory nuclei may be mediated through an increase in aromatase enzyme activity rather than an increase in enzyme expression. Overall, it can be concluded that, in some brain regions, a local response to ischemic damage may occur through local release or increased synthesis of estrogen.

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AF- activation function
AI - agranular insular cortex
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANS - autonomic nervous system
AP - arterial pressure
apoE - apolipoprotein E
ATP - adenosine triphosphate
BRS - baroreceptor reflex sensitivity
cAMP- cyclic 3',5'-andenosine monophosphate
CeC - capsular part of the CNA
CeL - lateral division of the CNA
CeM - medial division of the CNA
CNA - central nucleus of the amygdala
CNS - central nervous system
CREB - cAMP-response-element binding protein
CSF - cerebrospinal fluid
CVD - cardiovascular disease
CVLM - caudoventrolateral medulla
DAB - diaminobenzidine
DI - dysgranular insular cortex
DNA - deoxyribonucleic acid
ECG - electrocardiogram
ER - estrogen receptor
ERα - estrogen receptor alpha
ERβ - estrogen receptor beta
ERE - estrogen response element
FAD - flavin adenine dinucleotide
FMN - flavin mononucleotide
GABA - gamma-aminobutyric acid
GAS - gamma activation site
GI - granular insular cortex
GRE - glucocorticoid response element
HR - heart rate
HRP - horseradish peroxidase
hsp - heat shock protein
IC - insular cortex
KF - Kölliker-Fuse nucleus
LBD - ligand binding domain
LHA - lateral hypothalamic area
LPBC - central part of the lateral PBN
LPBCr -crescent part of the lateral PBN
LPBD - dorsal part of the lateral PBN

LPBI - internal part of the lateral PBN
LPBS - superior part of the lateral PBN
LPBV - ventral part of the lateral PBN
MAP - mean arterial pressure
MAPK - mitogen activated protein kinase
MCA - middle cerebral artery
MCAO - middle cerebral artery occlusion
MPB - medial PBN
MPBE - external part of the medial PBN
NADPH - nicotinamide adenine dinucleotide phosphate
NF-κB - nuclear factor κB
NGF - nerve growth factor
NTS - nucleus tractus solitarius
OVX - ovariectomized
PBN - parabrachial nucleus
PI3K - phosphoinositide-3 kinase
PKA - protein kinase A
PKC - protein kinase C
PKG - protein kinase G
RVLM - rostroventrolateral medulla
Scp - superior cerebellar peduncle
SNS - sympathetic nervous system
TIA - transient ischemic attack
TNFα - tumour necrosis factor α
TTC - 2,3,5-triphenoltetrazolium chloride
VPLpc - ventroposterior lateral parvicellular thalamic nucleus
VPMpc - ventroposterior medial parvicellular thalamic nucleus

Chapter 1. Introduction

1.1 Cardiovascular disease

Cardiovascular diseases are injuries and diseases of the cardiovascular system: the heart, the blood vessels of the heart, the peripheral venous and arterial system of the body and the venous and arterial system of the brain (83).

Cardiovascular disease (CVD) accounts for the death of more Canadians than any other disease. In 1999 alone, CVD accounted for 78,942 Canadian deaths, (83) of those, 54% were due to coronary artery disease, 20% to stroke, and 16% to other forms of heart disease (83).

1.2 Cerebrovascular disease (Stroke)

Cerebrovascular disease (stroke) accounts for 20% of all cardiovascular deaths in Canada (83). In 1997, strokes accounted for 7% of all deaths that occurred in Canada (207), and, in 2002, strokes became the 3rd leading cause of death in Canada (83;207) and the USA (9). The high mortality and morbidity rate associated with stroke cost the Canadian economy an estimated 1.8 billion dollars in 1994 (83).

Stroke can be described as a sudden or gradual disruption of blood flow to certain areas of the brain causing cerebrovascular brain injury or various neurologic dysfunctions (27). This interruption of perfusion to the brain results in acute hypoxia. Short term hypoxia may result in reversible neurologic deficits, while long-lasting hypoxia may lead to permanent neurologic deficits and cerebral infarction (27).

There are three types of strokes: occlusive or ischemic, transient ischemic attack, and hemorrhagic(27). Hemorrhagic strokes account for 16% of all strokes (27;83), and occur when a blood vessel around or in the brain ruptures. The resulting hemorrhage within the subarachnoid or the brain (intracerebral) (27;73) compresses the local brain tissue (73) and disrupts the tissue microenvironment (67). When blood comes into direct contact with brain tissue through loss of integrity of the blood brain barrier, it results in the subsequent loss of cellular homeostasis (67).

The majority of ischemic or occlusive strokes occur when arterial blood flow is blocked (73). This is often caused by artherosclerotic plaques that activate the clotting cascade, resulting in the development of a clot and subsequent blockage within one or more of the arteries supplying the brain (73). Some blood clots may form elsewhere in the peripheral blood stream and then travel to the brain via arteries (27). The ischemic stroke accounts for 84% of all strokes (27). Similar to the occlusive stroke, the transient ischemic attack (TIA) occurs when there is a transient interruption of blood flow in the brain (27). TIAs usually result in minor and reversible damage, but are often warnings of a potential ischemic stroke (27).

The carotid and cerebral arteries can become blocked resulting in ischemic stroke. The middle cerebral artery (MCA) (figure 1) by far is the most commonly occluded vessel (27). The MCA supplies a region of the prefrontal cortex involved in somatovisceral regulation known as the insular cortex (IC) (figure 1) (116). Once the vessel becomes blocked there is a reduction in perfusion of the insular cortex. This results in the

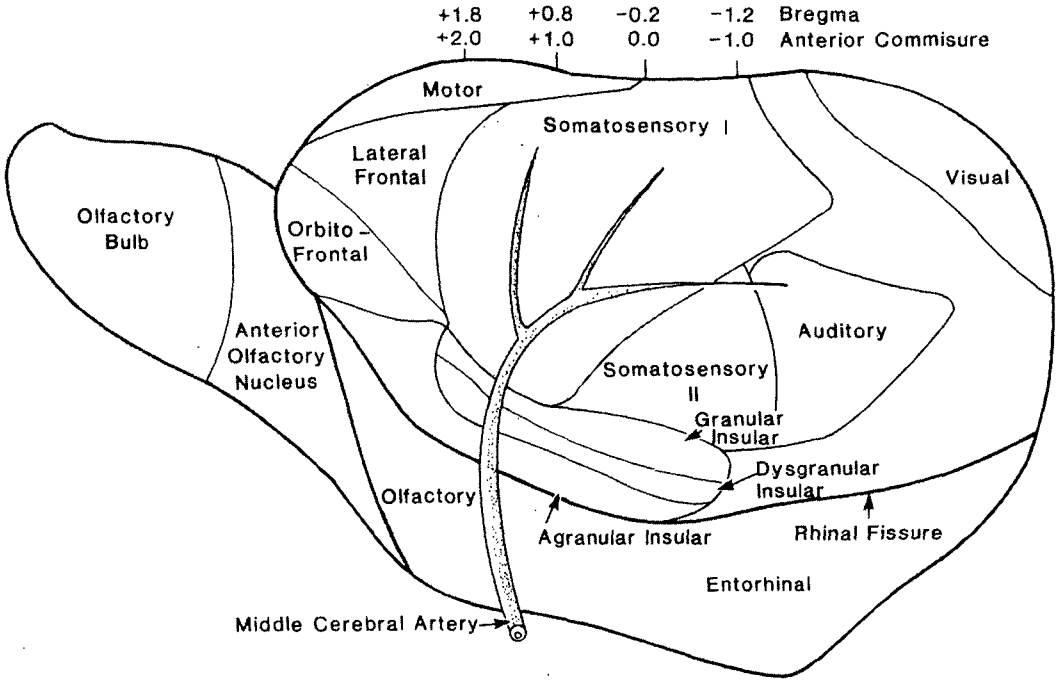


Figure 1. Lateral view of the rat forebrain to illustrate the position of the middle cerebral artery and the visceral sensory cortex and other cortical regions. The distances in millimeters at the top of the drawing are given with respect to bregma according to the atlas of Paxinos and Watson (modified from (174)).

development of three regions with varying degrees of perfusion: the core, the penumbra, and the peripheral zone (211).

In the ischemic core region or infarcted area, perfusion is almost non-existent. This area receives less than 15% of normal blood flow (114). The molecular and cellular effects of ischemia are outlined in figure 2 (27). Within the first 10 seconds of occlusion all oxygen in the cells of this region is depleted, which results in rapid anoxia (27). Four minutes following occlusion, glucose is depleted and anaerobic metabolism begins (27). Within four to six minutes, mitochondria can no longer produce ATP. As a result of anaerobic glucose metabolism, lactic acid and free fatty acids begin to build up in the cell (27). At this point, the cells in the core region are irreversibly damaged (211). Ionic gradients within the cell are disrupted resulting in influx of sodium, chloride, calcium, and water. Initially, the disruption of ionic gradients leads to repeated depolarizations in the core region. The further disruption of ionic gradients leads to the permanent depolarization and death of cells in the infarct region (27).

The penumbra region is a moderately hypoperfused region where blood flow is slightly greater than 40% of normal (211). Since there is some perfusion of this region, cellular ATP does not become depleted to the same degree as found in the core region (27). ATP concentration is maintained at lower levels and results in a cellular influx of sodium, chloride, calcium, and water (27). The disruption of ionic gradients leads to the depolarization of cells in this region (27). In addition, the increase in extracellular potassium concentration is related to the calcium-dependent exocytotic release of neurotransmitters from depolarized nerve terminals (98).

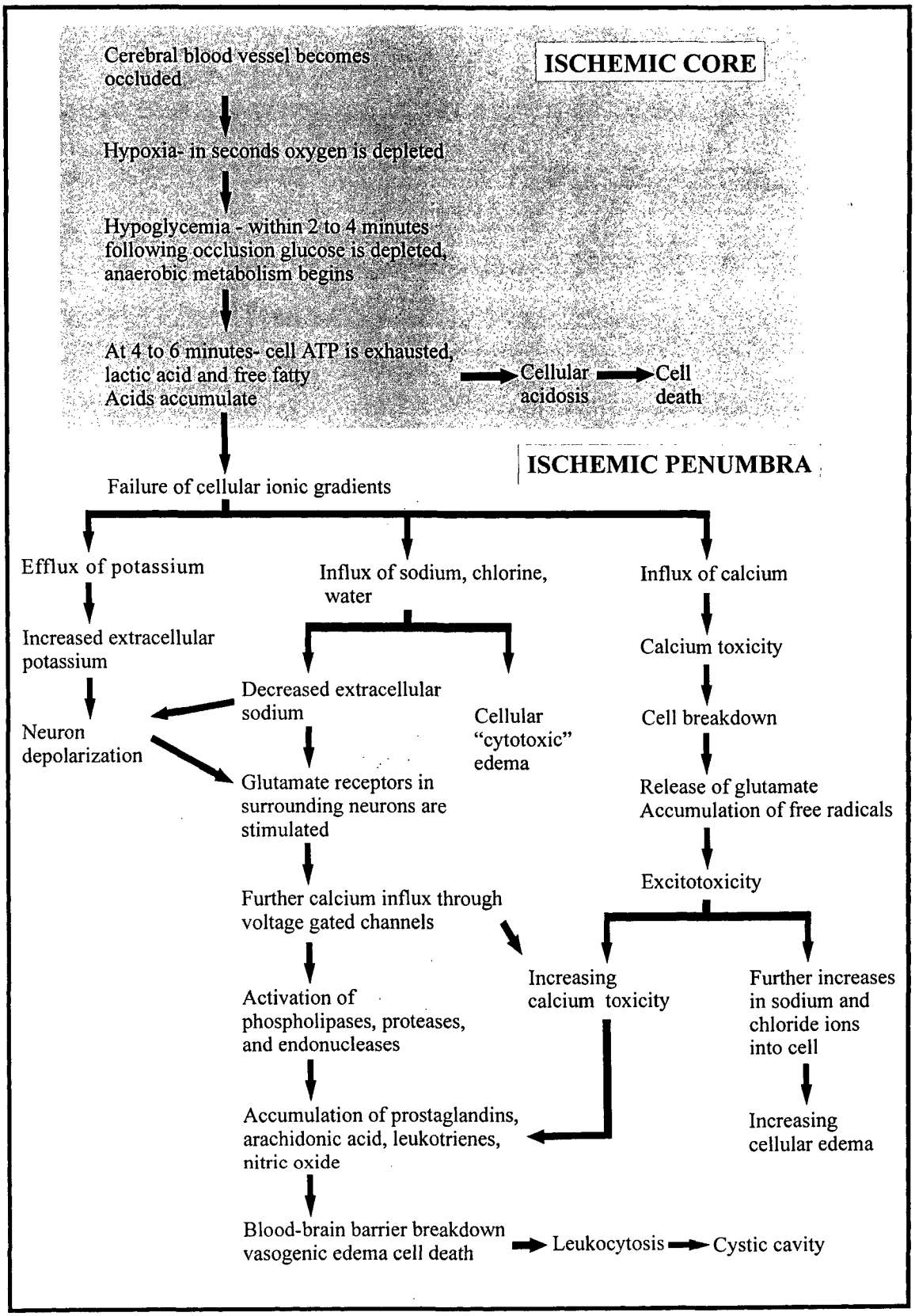


Figure 2. Molecular and cellular effects of ischemia (modified from (27)).

Upon depolarization, glutamate is released from neuronal terminals, but the loss of the transmembrane gradient prevents the uptake of glutamate through the sodium reuptake system in neurons and glia (27). Some of the glutamate released into the penumbra region may be from the dying neurons in the adjacent core region (27). Accumulation of glutamate within the penumbra region activates ionotropic glutamate receptors of the surrounding neurons to allow calcium and sodium into the cell (27). This results in sporadic transient depolarizations, and even more calcium influx through voltage-gated calcium channels (27).

Excessive intracellular calcium causes severe excitotoxic cell damage via activation of phospholipases, proteases, ATPases, and endonucleases (figure 2) (27). Free radicals are also associated with hypoxic neuronal damage and breakdown. These free radicals, produced primarily in the mitochondria, also increase intracellular calcium levels by damaging the ATP-dependent calcium transporter proteins involved in calcium regulation. Increased levels of intracellular calcium stimulates the further production of free radicals and build up of nitric oxide, also contributing to cellular break down (27). Typically, cellular injury within the penumbra region results in the retention of structural integrity but a loss of cellular function (211). This region can become infarcted if increased perfusion to this region does not occur (211).

The peripheral region is an area with slightly reduced perfusion (compared to normal perfusion) surrounding the penumbra (211). The cells of this region are less likely to die as a result of ischemia due to maintenance of blood flow.

The infarct begins in the core region, but reaches maximum size (including core and penumbra) between six and 24 hours following the onset of ischemia (211). The volume

of the infarct continues to increase an additional 25% between 12 and 24 hours, and grows an additional 30% between 24 and 72 hours (211). Each depolarization produced by the ischemic core leads to an approximate 20% increase in total infarct volume during the first 3 hours following occlusion. Since the IC is responsible for regulating autonomic tone within the body, the repeated depolarizations emanating from the growing infarct within the IC may cause an imbalance in the autonomic nervous system (ANS). The ANS provides innervation for the endocrine and exocrine glands, the viscera, and smooth muscles in all organs of the body (96). The ANS has two divisions: the sympathetic (SNS) and parasympathetic (PNS) systems (96). The SNS often mediates the response of the body to stress: it speeds up heart rate, increases blood pressure, and mobilizes the body's energy stores for energy (96). In contrast, the PNS acts to conserve the body's resources and restore homeostasis: it slows the heart rate, reduces blood pressure, and prepares the body for maintenance and rest (96). An imbalance in the ANS following a cerebrovascular event in the region of the IC may contribute to the cardiovascular pathology through altering the bodies ability to react to changes in blood pressure and heart rate.

1.2.1 Cardiovascular consequences of stroke

Unfortunately, ischemic heart and cerebrovascular diseases frequently coexist in the same patient (148). Any electrocardiogram (ECG) changes following stroke could therefore be caused by exacerbation of coincident coronary artery disease (148). Dimant and Grob (48) found that patients with acute cerebrovascular accidents (ischemic stroke, intracerebral and subarachnoid hemorrhage) had a 7- to 10-fold higher incidence of ST depression and

prolongation of the QT interval when compared with control patients (48). Both T wave inversion and ventricular premature beats were four times more common in the acute stroke group (48). This indicates an association between stroke and ECG changes, however, ischemic heart disease was three times more prevalent in the stroke group than among controls.

In contrast, Lavy and colleagues (110) specifically attempted to determine if the ECG changes observed following stroke were the result of cardiac disease (110). Out of 52 consecutive patients with acute ischemic stroke or intracerebral hemorrhage, no history of heart disease and a normal recent recording, 25 had no evidence of previous cardiovascular disease. From those 25 patients, 18 suffered an ischemic stroke, and 8 (44%) showed either a recent onset ischemia-pattern in their ECG or a cardiac arrhythmia (110). Similarly, out of the seven patients with hemorrhagic stroke and those with no evidence of previous cardiac disease, five demonstrated new onset cardiac arrhythmias.

Goldstein (68) attempted to control for the effects accompanying coronary artery disease. Within 24 hours of admission, a 12-lead ECG recording was performed on 53 acute stroke patients (subarachnoid and intracerebral hemorrhage, and ischemic stroke) while in the emergency room. Abnormal prolongation of the QT interval not observed in previous recordings was evident in 32% of the stroke group and in 2% of the controls (68). New T wave inversions were apparent in 15% of the stroke patients, and abnormal U waves in 13%; neither appeared as a new feature in the admission ECGs of the control group (68).

Direct evidence confirms that ECG changes may occur even in the presence of normal coronary arteries and in the absence of acute myocardial ischemic changes. In one

study of 29 patients who developed ECG changes following subarachnoid hemorrhage (47), 8 patients died, 5 of whom were autopsied following death (47). None of the autopsy patients showed evidence of coronary artery disease or myocardial infarction. Similar findings were noted by Shuster (195) and Tobias and colleagues (214). In a series of acute cerebral infarctions, confirmed on autopsy, none of the patients who died and who had ECG abnormalities showed evidence of coronary occlusion (57). Connor (46) also reported a patient who died following stroke without autopsy evidence of coronary stenosis.

The ECG effects described above indicate changes in ventricular depolarization and may be associated with the tendency to develop cardiac arrhythmias, some of a ventricular nature (148). The most commonly observed arrhythmia following a cerebrovascular event is atrial fibrillation, occurring at a frequency of 9% (68). Post-admission, recent-onset atrial fibrillation was not found in the control group. The incidence of ventricular arrhythmias in intracerebral hemorrhage patients with no history of heart disease was 10% (235). Atrial fibrillation was the most common arrhythmia (21%), and ventricular arrhythmias occurred at a frequency of 13% in patients with acute cerebrovascular accident (48). For the control group, the figures were 2% for atrial fibrillation and 3% for ventricular arrhythmias (48). Unfortunately, there was no allowance made for preexisting heart disease. More recently, it was found that acute thromboembolic stroke in the IC resulted in the occurrence of a pathological nighttime blood pressure increase that was also associated with a brief increase in serum norepinephrine concentrations (179). This indicates that sympathetic activation occurs following acute stroke involving the insular cortex (179). This will be discussed in more mechanistic detail in chapter 4.

The ECG abnormalities induced by acute stroke are often short lived, resolving with little residual effects over a period of days to months following cerebrovascular accidents (57;195). The hypothesis was proposed that if stroke-induced ECG changes result in an increased incidence of cardiac arrhythmias, then the prognosis should be adversely affected by their presence (148). In Goldstein's study (68), a mortality of 80% was seen in those patients demonstrating malignant ventricular arrhythmias (tachycardia, fibrillation, asystole), compared with a mortality rate of 23% in stroke patients not showing these changes. Similarly, Lavy and colleagues (110) found a mortality of 69% in patients with ischemic or intracerebral hemorrhagic stroke showing recent-onset ST segment or T wave changes, or arrhythmias on admission. This compared with a mortality of 0% in stroke patients without ECG changes (110). It has also been observed that following admission to the hospital, approximately 6% of stroke patients died suddenly and unexpectedly over the ensuing month (196). Myers and colleagues (136) noted elevated plasma norepinephrine concentrations in the stroke group was consistent with an increase in peripheral sympathetic activity. The increase in peripheral sympathetic activity could be responsible for the cardiac abnormalities associated with cerebral infarction. The evidence presented so far indicates that those stroke patients developing changes in their ECGs may be at risk for sudden death and should be monitored until these changes regress (148).

1.3 Effects of gender on stroke outcome

Each year more men than women under the age of 45 die as a result of stroke (1). After the age of 45 more women than men die as a result of stroke annually (1). This

gender-associated difference in stroke-induced death has been noted as a risk factor for stroke. There are several other stroke risk factors; age, family history, race, smoking, lack of exercise, high blood pressure, diet, obesity, diabetes mellitus, and stress (27;83). The gender differences in stroke outcome suggest that women are protected from stroke prior to the age 45 (73). Women go through menopause around the age of 45 and this is associated with a reduction in estrogen producing follicles in the ovaries and a subsequent reduction in circulating estrogen (73). This suggests that estrogen may act as a protective measure and/or therapy against CVD and in particular, stroke.

1.3.1 Neuroprotection and gender study

Animal studies have investigated the neuroprotective abilities of various hormones, including estrogen, progesterone and testosterone. In cats progesterone treatment did not reduce brain injury after vascular occlusion (133). Systemically circulating testosterone increased lesion size following ischemic brain injury in male rats (82). However, Toung and colleagues (217) found that loss of testosterone did not affect stroke-induced lesion size. Unlike testosterone and progesterone, estrogen has been proven to reduce stroke-induced cell death in animal models of stroke. Estrogen administered prior to middle cerebral artery occlusion (MCAO), either intravenously or via subcutaneous implants, reduces infarct size by approximately 50% (82;162;172;217).

Studies comparing gender for neuroprotection against ischemic insult show that the size of the MCAO-induced lesion is correlated with gender. Male and ovariectomized female (OVX) rats developed larger lesions than intact female rats (6). A temporary decline

in circulating estrogen levels during the normal cycling of female rats was associated with a decreased resistance to cerebral ischemia (113). The decline in circulating estrogen levels in the normal cycling of women is associated with increased sympathetic tone and increased arrhythmias (134). In contrast, elevated systemic estrogen concentrations have also been associated with a reduction in sympathetic tone and a reduced occurrence of arrhythmias in cycling women (134). This supports the suggestion that a lower systemic estrogen levels in men and postmenopausal women (as simulated by ovariectomized rats), compared to premenopausal women, increases risk of brain injury and cardiovascular dysfunction following stroke. Not only has pretreatment with estrogen been proven to protect the brain from ischemic insult, but when estrogen is administered intravenously (171) either immediately before MCAO to intact males or up to 40 - 90 minutes after MCAO in OVX rats, lesion size decreases from 45 to 90% (199). Estrogen can provide therapeutic effects when administered after MCAO induced ischemia in OVX rats, intact females (236), and in male rats (171). This may indicate a protective role of estrogen against stroke-induced autonomic dysfunction. However, local injection of estrogen into the IC reduced infarct size in rats, but did not prevent the stroke-induced cardiovascular dysfunction (172). This suggests that the stroke-induced cardiovascular dysfunction observed following MCAO is not correlated with infarct size. It also points out the possibility that other subcortical nuclei along the autonomic pathway may be mediating the cardiovascular dysfunction that is initiated following infarction of the IC. Exactly how estrogen mediates neuroprotection is still up for debate. Many feel that estrogen may mediate its neuroprotective effects through an estrogen receptor (51;52;177;230).

1.4 Estrogen

1.4.1 Estrogen receptors

Estrogen receptors (ER) are a part of the steroid hormone nuclear receptor superfamily, which has approximately 150 members (105). This family of receptors includes those for androgens, glucocorticoids, mineralocorticoids, and progestins (17;115).

The classical ER is a phosphoprotein that becomes hyperphosphorylated by its ligand. There are at least two known subtypes; ERα and ERβ. The receptors are about 47% identical (54) and share approximately 55% amino acid sequence homology (231). The ERα gene has been mapped to chromosome 6, whereas the ERβ gene is located on chromosome 14 in humans (70). The functional ER exists as either a homodimer or heterodimer (figure 3). Each ER has a DNA binding domain (C) and ligand binding domain (E) (figure 4 and 5). The C domain is also involved in dimerization (70). The E domain is also involved in dimerization and interacts with heat shock proteins (71). The A/B region and the E/F regions contain activation function domains (AF-1 and AF-2, respectively) that are responsible for activating gene transcription following binding of the ligand (20). There are substantial differences in the N-terminal of the A/B domain between the ER subtypes (156) that possibly lead to differences in binding affinities for different ligands (70). The binding of a structurally unique ligands can alter the tertiary structure of the ER and subsequently reposition the AF-2 domain (22). Repositioning of the AF-2 domain is associated with gene transcription and may affect (156) binding of coactivators (22). This could explain how estrogen receptors are able to initiate many different responses,

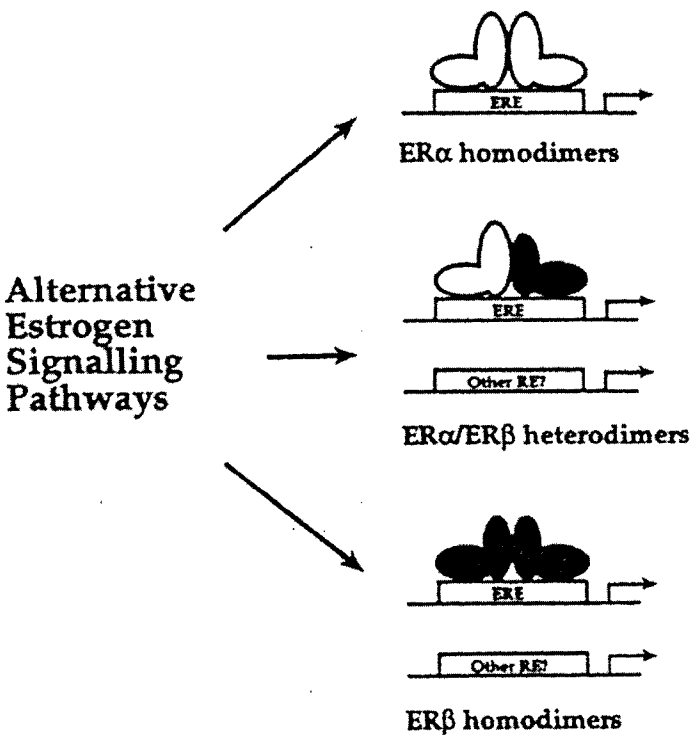


Figure 3. Alternative ER-medaited estrogen signalling pathways. The existence of two ER subtype proteins and their ability to form DNA-binding heterodimers suggest the existenceof three potential pathways of ER-mediated estrogen signalling. In cells ezpressing only ERα or Erβ protein, homodimers of either subtype interact with response elements in target gene promoters and influence transcription levels. Cells expressing both ER subtype proteins, heterodimers can be formed depending on the ratio of ER subtype proteins (113).

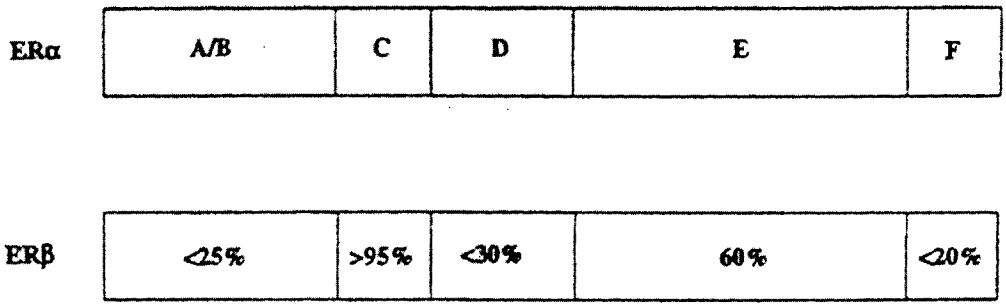


Figure 4. Comparison between ERα and ERβ amino acid sequence homology (113).

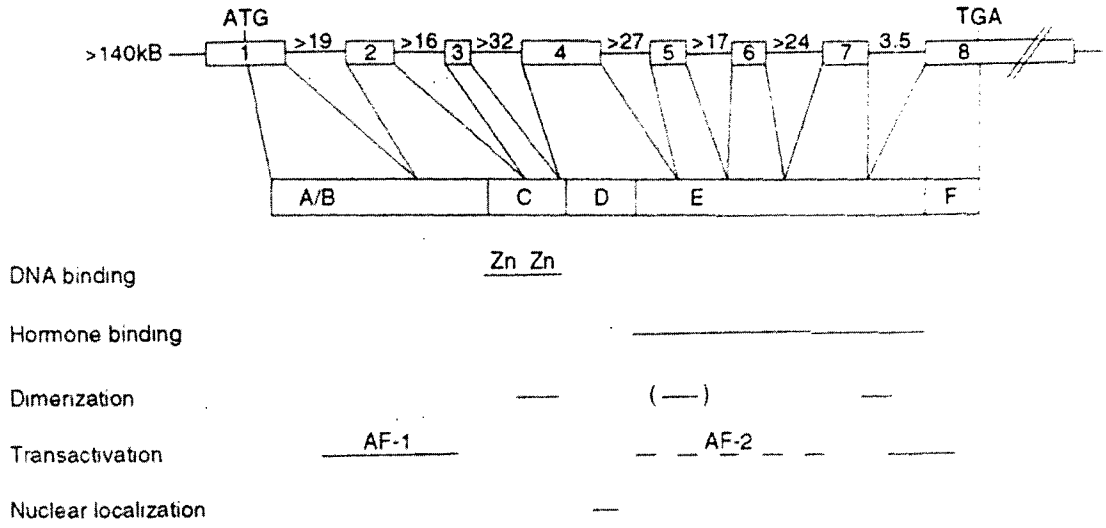


Figure 5. Schematic diagram of the genomic organization, domain structure, and functional characteristics of the estrogen receptor. The ER gene spans over 140 kb of DNA. The DNA-binding domain (C) is composed of two zinc fingers. The ligand binding domain is found in the E region. Two dimerization domains on the ER have been detected in domains C and E. The hinge domain (D) codes for nuclear localization signal. Two transactivation domains in the A/B and E/F regions act independently and confer on the ER both promoter and target organ specificity (162).

especially in different tissues. Finally, the hinge domain (D) codes for nuclear localization, and separates the C and E domain (156).

The genomic mechanism of ER action is considered the classical mode of action (figure 6). When an estrogen molecule diffuses into the cell, it binds to the E domain of the ER, which results in dissociation of the heat shock protein (chaperone) from the receptor and receptor dimerization (70). The liganded ER dimer then binds to specific sequences of DNA called estrogen-response elements (EREs), and also bind to nuclear-receptor coactivators or repressors (70) resulting in initiation or prevention of transcription (figure 6).

The exact location of ERs within the cell is not entirely clear. They may be in an equilibrium distribution between the nucleus and the cytoplasm; this equilibrium is then shifted upon ligand binding (70). The traditional estrogen-signaling pathway involving nuclear interaction takes minutes to hours to increase protein synthesis by transcription activation (70).

Estrogens have other more immediate effects that cannot be explained by a transcription mechanism (70). Estrogen may mediate these rapid actions through novel cell-membrane bound forms of the ER, that are similar to ERα (37;44;130;141;142). Although these receptors remain largely uncharacterized, they are thought to resemble their intracellular counterparts (221). This localization is consistent with the role of ERα-like ER as a transducer of rapid, nongenomic estrogen responses in neurons (44). Recently, both ERα and β have been shown to rapidly (within 15 minutes) activate the mitogen-activated protein kinase (MAPK) signaling pathway (220). The application of 17β-estradiol rapidly

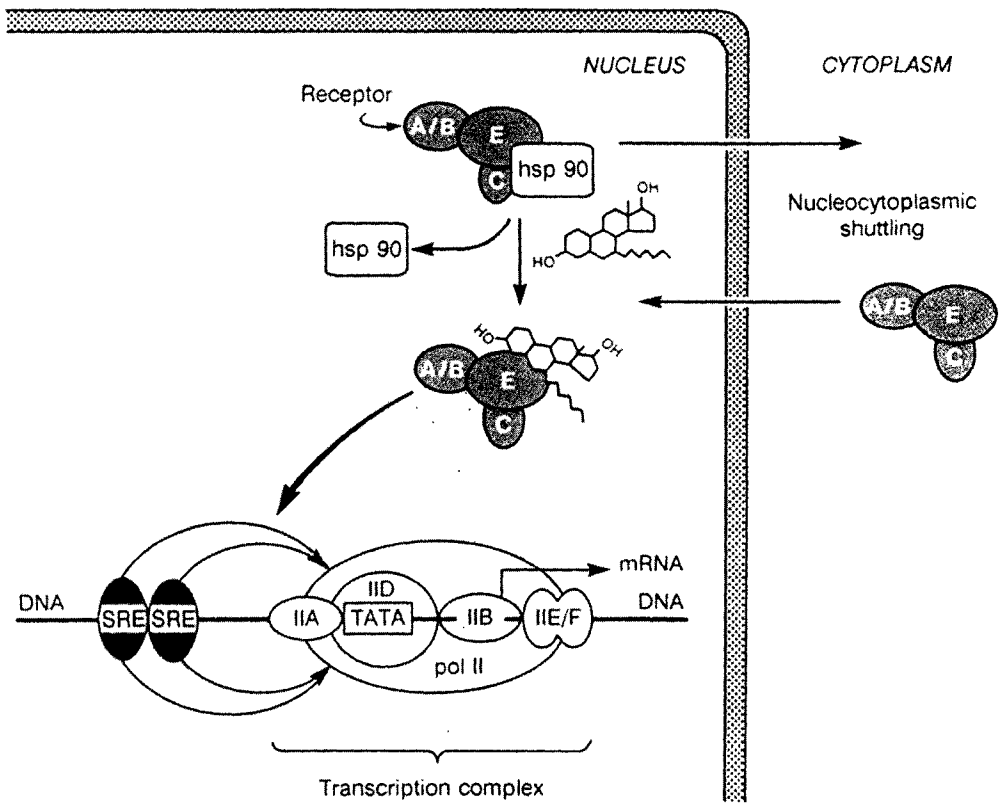


Figure 6. Schematic diagram of estrogen receptor action. Although the ER is predominantly found in the nucleus, it consistently shuttles between the nucleus and the cytoplasmic compartments. After the ER binds to the ligand, it then dissociates from the inactive hsp 90-ER complex and forms a homodimer or heterodimer (ER). The liganded ER dimer then seeks out estrogen-specific hormone response elements (SRE), and forms a DNA-receptor complex that stabilizes the formation of a transcription complex (TC). The TC comprises RNA polymerase II (pol II), transcription factors IIA, IIB, IID, and IIE/F, and a TATA box, all of which are located on the promoter gene that will be transcribed. The A/B, C, and E shown on the receptor refer to the domains of the ER (162).

increases kainate-induced currents of CA1 neurons as a result of a cyclic adenosine monophosphate (cAMP)-dependent phosphorylation (130). To initiate this potentiation, estrogen is required on both sides of the plasma membrane (130). Extracellularly, estrogen appears to activate a G- protein-coupled receptor, whereas the intracellular action of estrogen appears to be a modulation of the balance between phosphorylation and dephosphorylation (130). Unlike neocortical ERα, which is intranuclear and developmentally regulated, and neocortical ERβ, which is intracellular and expressed throughout life, the functional plasma membrane-associated receptor concentrates in caveolar-like microdomains of postnatal mouse neocortical and uterine plasma membrane (216). Association with plasma membrane positions the novel ER uniquely to interact rapidly with second messenger signaling mechanisms (216). This evidence further supports the theory that estrogen's rapid actions may be through a non-genomic mechanism mediated via a membrane-bound receptor.

1.4.2 Distribution of estrogen receptors in the CNS

ERs and ER mRNA have been found throughout the CNS in the rat (194;194). Significant ER mRNA labeling has been found in many of the nuclei responsible for autonomic cardiovascular regulation, such as the rat parabrachial nucleus (PBN), nucleus tractus solitarius (NTS) of the rat (191;197) and the golden hamster (23), and the rat central nucleus of the amygdala (CNA) and cerebral cortex (191;243). Many ER positive cells have been detected in sheep lateral PBN and are involved in regulation of cardiovascular parameters (187) and also in cells of the CNA and NTS of mice (5). The different ER

subtypes are not only distributed throughout the CNS, but they are also differentially distributed within a variety of nuclei.

ERα has been localized in the CNA and NTS of mice (5), as well as the amygdala in humans (49) and in rats (11;62;125;192). Recently, it has been shown that ERα is the predominant ER in cholinergic neurons of the rat basal forebrain, with only a few cells containing ERβ (192). The distributions of ERα and ERβ largely overlap (112;112;191;191). Embryonic and adult rat neural stem cells express both ERα and ERβ (25). However, recent observations reveal that ERα is expressed in the cerebral cortex early in development of female rats, ERα and ERβ are co-expressed at later developmental times, and only ERβ is expressed in the cerebral cortex of adult rats (243). In other cases ERα and ERβ are expressed in the same cells in some regions (193).

ERα have been observed in astrocyte-like cells in the brain of humans (49) and male rats (62). ERα-immunoreactivity was also affiliated with the plasma membrane of select interneurons and with endosomes of a subset of principal (pyramidal and granule) cells (5). Moreover, ERα immunolabeling was localized in nuclei of the pyramidal cells of adult male rats (11). ERβ was observed in the perikarya, apical dendrites, and nuclei of pyramidal neurons (11). Furthermore, ERβ-immunoreactivity was observed in astrocytes but seem to be localized in astrocyte processes and perikarya and, in some cases, in astrocyte nuclei of male and female rats (11). This evidence does not confirm the location of the ERs as being nuclear or membrane bound. The presence of ERs in many brain regions and in many cell types suggests that estrogen may play an important role in different regulatory CNS functions.

1.4.3 Regulation of estrogen receptors in the CNS

ER mRNA expression changes with varying estrogen concentrations in the brain due to change in ovarian production of endogenous estrogen. Shughrue and colleagues (190) demonstrated that ER mRNA levels are sexually dimorphic, vary during the estrus cycle, and increase after ovariectomy. Chakraborty and colleagues (41) found a regional specificity to estrogen sensitivity in the rat brain. The magnitude and direction of change in ER mRNA concentrations observed during the estrous cycle are also region-specific and it is suggested that factors other than endogenous estrogen levels differentially modulate ER mRNA expression in the hypothalamus (190). Perhaps this is true for other brain regions. An apparent down regulation of the ERα subtype occurring in conjunction with an up-regulation of the ERβ subtype in the parabrachial nucleus (PBN) was observed during the seven days following bilateral ovariectomy of the female rat (175). Peripheral estrogen concentrations may be responsible for the differential regulation of estrogen receptor gene expression within the PBN (175).

The discovery of alternative ERα promoter expression in distinct neuronal populations suggests that multiple promoter usage is a possible mechanism to achieve differentiated regulation of ERα expression, dependent on the cell phenotype and consequently the functions mediated by the specific cell (152). Similarly, it has been suggested that gonadal steroid hormones may regulate ERβ protein in male mice and ERα may be involved in the expression and regulation of ERβ in a region- specific manner (140).

1.4.4 The function of ERα vs ERβ in neuroprotection

According to Wise and colleagues (229), ERβ may act via a genomic mechanism by activating EREs and subsequently inducing the transcription and translation of proteins. On the other hand, it is speculated that ERα is mostly involved in cross-talk with second messenger systems (230), possibly through the existence of an ER-containing, multimeric complex consisting of hsp90, *src*, and B-Raf (216). However, activation of either ERα or ERβ has been shown to result in the activation of the MAPK pathway (126).

The role of different ER subtypes in neuroprotective mechanisms is not clear. The deletion of ERα completely abolishes the protective actions of estrogen in experimental brain ischemia in mice; whereas the ability of estrogen to protect against brain injury was totally preserved in the absence of ERβ (52) This suggests that ERα may mediate neuroprotection of the cerebral cortex following ischemia (52). ERβ mRNA in the cortex decreases by middle-age in estradiol treated ovariectomized female rats (227). Pretreatment with 17β-estradiol prevents MCAO-induced or injury-induced downregulation of ERβ mRNA (228). Dubal and colleagues (51) also found that both ERβ and ERα were expressed in normal and ischemic brains of rats, but estradiol increased the ratio of ERβ to ERα after cerebral ischemia. This suggests a role for ERβ in mediating estrogens neuroprotective mechanism following ischemia. Some of these neuroprotective mechanisms are discussed in section 1.4.5 and section 1.4.6.

1.4.5 Nuclear/cytoplasmic ERs mediating genomic effects of estrogen in the CNS

Genomic mechanism of estrogen action in the brain result in long-term effects such as the regulation of nerve cell development and protection of the brain during aging (122), which helps to preserve memory and other cognitive functions (234). McEwen and Woolley (123) noted that cyclic fluctuations in gonadal hormones are associated with cyclic changes in performance on a variety of cognitive and motor tasks in human subjects. Similarly, there is an association between serum estrogen level and ischemic neuroprotection (113).

The genomic neuroprotective actions of estrogen promote axonal sprouting (218), neurogenesis (25), synaptogenesis (24;111;123), expression of neurotrophic factors such as nerve growth factor, modulate the activity of cholinergic neurons (194), and increase expression of bcl-2 (81;139). Estrogen has also been shown to increase production of apolipoprotein E (apoE) in both astrocytes and microglia (212). ApoE is believed to be involved in neurogeneration and remyelination.

Estrogen may also promote gene transcription by cross-talk with intracellular signaling mechanisms such as the cAMP pathway via CREB (222), or promote neuronal differentiation, survival, and plasticity via rapid interaction with kinases of the MAPK cascade and other signaling pathways (216). Estrogen interaction with the MAPK pathway has been suggested in neuronal cells (126;203) and this cross-talk may be mediated as a result of signal transduction by G-proteins, kinases or the redox-sensitive transcription factor nuclear factor-κB (NF-κB) (61;210). Therefore, the estrogen-ER complex may not be required to bind directly with DNA to mediate genomic mechanisms of action (52).

1.4.6 Non-genomic actions of estrogen in the CNS

Estrogen has also been shown to have non-genomic mechanisms of action in the CNS. Estrogen has direct antioxidant effects (223;233;242), and vasodilatory effects (84;198). The short-term vasodilatory effects are mediated through triggering a rapid endothelial nitric oxide release via a MAPK-dependent mechanism perhaps through interaction with a membrane bound receptor (198). In addition, evidence has been found supporting estrogen's neuroprotective effects during glutamate toxicity (43;81;204). It has been proposed that estrogen produces a rapid, transient activation of *src*-family tyrosine kinases and tyrosine phosphorylation of p21(ras)-guanine nucleotide activating protein resulting in activation of the MAPK pathway following glutamate toxicity (204). Intravenous injection of estrogen immediately before MCAO-induced ischemia provides protection that is equivalent to that of chronic estrogen treatments over time (217). This suggests that both non-genomic and genomic mechanisms may not be required for immediate neuroprotective effects of estrogen (217).

1.5 Estrogen biosynthesis and aromatase

1.5.1 Aromatase

Estrogens are C18 steroid hormones, which circulate predominantly bound to plasma albumin and specific estrogen-binding-globulin (figure 7) (70;73). In addition to estradiol, other natural steroidal estrogens include estrone, estriol and their conjugates. The enzyme

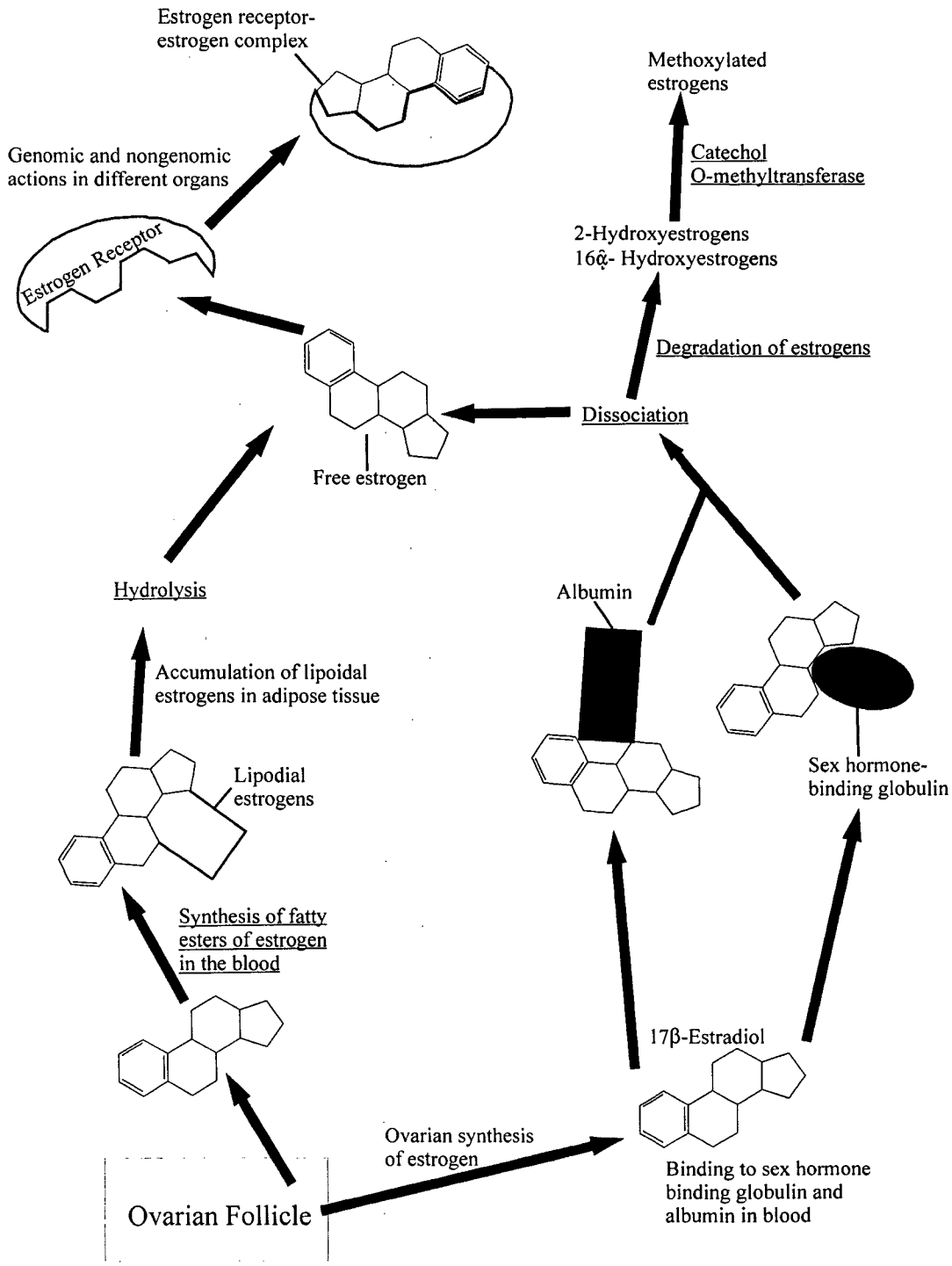


Figure 7. Ovarian synthesis, transport, and metabolism of estrogens. Following synthesis, mainly in the ovary, 17β-estradiol is secreted into target tissues to exert a specific genomic or nongenomic effects. Lipoidal estrogens are synthesized in the blood and in other tissues but accumulate predominantly in fat. Enzymatic catabolism of estrogens yields the hydroxyestrogens and methoxyestrogens (76).

aromatase (cytochrome P450-19 or CYP19) catalyzes the biosynthesis of estrogen from androgens (C19 steroid hormones) (138).

Aromatase belongs to a superfamily of heme-containing enzymes that are responsible for the oxidative metabolism of prostaglandins, fatty acids, biogenic amines, plant metabolites and steroids, as well as chemical carcinogens, mutagens, many drugs and other environmental pollutants.

CYP19 coupled with cytochrome 17-oxidoreductase enzyme mediates the aromatization of testosterone (a C19 hormone) creating estrogen (201). CYP19 is localized in the smooth endoplasmic reticulum of various tissues of both males and females (70;201) and is found in many species from invertebrates to mammals.

1.5.2 Regulation of aromatase expression

In humans, a number of tissues have the capacity to express aromatase and synthesize estrogens. These include the ovaries and testes, the placenta, and fetal (but not adult) liver, adult adipose tissue, chondrocytes and osteoblasts of bone, vascular smooth muscle, and numerous sites in the brain, including several areas of the hypothalamus, limbic system, and cerebral cortex (200).

The regulation of aromatase expression occurs through the use of tissue-specific promoters and by alterative splicing mechanisms, which regulate the transcription of a single sequence encoding human aromatase (45).

A brain-specific promoter (If) (89) is the major promoter-specific transcript found in the rat amygdala (182), although promoter II-specific transcripts and I.4-specific

transcripts have also been detected in the rat brain (182). This indicates that different promoters are employed in the various brain loci of expression and that consequently the regulation differs widely in these particular brain sites (200).

Regulation of aromatase in the brain appears to be increased by androgens and either suppressed or not affected by cAMP (2). Aromatase expression was elevated by a number of stimuli including α_1-adrenergic receptor agonists; substance P, cholecystokinin, neurotensin, brain natiuretic peptide, as well as phorbol esters and dibutyryl cGMP (3). This suggests a major role of protein kinase C (PKC) and protein kinase G (PKG) pathways in this regulation, which is presumably mediated via the brain-specific promoter (3). The inhibition of aromatase activity is blocked by kinase inhibitors, which supports the impression that phosphorylation processes are involved (14). It is not known whether estrogens regulate aromatase synthesis directly or by affecting different inputs to aromatase producing cells (14). Aromatase activity in hypothalamic homogenates is rapidly decreased by exposure to conditions (addition of Ca^{2+}, Mg^{2+}, ATP) that enhance protein phosphorylation (14). The local production and availability of estrogens in the brain can therefore be rapidly changed by Ca^{2+} based on variation in neurotransmitter activity (14).

1.5.3 Systemic estrogen production

Natural estrogens are secreted principally by the granulosa cells in ovarian follicles and by the adrenals, corpus luteum, and placenta in women, and by the testes in men (138).

The regulation of estrogen production in the female, which is similar to that of the male, consists of hypothalamic gonadotropin releasing hormone stimulating the release of

follicle-stimulating hormone and lutenizing hormone from the anterior pituitary, which stimulate the secretion of estrogen from the ovaries. All these hormones are regulated through a complex feedback system (73). The physiological function of estrogen in females include development of secondary sexual characteristics, regulation of gonadotropin secretion for ovulation, preparation of tissues for progesterone response, maintenance of bone mass, regulation of lipoprotein synthesis, prevention of urogenital atrophy, regulation of insulin responsiveness, and maintenance of cognitive function (30). Serum concentrations of estrogens in the menstrual cycle in normal women is shown in table 1. During pregnancy, the placenta becomes the main supply of estrogens (73). During menopause, ovarian secretion of estrogen declines at varying rates (73). In young adult men, estrogen is essential for fusion of epiphyses and maintenance of bone mass (31;129;206).

Estrogens are synthesized in several different tissues throughout the body. Localized expression of aromatase in vascular smooth muscle cells suggests local synthesis of estrogen (80;132). Aromatization of testosterone by skeletal muscle indicates a possible additional source of extraglandular estrogen for men and women (121). Aromatase is expressed in the ovary (145;200), placenta (151), skin (201), adipose tissue (59;183;189) and human bone tissue where it may play important roles in the maintenance of bone tissue (183;189).

In men and in postmenopausal women, aromatization of C19 steroids in adipose and skin is the primary mechanism for estrogen formation (69;118). In men, it has been estimated that testicular steroidogenesis accounts for 15% of the circulating levels of estrogen (85). Aromatase protein is localized in human spermatozoa (10), while aromatase

Table 1. Production rates and serum concentrations of estrogens in the menstrual cycle in premenopusal and postmenopausal women

Phase	17β-Estradiol		Estrone		Estriol	
	Serum Conc.	Daily Production	Serum Conc.	Daily Production	Serum Conc.	Daily Production
	pg/ml	μg	pg/ml	μg	pg/ml	μg
Follicular	40-200	60-150	30-100	50-100	37690	37794
Preovulatory	25-500	200-400	50-200	200-350	-	-
Luteal	100-150	150-300	50-115	120-250	37787	37984
Premenstrual	40-50	50-70	15-40	30-60	-	-
Postmenopausal	<20	5-25	15-80	30-80	37690	37762

Data from Baird and Fraser (12) and Flood et al., (58) (modified from (70)).

mRNA can be detected in Leydig, Sertoli and germ cells of the rat testis (108). Aromatase protein and mRNA have also been isolated in epithelial and stromal cells of the normal human breast (26). In the placenta, the principle substrate 16α-hydroxyandrostenedione is produced by the fetal adrenal and liver (138). In ovarian granulosa cells the substrate testosterone is derived primarily from the adjacent theca cells (201). In all of these sites of estrogen production, the aromatase substrate (C19 steroid precursor) is synthesized in a cell type different from that expressing aromatase (138).

The extragonadal sites of estrogen biosynthesis are dependent on circulating precursor C19 steroids for estrogen biosynthesis (202). Although they have the capacity to convert C19 steroids to C18 steroids, some of these extragonadal tissues lack the ability to synthesize C19 (202), with the exception of the brain (244). The estrogen synthesized within bone, breast, and brain is probably only biologically active at a local tissue level in a paracrine or autocrine fashion (107). Thus, the total amount of estrogen synthesized by these extragonadal sites may be small, but local tissue concentrations achieved are probably quite high, and exert significant biological influence locally (202). Since these tissues are largely dependent on circulating precursor hormones for the local production of estrogen, then the local tissue levels may be dramatically different between men and women throughout life and especially later in life.

Compared with women, men maintain a high circulating level of testosterone throughout life (table 2), which is available for conversion to the active estrogen (estradiol)

Table 2. Plasma steroid levels in men and in postmenopausal women.

Steroid	Women (pg/ml)	Men (pg/ml)
Testosterone	144.1	3461.1
Estrone	27.0	35.2
Estradiol	10.9	27.2
Dehydroepiandrosterone	4326.4	288.3
Dehydroepiandrosterone Sulfate	921218.0	736974.4

(modified from (224))

in extragonadal sites (202). The average circulating testosterone levels of men are 2884.3 to 8652.9 pg/ml whereas postmenopausal women have circulating testosterone levels of 173.1 pg/ml (202). Plasma estrogen levels in postmenopausal women (~6.8 - 35.4 pg/ml) are in the range of adult men (202). The uninterrupted supply of testosterone in men throughout life supports local production of estradiol by aromatization of testosterone in estrogen-dependent tissues, and thus affords ongoing physiological activity. Estrogen concentrations vary from tissue to tissue. Schleicher and colleagues (184) showed peak estrogen levels of 16 ± 3.4 ng/ml in the plasma following intravenous estrogen administration however estrogen levels in the liver, kidney, heart and brain exceeded plasma levels. Perhaps this indicates the importance of local estrogen production to the maintenance of these tissues.

1.5.4 Production of estrogen in the CNS

The presence of aromatase is of potential importance in the brain for aromatizing androgens, such as testosterone, into estrogen. In the brain, estrogen synthesis appears to be confined to certain cell types. For example, 17β-estradiol was isolated in embryonic rat neural stem cells (25) and in astrocytes and neurons from the cerebral cortex of neonatal rats (244). Astrocytes appear to be the most active steroidogenic (244). Other cell types such as oligodendrocytes produce pregnenolone, progesterone, and androstenedione but not estrogen (244). It has been suggested that the brain is capable of neurosteroidogenesis via a tripartite contribution astrocytes, oligodendrocytes and neurons not only in the production of estrogen, but also the parent hormones of estrogen (244).

Astrocytes in the amygdaloid complex do not normally express aromatase however enzyme expression is induced in the glial cells by different forms of brain injury suggesting a role for local astroglial estrogen formation in brain repair (64). Thus, potent neuroprotective effects induced by testosterone may be mediated by 17β-estradiol following local aromatization of testosterone, and not through direct activation of the androgen receptor. It is difficult to link the slow regulation of estrogen synthesis that occurs via changes in aromatase concentration with swift actions of estrogen observed at the membrane level (15). Even if fast transduction mechanisms are available, this will not result in rapid changes in brain function if the availability of estrogen does not also change rapidly (15). Given that aromatase is present in presynaptic boutons, it is possible that rapidly changing levels of locally produced estrogen are available for non-genomic
regulation of neuronal physiology in a manner more analogous to the action of a neuropeptide than previously speculated (15).

Estrogen biosynthesis in the brain is dependent on circulating C-19 precusor steroids (202). Postmenopausal women have low circulating testosterone levels due to a lack of functioning ovaries (202) and therefore have significantly lower testosterone levels in their brains than their premenoupausal counterparts (21).

In the brain, estrogen has been detected in the cerebrospinal fluid (185) and in several other regions (123;137;244) such as the cerebral cortex (244) and the amygdala (137). Similarly, aromatase has also been detected in the cerebral cortex (208;244), and in other brains areas (208;232). Perhaps estrogen can be synthesized in one brain region and subsequently released in another via neuronal projections. As discussed earlier in (section

1.3.1), estrogen has been shown to prevent autonomic dysfunction following ischemic insult to the IC, but not at the level of the IC. This means that estrogen could be potentially released in other nuclei, downstream from the IC in the central autonomic network, to prevent the cardiovascular dysfunction associated with ischemic insult to the insular cortex.

1.5.5 Estrogen metabolism

Total clearance of 17β-estradiol from different tissues varies. The plasma clearance is 154 ml/min/kg in the female rat, with a half-life of approximately 26 minutes, while estrogen levels in the kidney, heart and brain decay roughly with the same rates as in the plasma (184). However, in the liver the half life of estrogen was 2.6 hours, much longer than in other organs (184).

Estrogens are metabolized by glucuronidation, and the conjugates are excreted into the bile or urine (70). Hydrolysis of the conjugates by intestinal flora and subsequent reabsorption of the estrogen results in enterohepatic circulation (70). Estrogens are also metabolized by hydroxylation and subsequent methylation to form catechol and methoxy-estrogens (143;151).

1.6 Central autonomic network

1.6.1 Insular cortex - Its role in autonomic regulation

The IC is responsible for the regulation of visceral functions such as cardiovascular, gastrointestinal, and respiratory. These different regulatory functions appear to be topographically organized into different regions of the IC.

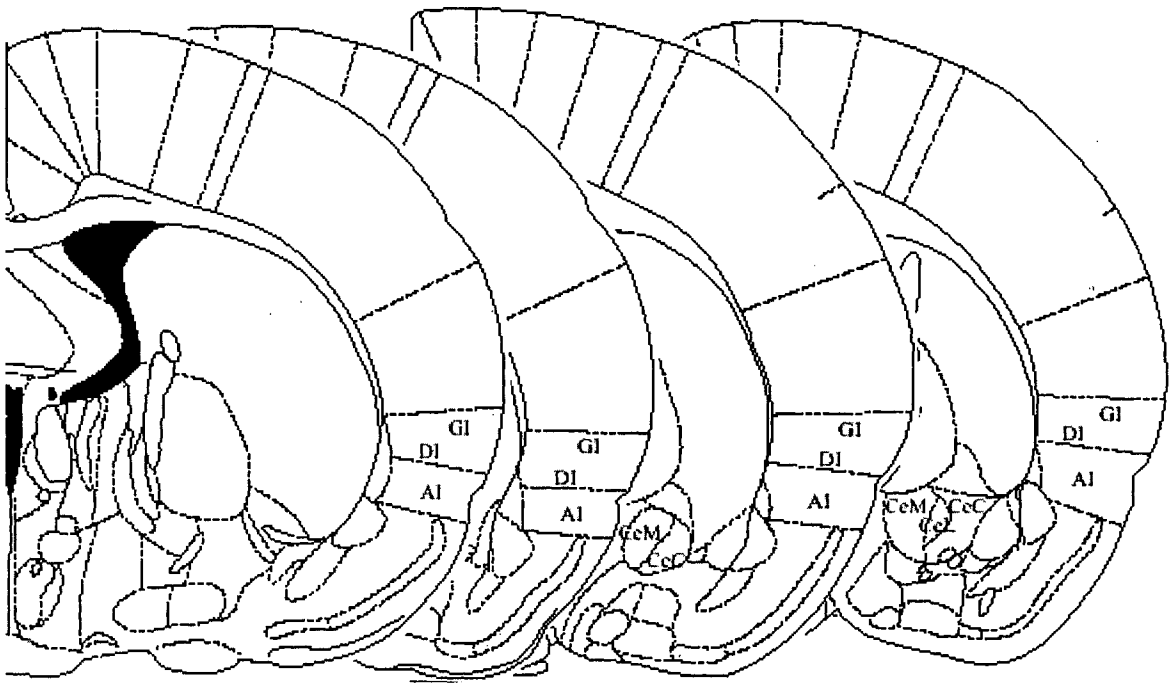
Certain regions of the IC have been shown to have autonomic regulatory functions, such as the agranular, granular (gastric mechanoreceptor-responsive units situated more dorsal and rostral, cardiopulmonary and respiratory inputs ventral and caudal) and dysgranular (taste cortex in the rostral half) regions (figures 1 and 8) (38;116).

Different regions of the IC mediate changes in blood pressure and heart rate when stimulated, and stimulation of the left and right IC produce different cardiovascular responses. In the rat, stimulation of the left IC induces a depressor response and bradycardia, while right IC stimulation produces tachycardia and a pressor response (240). This indicates that the two insulae may communicate with one another to integrate and balance cardiovascular function between the two hemispheres in rats (241).

Individual areas within the right IC have been shown to produce tachycardia and a pressor response, while an adjacent area within the right IC has been shown to produce a depressor response and bradycardia when electrically stimulated. These areas are called cardiac chronotropic sites and they were identified primarily in the granular region of the rat IC (146). The rostral posterior granular IC produces tachycardia upon electrical stimulation, while the caudal posterior region of the granular IC produces bradycardia (146). This demonstrates the complexity of this region in cardiovascular and ANS regulation.

1.6.1.1 Insular cortex - efferent connections

Pressor sites in the IC project efferent connections bilaterally to the CNA (237), and an area overlapping in the terminal field of the aortic baroreceptor nerve (161). The IC has also been shown to have direct efferent projections to the PBN (116;181;188). The efferent



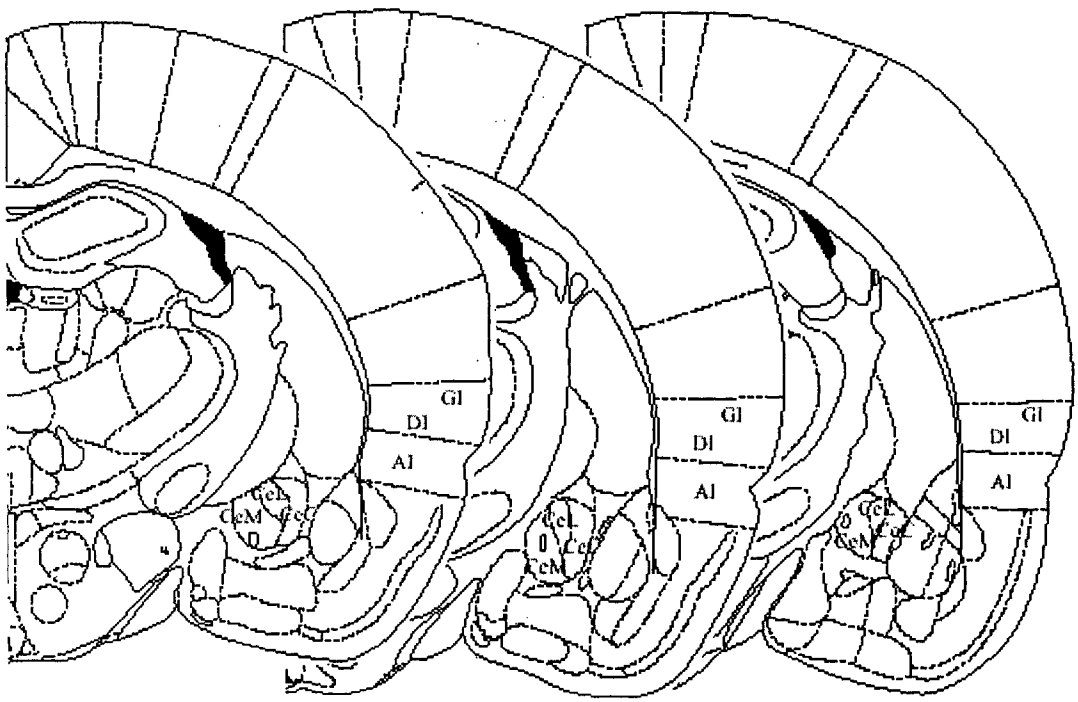


Figure 8: Insular cortex and subnuclei at levels -0.80 to -2.56 caudal to bregma. Abbreviations: AI=agranular insular cortex; DI=dysgranular insular cortex; GI=granular insular cortex; CeC=capsular part of the CNA; CeL=lateral division of the CNA; CeM=medial division of the CNA (modified from Paxinos and Watson (154)).

projections of the IC are shown in figure 9. Similarly, cardiovascular depressor sites of the IC have efferent connections with the (ipsilateral) CNA, and (bilateral) PBN, similar to that described for the cardiovascular pressor sites (237).

1.6.1.2 Insular cortex - afferent connections

The major afferent connections of the IC involved in autonomic regulation are shown in figure 9. The stellate ganglion (which regulates the heart) also has projections to the IC via extension to the lateral hypothalamic area (LHA) and CNA (225). This suggests that the heart has afferent connections to the IC via the CNA or LHA.

1.6.1.3 Cardiovascular regulatory function of the insular cortex

The IC plays a significant role in the autonomic regulation of blood pressure and heart rate (161). Electrical stimulation of the rat IC elicits cardiovascular responses such as changes in blood pressure and heart rate (161). Selective chemical lesions of the right IC results in a long-term elevation in mean arterial pressure as early as four hours post-injection (28). Also, elevation in sympathetic nervous system (SNS) tone following IC stimulation results in increasing degrees of heart block due to the development of an imbalance in the ANS (150). An increased degree of heart block is associated with increased plasma norepinephrine levels (indicator of increased sympathetic tone) after one hour of microstimulation of the granular IC (150). This stimulation-induced increase in SNS tone leads to escape rhythms, ventricular ectopies and ultimately asystole in rats (150).

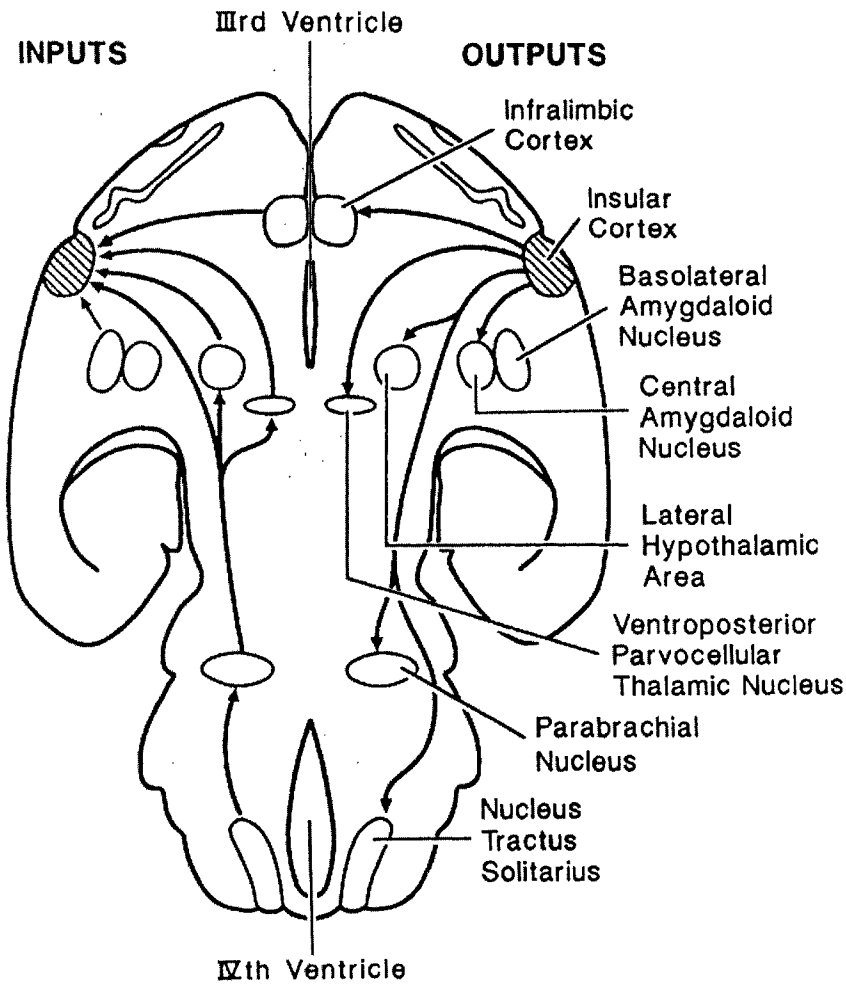


Figure 9. A schematic diagram of a horizontal section through a rat brain, illustrating the afferent (left) and efferent (right) connections of the insular cortex with subcortical autonomic regulatory nuclei (116).

1.6.1.4 Cardiovascular consequences of stroke, involving the insular cortex, in humans

Similar to rats, humans appear to have dominance of the right IC for IC-induced sympathetic effects. Electrical stimulation of the right IC more frequently produces a pressor response and tachycardia (147). Inactivation of the right hemisphere decreased heart rates, and vice versa when the left hemisphere was inactivated in humans. This indicates that the right IC primarily increases sympathetic tone, while the left IC increases parasympathetic tone in humans (239). Therefore, in human stroke patients an imbalance of the autonomic nervous system, or SNS activation can trigger malignant arrhythmias (186) and myocardial cell damage (135).

Similar to animal studies, increased plasma norepinephrine levels are associated with decreased baroreceptor reflex sensitivity (BRS) in humans (160). Impairment of BRS is significantly greater in stroke patients than in controls, and patients with right-sided strokes may have elevated sympathetic output (159). Decreased BRS is associated with a greater risk of cardiac mortality in human patients (106), similar to that observed in experimental stroke in rats. Some studies indicate that the IC may play a significant role in the pathogenesis of hypertension (178) and hypertension is more frequently found in stroke patients (144). In the clinical setting normotensive patients who experienced right-sided infarction exhibited an increased incidence of arrhythmias (144;178), myocardial complications after stroke (178). The hospital based study by Tokgözoglu and colleagues (215) found that patients with right MCAOs and insular lesions had depressed heart rate variability, or reduced BRS, when compared to controls. During this study, they observed

seven patients die suddenly and all seven patients had lesions involving the IC (five patients had lesions in the right insulae). Three of the seven patients that died suddenly were monitored prior to death, and it was found that all three patients developed ventricular tachycardia that deteriorated into ventricular fibrillation. This suggests that autonomic and cardiovascular changes may be responsible for the large number of stroke-induced deaths in the clinical setting (215). For a summary of the cardiovascular changes associated with either stroke, stimulation or lesion of the IC see table 3.

1.6.1.5 Experimental stroke studies in animals

Similar to the microstimulation studies, experimentally-induced stroke damage of the IC leads to a number of autonomic cardiovascular dysfunctions in different animal models, including increased sympathetic nerve activity (74) and a decline in BRS (159). Experimentally induced stroke results in a significant increase in MAP at 10, 60, and 90 minutes post-MCAO and a significant increase in heart rate of male rats at 10 minutes post-MCAO of the left common carotid artery when compared to the sham group (155). These cardiovascular consequences of experimentally-induced stroke are similar to those observed in stimulation studies in rats. Given these results it could be hypothesize that decreasing the stroke-induced damage to the IC could prevent the cardiovascular dysfunction and functional deficits associated with stroke. In ovariectomized female rats, intravenous estrogen injection significantly reduced sympathetic tone within 30 minutes and significantly increased parasympathetic tone within 5 minutes of estrogen administration (170). Both of these effects on autonomic tone were blocked by the prior administration of an ER antagonist, ICI

Table 3. Cardiovascular effects of stimulation vs. lesion vs. stroke involving the rat insular cortex.

Treatment	Hemisphere	MAP	HR	RSNA	Animal Model	Reference
Electrical Stimulation	Right	Increased	Increased		Rat	(161)
	Right	Increased	Increased		Rat	(240)
	Left	Decreased	Decreased		Rat	(240)
	Right - Pressor site	Increase	Increase		Rat	(146)
	Depressor site	Decrease	Decrease			(147)
	Right	Increase	Increase		Human	
Lesion	Right	Long-term Increase		Initial Increase (10 min)	Rat	(28)
	Right		Decrease		Human	(239)
	Left		Increase		Human	(239)
Artery Occlusion	Right			Increase	Rat	(159)
	Right	No change	No change		Rat	(172)
	Left	Increase	Increase		Rat	(155)

A blank space indicates that the variables was not measured.

182,780 (170). Only the estrogen-induced increase in parasympathetic tone was observed in male rats (164;170;175). This evidence indicates a role for estrogen in protecting against the autonomic dysfunction observed in the presence of an underlying cerebrovascular injury (175). Some studies have shown that neuroprotective agents such as isradipine,injected subcutaneously (158), or estrogen, injected intravenously or via subcutaneous implant (19;82;162;217) administered prior to MCAO reduces infarct size by 40% and ~ 50%, respectively. The induction of aromatase expression in astrocytes in regions of the brain, like the amygdaloid complex, in response to a variety of brain injuries (64) suggests there is a role for local astroglial estrogen formation in brain repair (63) and a possible neuroprotective mechanism activated by the brain in response to trauma. Local injection of estrogen into the IC reduces the size of the infarct that is developed following MCAO when compared with saline treated controls (figure 10), but does not prevent the stroke-induced cardiovascular dysfunction (172). Perhaps the stroke-induced cardiovascular dysfunction is not dependent on infarct size and other subcortical nuclei in the autonomic pathway may mediate the cardiovascular dysfunction observed following infarction in the IC.

1.6.2 Central nucleus of the amygdala - Its role in autonomic regulation

The CNA mediates changes in behavior (77), control of peripheral airways (76), cardiovascular (13;176), and gastrointestinal functions, and may be part of the nociceptive pathway (19). Some have suggested that the CNA is differentially involved in cardiovascular and behavioral responses (226). These different functions of the CNA may be controlled by regions within the CNA (119;219).

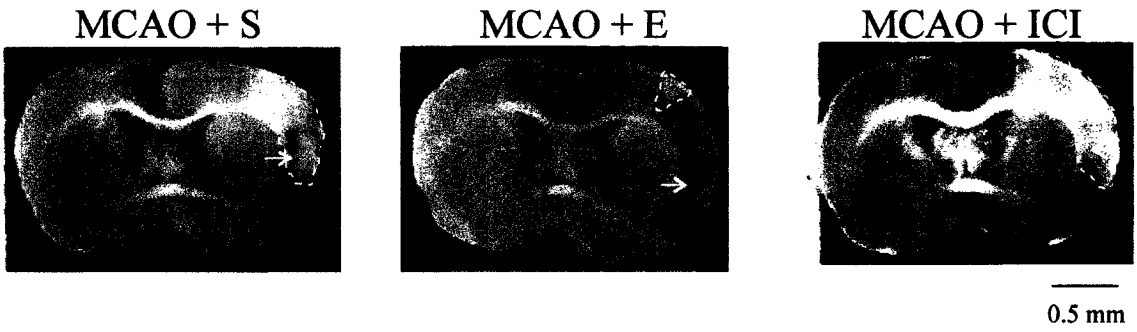


Figure 10. Digital photomicrograph illustrating representative examples of the extent of the infarct size within the insular cortex after MCAO and either saline, estrogen (30 minutes prior to MCAO), or ICI-182,780 (ICI) injections into the insular cortex. Infarct zones are outlined in each photomicrograph, and arrow indicates tip of injection cannulas in the region of the insular cortex (provided by T. Saleh;172).

The CNA is subdivided into four major regions (figure 8) the medial, lateral, lateral capsular, and the intermediate subdivision (117). In the rat, the detailed cytoarchitecture of the CNA likely reflects the functional integration of afferent neurons rather than the organization of the CNA output neurons, since peptidergic and afferent terminal distribution patterns are more restricted to individual cytoarchitecturally defined subregions of the CNA (33).

1.6.2.1 Central nucleus of the amygdala - efferent connections

The CNA sends efferent projections to many regions of the CNS (29;35;37;65;78;90;176;219). Some in particlar are the PBN (35;78;90;219), the NTS, and the dorsal motor nucleus of the vagus nerve (90;219). Saha and colleagues (163) recently provided evidence of a GABAergic pathway from the CNA to the NTS. This pathway may provide a substrate for inhibition of lower brainstem visceral reflexes, including baroreflex inhibition, through which the CNA could participate in cardiovascular regulation related to emotional behavior and the defense reaction (163).

1.6.2.2 Central nucleus of the amygdala - afferent connections

The CNA receives afferent information from many regions of the CNS (7;29;35;36;94;95;97;102;127;157). Some of particular concern are the ipsilateral PBN (36;94;97;127), ipsilateral NTS (102). It can be observed that some of these connections are reciprocal connections. It has been demonstrated that the CNA has a direct reciprocal connection with the IC (97;180;209;238), the PBN (35;90;94;97;127;213;219), and the NTS

(90;103;219). Since some of these nuclei have cardiovascular regulatory functions (i.e., IC and PBN), then it can be theorized that the CNA could participate in cardiovascular regulation via its anatomical connectivity with these other autonomic nuclei.

1.6.2.3 Central nucleus of the amygdala - cardiovascular regulatory function

It has been argued that the CNA has a strong influence on cardiovascular regulation. Bilateral lesions of the CNA prevent the development of hypertension induced by daily stress in rats (13). Efferent projections from the IC begin in pressor sites and project, bilaterally, to connections in the CNA, while depressors sites have efferent connections with only the ipsilateral CNA (237). Those connections between the IC and the CNA are reciprocal (97) and it has been proposed that the IC may participate in autonomic regulatory processes by way of its direct projections to the CNA (153).

Chemical stimulation of the CNA subsequently alters cardiovascular regulatory parameters such as induce a decrease of arterial blood pressure in the rat (176). Some research suggests that subnuclei within the CNA may mediate cardiovascular changes through interactions with other cardiovascular regulatory subnuclei such as the PBN (19;127;163) and estrogen may be involved in modulating the activity of the CNA or the CNA's effects on other nuclei (174). The CNA may mediate the ANS imbalance and subsequent stroke-induced cardiovascular dysfunction following ischemia within the IC. If this is true, ischemia in the IC may result in an increase of estrogen synthesis or release within the CNA in order to inhibit the development of cardiovascular dysfunction.

1.6.3 Parabrachial nucleus - Its role in autonomic regulation

The PBN is comprised of a group of neurons surrounding the superior cerebellar peduncle (Scp) in the rostral pons (figure 11), it participates in a variety of visceral regulatory functions that range from mediating the convergence of pain, chemosensory, and temperature (39) sensibilities with gustatory (88), cardiovascular (39) and respiratory systems (28;39;42;109;161;240). It has also been speculated that nociceptive, visceral, and gustatory signals may reach cortical and other forebrain sites via a PBN-thalamic pathway (101). The functional heterogeneity of the PBN is reflected in its anatomy (42;60;87).

Chemical stimulation (L-glutamate or electrical current pulses) of the PBN caused pressor-tachycardic responses that mapped to the outer edge of the external lateral subnucleus while depressor-bradycardic responses were elicited from stimuli near the dorsal lateral subnucleus (42). Extracellular recordings in anesthetized rats indicate the presence of cells within the lateral PBN, that increase and decrease their excitability following to baroreceptor activation (92). L-glutamate injection into the ventral and medial regions of the PBN in the cat also resulted in a significant pressor response (32). The locations of the sites giving the most potent responses implicate specific ascending and descending pathways as substrates for cardiovascular responses in the rat (42).

1.6.3.1 Parabrachial nucleus - efferent projections

The external lateral PBN (LPBE) sends projections to the external medial PBN (19), the lateral CNA (102), and the caudomedial CNA (19). Similar to the LPBE, the caudal two

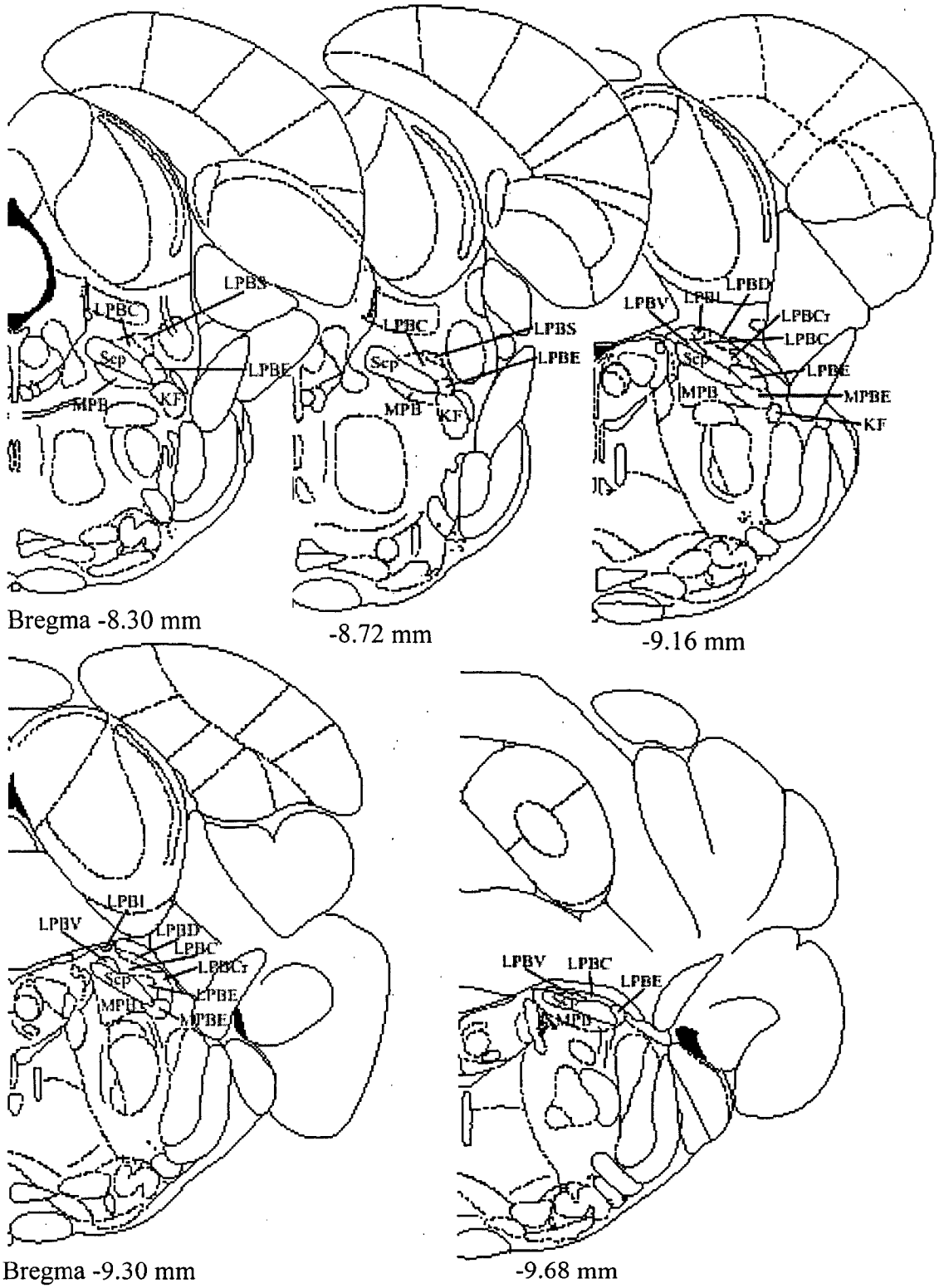


Figure 11: Parabrachial nucleus subnuclei shown at levels -8.80 to -9.68 mm caudal to bregma. Abbreviations: KF=Kolliker-Fuse nucleus; LPBC=central part of lateral PBN; LPBCr=cresent part of lateral PBN; LPBD=dorsal part of lateral PBN; LPBE=external part of lateral PBN; LPBI=internal part of the lateral PBN; LPBS=superior part of the lateral PBN; LPBV=ventral part of lateralPBN; MPB=medial PBN, MPBE= external part of medial PBN; Scp=superior cerebellar peduncle (modified from Paxinos and Watson (154)).

thirds of the external PBN mainly give rise to a dense projection to the caudomedial subdivision of the ipsilateral CNA (19).

The lateral PBN (LPB) sends efferent projections in the IC (8). Efferents from the medial PBN (MPB) project to the dysgranular cortex and other regions of the IC (8;102). The external medial (MPBE) subnuclei mainly project to the rostrolateral subdivision of the CNA (19). Corticopetal axons ran laterally from the LHA, through the ansa peduncularis, transversing the amygdala and ventral striatum to reach the external capsule and, predominantly ipsilateral IC (8). Descending projections from the MPB target the NTS; the MPB projects to the more rostral NTS (102).

1.6.3.2 Parabrachial nucleus - afferent projections

Afferent projections of the PBN, were observed in the amygdala, and a few cells were labeled in both the ipsilateral and contralateral IC (78). The PBN has also been shown to receive afferent projections from the NTS (e.g. lateral commissural area; previously shown to receive primary barosensory input) (79;87), and the vagus (66).

The LPB has been shown to project to the IC (8), the CNA (60;86;94;95). The LPB also receives afferent information from the NTS (the general visceral part of the NTS). Similarly, in the LPB there is a zone containing only spinal input, in the more dorsal regions. The zone in which termination from the NTS contains inputs only from neurons projecting to the amygdala (18).

The LPBE sends afferent projections to the external medial PBN (MPBE), and the caudomedial CNA (19). The LPBE also receives afferent input from the NTS (the general

visceral part of the NTS) (87). Similarly, central (CPB), dorsal (DPB), and dorsal lateral PBN (LPBD) also receives afferent projections from the NTS (87). Crossing projections from the medial NTS/dorsal motor nucleus of the vagus favor the LPBD in the cat (100). Milner et al. (124) determined that two distinct populations of afferents, which may originate from the caudal medial NTS, terminate in the ventrolateral quadrant of the PBN (LPBV) in the rat.

The caudal lateral part of MPBE is innervated by the medial NTS (87) and the spinal trigeminal nucleus (55). This region also sends afferent projections to the rostrolateral CNA (19). The medial parts of the PBN in the cat receive afferent innervation from medial areas of the medial NTS/dorsal motor nucleus of the vagus (100).

Shipley and Sanders (188) found that there are reciprocal connections between the IC and the PBN. Like the IC, the CNA has also been shown to send reciprocal projections from the CNA to the PBN (19;90;95;128;213). Supporting this finding, Jhamandas and colleagues (94) observed neurons in the CNA that were synaptically activated by electrical stimulation of the PBN. Other areas such as the NTS have reciprocal connections with the PBN (93;104).

1.6.3.3 Parabrachial nucleus - cardiovascular regulatory function

Several studies have provided evidence to support the fact that the PBN plays an important role in the organization of cardiovascular regulatory function by the brain (131). Electrical stimulation of the PBN increases arterial pressure in the cat (75) and in the rat (16;104;131) and increases heart rate (131) in the rat. The increase in both arterial blood

pressure and heart rate is the result of inhibition of the heart rate component of the baroreflex and/or an increase in cardiac sympathetic drive (109). It was concluded that the PBN is a necessary component of the baroreflex pathway in the cat (72), and a powerful cardiovascular response pattern is organized within intrinsic neurons of this nucleus (131). The most excitable sites were concentrated within the intermediate one-third of the PBN in its medial and lateral subdivisions in both the rat and the cat (92;131). Results from a vagal stimulation study suggest that estrogen is released into the PBN in response to an increase in visceral afferent traffic (175) and has the ability to inhibit neurotransmission within the PBN following local administration (174). Estrogen, therefore, could act to inhibit the increase in sympathetic tone at the level of the PBN, following ischemic damage to the IC.

1.6.3.4 Parabrachial nucleus - influence over other cardiovascular regulatory nuclei

The PBN plays a major role in mediating ascending and descending cardiovascular control signals. This nuclei projects to and/or appears to influence other brain regions (16;37;56;79;93;102;103;168) that receive barosensory input (37;102;103), modulate heart rate (like the CNA) (79;168) and blood pressure (102).

1.7 Hypothesis and objectives

From the body of literature reviewed above it is evident that female hormones may act as a protective measure and/or therapy against stroke. Local injection of estrogen into the IC results in reduced infarct size compared with saline treated controls, but does not prevent the stroke-induced cardiovascular dysfunction (172). This suggests that subcortical

nuclei along the autonomic pathway may be mediating the cardiovascular dysfunction that is initiated following hypoxic insult to the IC. The presence of ERs in subcortical nuclei indicates that estrogen's role in preventing ischemia-induced cardiovascular dysfunction may involve effects on these nuclei. What is not known is the source of the estrogen that acts as a neuroprotective agent; is it supplied by peripheral sources or through local endogenous synthesis? Aromatase expression is induced in the hippocampus and in amygdaloid complex in response to a variety of brain injuries (64). This indicates that estrogen synthesis or release could be increased in some autonomic regulatory nuclei in response to ischemia in the IC. Changes in endogenous estrogen concentrations within cardiovascular regulatory nuclei (IC, CNA, and PBN) in response to ischemic insult to the IC are yet to be determined.

Therefore, taken together, this evidence has led us to **hypothesize that local endogenous estrogen production in the central nervous system (CNS) is elevated in response to MCAO**. To provide support for this hypothesis, the objectives of the work presented in this thesis were:

1) To determine if peripheral circulating plasma estrogen concentrations increase in response to MCAO,

2) To determine if estrogen concentration increases both at the site of the infarct as well as in subcortical autonomic nuclei (PBN and CNA), following MCAO,

3) To determine if local inhibition of aromatase prevents the rise in CNS estrogen following MCAO in these identified sites,

4) To determine if those nuclei in which an increased synthesis of local estrogen is observed following MCAO also demonstrate an increase in aromatase protein levels.

Chapter 2. Materials and methods

All experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee (protocol No. 00-023).

2.1 General surgical procedures

Male Sprague-Dawley rats (Charles River; Montreal, PQ, Canada) between 250 - 275 g, were obtained seven days prior to surgical procedures. The animals were group housed, supplied with food and water *ad libitum*, and given seven days to become acclimatized to their environment. After the acclimation period, each animal was anaesthetized with sodium thiobutabarbital (Inactin; Research Biochemicals International, Natick, MA; 100 mg/kg; ip). When the rat had reached a surgical plane of anaesthesia, an incision was made in the right loin of the rat, exposing the right femoral artery. A 20 cm length of polyethylene tubing (PE-50; Clay Adams, Parsippany, NJ) was connected to another 20 cm length of tubing via a metal shaft from a 23-gauge hypodermic needle (Precision Glide, Becton Dickinson and Co.; Fisher Scientific, Nepean, Ont., Canada). The jointed polyethylene catheter was connected to a pressure transducer (Gould P-23 ID; Cleveland, OH, USA) containing heparinized (1:1000; Sigma-Aldrich, St. Louis, MO, USA) physiological saline (0.9%). The bevelled tip was inserted into the right femoral artery. The mean arterial blood pressure was measured using a pressure transducer, which was connected to a Gould model 2200S polygraph. The heart rate was determined from the pulse pressure using a Gould tachograph

(Biotach, Cleveland, OH, USA). A digital rectal thermometer (Fisher Scientific, Nepean, Ontario, Canada) was employed to measure body temperature. A heated circulatory feedback water blanket system was used to maintain body temperatures at 37°C during all experimental periods (actual recorded body temperatures were 36.4°C ± 0.04°C). A tracheotomy was performed and a tube placed into the trachea. The animals were then placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA), in the prone position, and secured firmly in place.

2.2 Microdialysis probe efficiency (recovery)

The dialysis probe was needle-shaped, with a stainless steel shaft (14 mm long, 0.38 mm outer diameter, 1 μl internal volume) and a semi-permeable cuprophane membrane (2 mm long, 0.24 mm outside diameter, molecular weight cutoff at 6000 daltons; CMA, Quebec, Canada). It has been previously determined in our lab that the microdialysis probe had an average recovery efficiency of approximately 23.0 ± 8.0% (169), similar to previous studies showing a recovery of 20% (120;243).

It should be noted that estrogen concentrations obtained using the dialysis probe technique do not provide true quantifications of estrogen concentrations in the extracellular fluid in the various brain nuclei. In addition, the estrogen concentrations obtained are an accumulation of estrogen in dialysate, via diffusion, over a set period of time period. However, the change in estrogen concentrations obtained using the microdialysis probe technique reflects estrogen concentration change during the set time period. True quantitative values can only be obtained using proper calculations for necessary conversions.

The concentrations obtained in this experiment have not undergone any conversions and should not be interpreted as actual quantitative estrogen measurements, but rather the values should be used only to indicate change in extracellular estrogen levels in each autonomic regulatory nuclei, in a more qualitative manner.

2.3 Microdialysis

Following placement in the stereotaxic frame, a 2.5 cm rostro-caudal incision was made down the midline of the rat's skull, exposing bregma (± 0.0 mm). Bregma is located at the intersection suture line dividing the left and right frontal and parietal bone and that suture line separating the frontal bone from the parietal bone. A microdialysis probe, mounted on a micro manipulator, was centered over bregma and its coordinates recorded. The coordinates for the PBN, CNA, and IC were obtained from a stereotaxic atlas of the rat brain (154). The microdialysis probe was moved these coordinates, using bregma as the reference point, to indicate where the burr hole was to be drilled. For the PBN, a small burr hole was drilled (- 9.0 mm posterior to bregma, ± 1.8 mm lateral to bregma) using a hand held drill in the left parietal bone. In the case of the IC and CNA, the right frontalis and temporalis muscles were dissected away to expose the squamosal bone. Again, using a hand held drill, a small burr hole was made in the rostro-dorsal region of the squamosal bone (IC infarct; + 0.8 mm posterior to bregma, ± 5.7 mm lateral to bregma; IC peri-infarct; + 0.7 mm posterior to bregma, ± 2.5 mm lateral to bregma; CNA; -1.4 mm posterior to bregma, ± 4.2 mm lateral to bregma). When the burr hole was completed, a 301/2-gauge hypodermic needle (Precision Glide; Becton Dickinson and Co., Fisher Scientific) was used to cut and

remove the dura mater covering the brain to permit stereotaxic insertion of the microdialysis probe. The dialysis probe was connected to a syringe pump (Harvard Apparatus, Saint Laurent, Quebec, Canada) via PE-50 in-flow tubing, 30 cm in length. A 20 cm length of PE-50 outflow tubing was connected to the outlet portion of the probe. The probe and tubing were flushed with 70% ethanol, and then with deionized water to ensure the interior of the probe was free of debris that might inhibit flow. Then the microdialysis probe was perfused with artificial cerebrospinal fluid (aCSF; pH 7.4; composition, NaCl 126.5 mM; $NaHCO_3$ 27.3 mM; KCl 2.4 mM; KH_2PO_4 0.5 mM; $CaCl_2$ 1.1 mM; $MgCl_2$ 0.85 mM; $NaSO_4$ 0.5 mM; glucose 5.9 mM; 0.8% bovine serum albumin and 0.03% bacitracin; all from Sigma-Aldrich, St. Louis, MO, USA) at a flow rate of 20 µl/min prior to insertion of the probe into the brain. The dialysis probe was stereotaxically lowered until the tip was in contact with the surface of the brain. The dorsal-ventral coordinates of the brain surface were recorded, and the probe was stereotaxically lowered into the brain tissue until the desired depth was reached (PBN, - 6.6 mm from surface of the brain; IC infarct,- 5.0 mm from surface of the brain; IC peri-infarct, - 2.5 mm from surface of the brain; CNA, - 7.5 mm from surface of the brain). When the probe was inserted, the flow rate of the aCSF was slowed to 5 µl/minute, determined as our optimal recovery rate. The probe was perfused for 30 minutes without sampling. In some groups a non-steroidal aromatase inhibitor was added to the aCSF (letrozole; Novartis International Pharmaceutical Ltd, Ringaskiddy, Ireland; 0.1µM). Dialysate samples (150 µl) were collected in disposable sample cups (Inter Medico, Markham, Ontario) via the outflow tubing.

2.4 *Middle cerebral artery occlusion*

The methods described below have been previously published in our lab (165;171;172). Briefly, subsequent to the rostro-caudal incision (dorso-ventral) was made in the frontalis muscle at the level of bregma the frontalis muscle and temporalis muscles were dissected away exposing the squamosal bone and the area where the zygoma fuses with the squamosal bone. Using a hand held drill, a small burr hole was made in the rostro-dorsal region of the squamosal bone. Beginning at the burr hole, the squamosal bone was dissected away using a pair of rongeurs and forceps, exposing the MCA. When the MCA was fully exposed (described previously in the general surgical procedures section), a 30 ½-gauge hypodermic needle with a bent tip was used to cut and remove the dura mater covering the MCA. In the occlusion group (n=6/nucleus/group) the MCA was permanently occluded using bipolar electrical coagulation (Cameron-Miller Inc; Chicago, IL, USA) at three points. The first occlusion was made just dorsal to the rhinal fissure, the second occlusion was made slightly ventral to the bifurcation of the MCA to the frontal and parietal cortices, and the third occlusion was made just before the bifurcation of the MCA to the parietal cortex. In the sham occlusion group (n=6/nucleus/group), all surgical procedures described above were performed except the MCA was not occluded.

2.5 *Sample collection*

The dialysate samples were collected at 30 minute intervals for one hour prior to middle cerebral artery occlusion/sham occlusion, and for four hours after occlusion or sham occlusion (one sample was taken at 10 minutes (50 µl) after middle cerebral artery

occlusion/sham occlusion). The samples were immediately placed into a -20 °C freezer, where they were kept until analysis (maximum of 4 weeks). Arterial blood samples (0.5 ml) were obtained via a small junction in the femoral artery catheter. Blood samples were collected in siliconized microcentrifuge tubes and then centrifuged at 2000 rpm for five minutes. The plasma was pipetted into another labeled siliconized microcentrifuge tube and was subsequently frozen at -20 °C. Both dialysate and blood samples were assayed for 17β-estradiol using a chemiluminescent enzyme immunoassay kit (Diagnostic Products Corporation, Los Angeles, CA) on an Immulite detector (Immulite®, Diagnostic Products Corporation, Los Angeles, CA; distributed by InterMedico, Ont, Canada).

2.6 Chemiluminescent enzyme immunoassay - analysis for 17β-estradiol

The Immulite® assay for 17β-estradiol is a solid-phase, chemiluminescent enzyme immunoassay designed for the quantitative measurement of estradiol in serum. Previously in our lab, this assay was validated for use in the rat for 17β-estradiol (175). The solid phase of the assay was made up of polystyrene beads enclosed within an Immulite® Test Unit, and was coated with a polyclonal rabbit antibody specific for estradiol. The sample and alkaline phosphatase-conjugated estradiol were simultaneously introduced into the Test Unit, and incubated for approximately 60 minutes at 37°C with intermittent agitation. During that time, 17β-estradiol in the sample competed with the enzyme-labeled estradiol for a limited number of antibody binding sites on the beads. Unbound enzyme conjugate was removed by a centrifugal wash. A chemiluminescent substrate (phosphate ester of adamantyl

dioxetane, which undergoes hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate) was added and the Test Unit was incubated for a further 10 minutes. The continuous production of the intermediate resulted in the sustained emission of light. The photon output of the bound complex was measured by the luminometer and the photon output was inversely proportional to the concentration of estradiol in the sample. The quantitation limit for estradiol was 10 pg/ml and the average intra-assay and inter-assay coefficients of variation were approximately 4.3 and 5.2%, respectively (Diagnostic Products Corporation). The detection limit of the assay was 8 pg/ml. Concentrations obtained between zero and 10 pg/ml were used in statistical analysis but were outside the limits of assay validation and should therefore be interpreted with care. The assay has less than a 0.009% cross-reactivity with any other steroid hormone that may be present in the samples and less than 0.09% cross-reactivity with any metabolite of the parent hormone (Diagnostic Products Corporation).

2.7 Determination of infarct size and probe placement

Four hours post-sham or MCA occlusion, the rat was transcardially perfused with phosphate buffered saline (0.1 M; 200 ml) and the brain was removed and placed in a rat brain matrix (Harvard Apparatus) and cut into 1 mm coronal slices. The coronal slices were transferred into 10 ml of 2 % 2,3,5-triphenoltetrazolium chloride (TTC; Sigma-Aldrich) solution. The stain is used to determine the effects of cerebral ischemia *in vivo*. While submerged in the TTC (a marker of mitochondrial enzyme activity, turning the living tissue a red color and leaving the ischemically damaged tissue unstained), the slices were incubated

for 10 minutes. The remaining portion of the brain and all the coronal slices were stored in 10 % formalin. Infarct size was determined by measurement of the unstained region of the TTC stained coronal section. The level in the brain where the anterior commissure is observed to join (approximately -0.26 mm to bregma) was the level at which the coronal sections were taken. Those sections were digitally photographed and used to measure infarct area. Infarct area was quantified using a computer-assisted image analysis program (Bioquant; R & M Biometrics Inc., Nashville, TE). Using the imaging system, the region without staining was outlined and the area within the outline was calculated following calibration of the image analysis program. The unstained area and the right hemisphere (to correct for any swelling post -MCAO that may have occurred) of the coronal section were measured three times and the average recorded. The infarct size was expressed as a percentage of the area of the right hemisphere. Microdialysis probe placement in the central nucleus of the amygdala and the parabrachial nucleus were verified using coronal sections (± 100 μm) cut through the extent of the central nucleus of the amygdala and the parabrachial nucleus using a vibratome. The sections were mounted onto gelatin-coated slides, and stained with thionin (nissel stain). The section containing the probe site (where the probe has reached its maximum depth) was compared with corresponding sections obtained from a stereotaxic atlas of the rat brain (154). Microdialysis probe placement in the insular cortex was verified by examination of the TTC stained coronal sections under a dissecting microscope and comparison with corresponding sections obtained from a stereotaxic atlas of the rat brain (154).

2.8 *Preparations for immunohistochemistry*

2.8.1 *Surgical procedures*

Male Sprague-Dawley rats (three animals per sham and MCAO groups) between 250 - 275g were used for the immunohistochemical study. Surgical procedures and monitoring were as described above except that the rats were anaesthetized with propofol (5 mg/kg/hr per rat) because sodium thiobutabarbital was temporarily unavailable. The HR and MAP were monitored for stability to ensure proper level of anesthesia. Four hours after MCAO or sham occlusion all animals were moved to a fume hood where they were transcardially perfused with phosphate buffered saline (0.1 M; 200 ml) and phosphate buffered paraformaldehyde (4 %, 200 ml). The brains were removed and stored in a solution of 20% sucrose and phosphate buffered paraformaldehyde (4%) at 4 °C.

2.8.2 *Dehydrating and embedding in paraffin*

The fixed brains were placed in a rat brain matrix (Harvard Apparatus) and cut into five coronal sections. Three serial sections containing the insular cortex, the central nucleus of the amygdala, and the parabrachial nucleus were cut. The trimmed tissue was then placed into labeled tissue cassettes (Histoprep; Fisher Scientific, Nepean, Ontario, Canada) and 70% ethanol for two hours (ethanol was replaced with fresh 70% ethanol for the second hour). This same step was repeated using 95% ethanol, then 100% ethanol. The cassettes were submerged in 50:50 (100% ethanol:xylene) for one hour, then xylene for two hours (xylene was replaced with fresh xylene for the second hour). The tissues were then infiltrated in paraffin over night and embedded in paraffin the next morning. The paraffin embedded

tissue was cut into 5 μm sections using a rotary microtome. Sections were floated in a water bath (distilled water maintained at 45 °C) before mounting on poly-L-lysine-coated slides. The mounted sections were allowed to dry on a slide warmer over night.

2.8.3 Deparaffinizing and rehydrating

The coronal sections (5 μm) containing the portions of the CNA, the PBN, and the IC were identified from a stereotaxic atlas of the rat brain (154). Sections containing the central nucleus of the CNA, the PBN, and the IC, were deparaffinized and rehydrated. Those sections that did not contain portions of the CNA, PBN or IC were stored in slide boxes for potential future use. The selected sections were deparaffinized in xylene and rehydrated through three changes of 100% ethanol each for five minutes and three changes of 95% ethanol each for five minutes. Finally, the slides were immersed in distilled water for five minutes. The tissue sections were then incubated in 0.05% saponin (in deionized water) for 30 minutes (antigen unmasking) and washed three times in phosphate buffered saline (0.1 M).

2.8.4 Immunohistochemistry

The primary antibody for the cytochrome P450 enzyme, aromatase, was obtained from Dr. Yoshio Osawa (Endocrine Biochemistry Department, Hauptman-Woodward Medical Research Institute, Inc., Buffalo, NY). The primary antibody was a rabbit polyclonal antibody, and was used in combination with the ABC staining system (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Slides were incubated for five to ten minutes

in 1% hydrogen peroxide (H_2O_2) then washed three times in phosphate buffered saline (PBS; 0.1 M, pH 7.4) each for five minutes. Next, the sections were incubated in 1.5% blocking serum (75 µl normal goat serum (NGS), 5 ml PBS) for one hour. The primary antibody was diluted to a ratio of 1:250 (diluted with blocking serum). This titre has been previously established in our laboratory to produce a good signal to background ratio of staining. The sections were incubated in the primary antibody overnight at 4 °C. Two randomly selected slides from each group undergoing processing for immunohistochemistry were chosen as controls and incubated in PBS overnight at 4 °C instead of primary antibody. The primary antibody was washed from the sections using three washes of PBS. The sections were incubated in biotin-conjugated goat anti-rabbit IgG secondary antibody (75 µl NGS, 5 ml PBS, 25 µl biotinylated secondary antibody) for 30 minutes. The sections were washed in PBS (three times each for five minutes) then incubated in avidin-biotin enzyme reagent (50 µl avidin reagent, 50 µl biotinylated HRP, 2.5 ml PBS) for 30 minutes. The sections were again washed three times in PBS each for five minutes. One drop of peroxidase substrate (1.6 ml distilled water, five drops of 10X substrate buffer, one drop 50X diaminobenzidine (DAB) chromagen, one drop 50X peroxidase substrate) was added to the surface of each section and allowed to incubate for ten minutes after which the slides were washed in distilled water for five minutes. The tissue sections were then quickly dehydrated through a series of ethanols (three times in 95%, three times 100% at 10 seconds each) and xylene (four changes at five seconds each).

Tissues were examined using photomicrographs (1.25 X and 16 X magnification), which were taken with a digital camera mounted on a microscope connected to a computer.

Cell counts were performed and analyzed using Bioquant imaging software ® (R& M Biometrics, Inc., Tujunga, CA). Three to five sections through the PBN and CNA were taken from the same levels in the brain (PBN, -9.80 mm; CNA, -1.80 mm) and were analyzed for positive staining of the aromatase enzyme in each animal. This process would ensure the accuracy of comparisons between the animals. Cells were considered positive if their cytoplasm (of the somas) was stained dark red-brown when compared to the lightly stained (pale orange) surroundings. Counts were averaged to determine a mean number of positively stained cells per nucleus in each animal. This mean was used to calculate the overall average number of cells from three separate animals per group (n=3).

2.8 Data analysis

All data are presented as a mean ± standard error of the mean (S.E.M.). The data entry and statistical analyses were carried out using a statistical software package (SigmaStat and SigmaPlot; Jandel Scientific; Tujunga, CA). In all cases, differences were considered significant if $p \leq 0.05$. Sequentially collected data were analyzed by a two-way analysis of variance (ANOVA) for repeated measures. If any significant differences were detected, a Student-Newman-Keul's post hoc analysis was used to determine where the differences occurred. Differences in baseline were determined using both an ANOVA and a student t-test. For the infarct size data, mean infarct area (mm^2, at the level -0.26 caudal to bregma) were compared between groups using a one-way ANOVA. Area of the left and right hemispheres from the infarct data were compared using paired t-test because the comparisons were made in the same animals. For the immunohistochemical data, the CNA

and PBN were analyzed for the number of aromatase enzyme immunoreactive cells and were compared between sham and MCAO groups using a t-test.

The letters located in the line graphs of the results section indicate significant differences between points, between the following treatment groups:

A = value is significantly different from baseline values of the same group

B = value is significantly different from sham values at the same time points

Chapter 3. Results

3.1 Changes in peripheral circulating estrogen concentrations in response to MCAO

To determine if MCAO leads to a change in systemic plasma estrogen concentrations, plasma estrogen concentrations were measured from one hour prior to MCAO or sham-MCAO, until four hours after MCAO. In the sham group (n=4), the mean plasma estrogen concentrations measured at -60 minutes and -30 minutes (20.0 pg/ml ± 0.862 and 19.5 pg/ml ± 0.862, respectively) were significantly lower ($P < 0.05$; figure 12) than the mean baseline plasma estrogen levels in the MCAO group (n=7; 23.6 pg/ml ± 0.651 and 22.9 pg/ml ± 0.651, respectively). Within the MCAO group, the baseline plasma estrogen concentration measured was significantly higher than the plasma estrogen concentrations measured at 30, 150, and 240 minutes post MCAO ($P < 0.05$; figure 12). Within the sham group, the baseline estrogen values were not significantly different from post-sham MCAO values, with the exception of the significant increase measured at 180 minutes post-sham MCAO ($P < 0.05$; figure 12). When comparing the MCAO and sham groups at each sampling time point, significant differences were observed at -60, -30, 10, 180, and 240 minutes ($P < 0.05$; figure 12), although there was a significant decrease with time indicated by the two-way ANOVA ($P < 0.05$).

3.2 Changes in blood pressure, heart rate, and dialysate estrogen concentrations within cardiovascular regulatory nuclei in response to MCAO

The following sections report results obtained by microdialysis of specific cardiovascular regulatory nuclei before and after MCAO or sham surgery in rats. MAP and

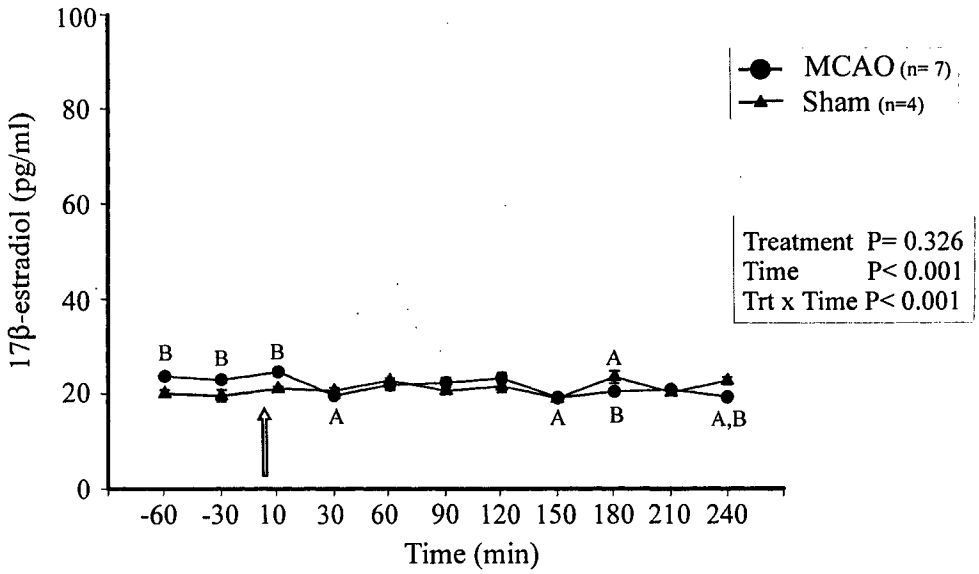


Figure 12. Plasma estrogen concentrations. The arrow indicates the time of MCAO and sham-MCAO.

HR were recorded over the course of the experiment in all rats. The dialysate estrogen concentration was measured using microdialysis. The results are reported for each brain nucleus (IC, PBN, and CNA).

3.2.1 IC Infarct

3.2.1.1 Changes in blood pressure and heart rate

Over the course of the experiment there were no significant differences in the MAP and mean HR between the MCAO (n=6) and sham (n=6) groups or when compared to baseline values within each group ($P > 0.05$; figures 13A and B), although there was a significant decrease in HR with time ($P = 0.002$).

3.1.1.2 Changes in dialysate estrogen concentrations

There was a significant difference in the estrogen concentration in the IC infarct in MCAO vs. sham treated rats with time ($P < 0.001$) for the interaction. Within the MCAO group (n=5), there was a gradual increase in dialysate estrogen concentrations over the early course of the experiment, reaching a maximum of 27.2 ± 5.2 pg/ml by 30 minutes post MCAO when compared to estrogen levels at -60 minutes only (16.8 ± 1.2 pg.ml; figure 13C). Following the increase, there was a gradual decrease in mean dialysate estrogen concentrations from 30 minutes to 240 minutes (figure 13C) post-MCAO, within the MCAO group. By 210 minutes post-MCAO, estrogen levels dropped below detectable levels (less than 10.0 pg/ml). Estrogen levels measured at 150, 180, 210, and 240 minutes were significantly different ($P < 0.05$; figure 13C) from -30 minutes (19.8 ± 1.4 pg/ml).

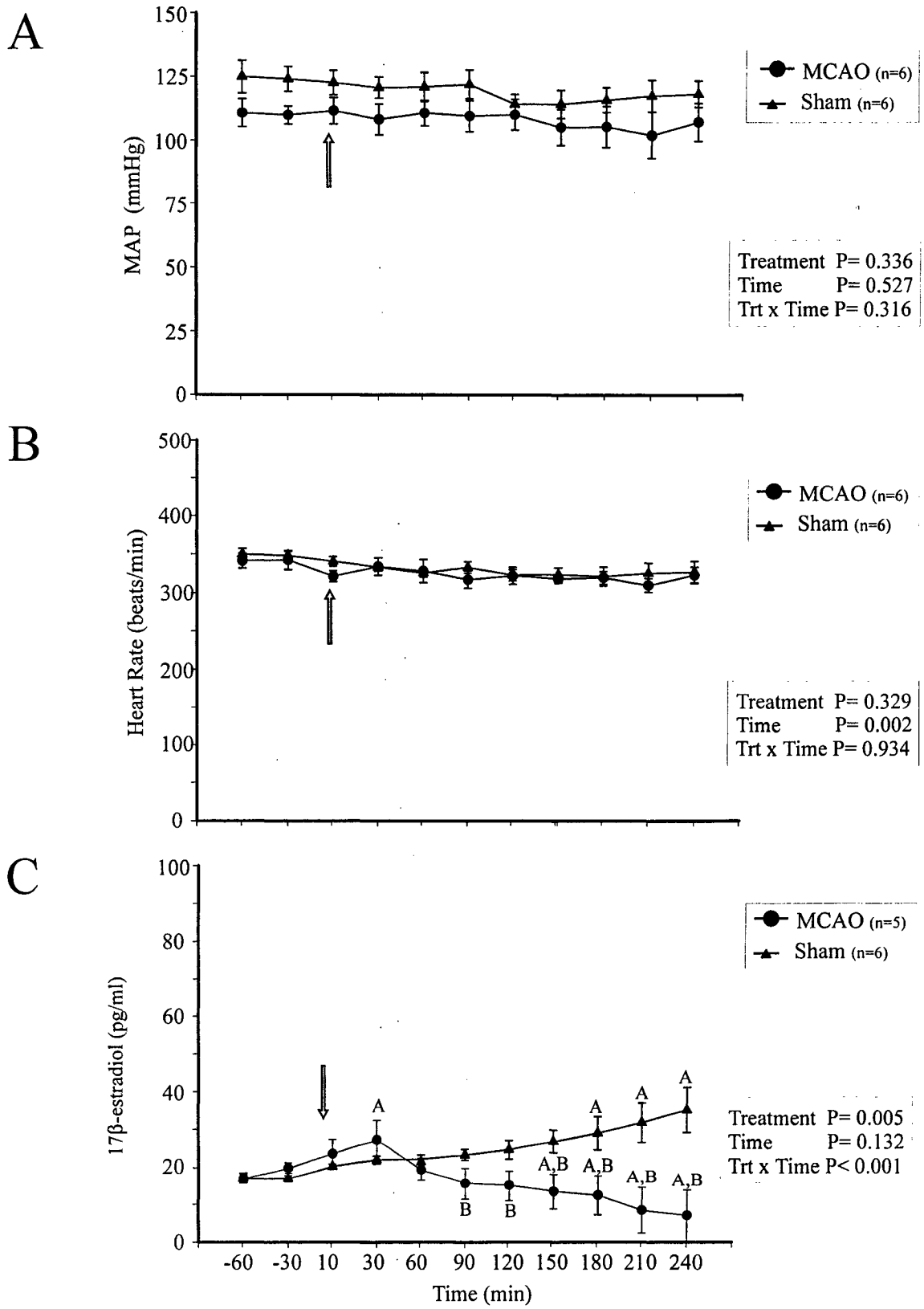


Figure 13. Dialysis in the IC infarct. A: mean arterial blood pressure (MAP), B:mean heart rate, C: mean estrogen concentrations. The arrow indicates the time of MCAO and sham-MCAO.

In the sham group (n=5), there was a significant increase in mean dialysate estrogen concentration after the sham MCAO. The mean estrogen values established at -60 and -30 minutes (16.8 ± 1.1 pg/ml and 16.8 ± 2.2 pg/ml, respectively) were significantly lower than estrogen concentrations measured at 180, 210 and 240 minutes post-sham MCAO ($P < 0.05$; figure 13C). Comparison of the MCAO and sham groups at each time point indicated there were significant differences observed at 90, 120, 150, 180, 210, 240 minutes post MCAO ($P < 0.05$; figure 13C).

3.2.2 IC Peri-infarct

3.2.2.1 Changes in blood pressure and heart rate

Over the course of the experiment there were no significant changes in the MAP and mean HR within the MCAO (n=6) or the sham (n=6) groups ($P > 0.05$; figures 14A and B). Similarly, there were no significant differences in MAP and mean HR between the MCAO and sham groups at each sampling time point ($P > 0.05$; figures 14A and B).

3.2.2.2 Changes in dialysate estrogen concentrations

There was a significant difference in the estrogen concentration in the IC peri-infarct in MCAO vs. sham treated rats with treatment ($P = 0.005$) and the treatment X time ($P < 0.001$) interaction. Prior to MCAO, dialysate estrogen concentrations in the IC peri-infarct were not significantly different between the sham (n=6) and MCAO (n=6) groups ($P > 0.05$; figure 14C). Within the MCAO group, there was a gradual increase in dialysate estrogen concentrations over the course of the experiment, beginning below detectable concentrations

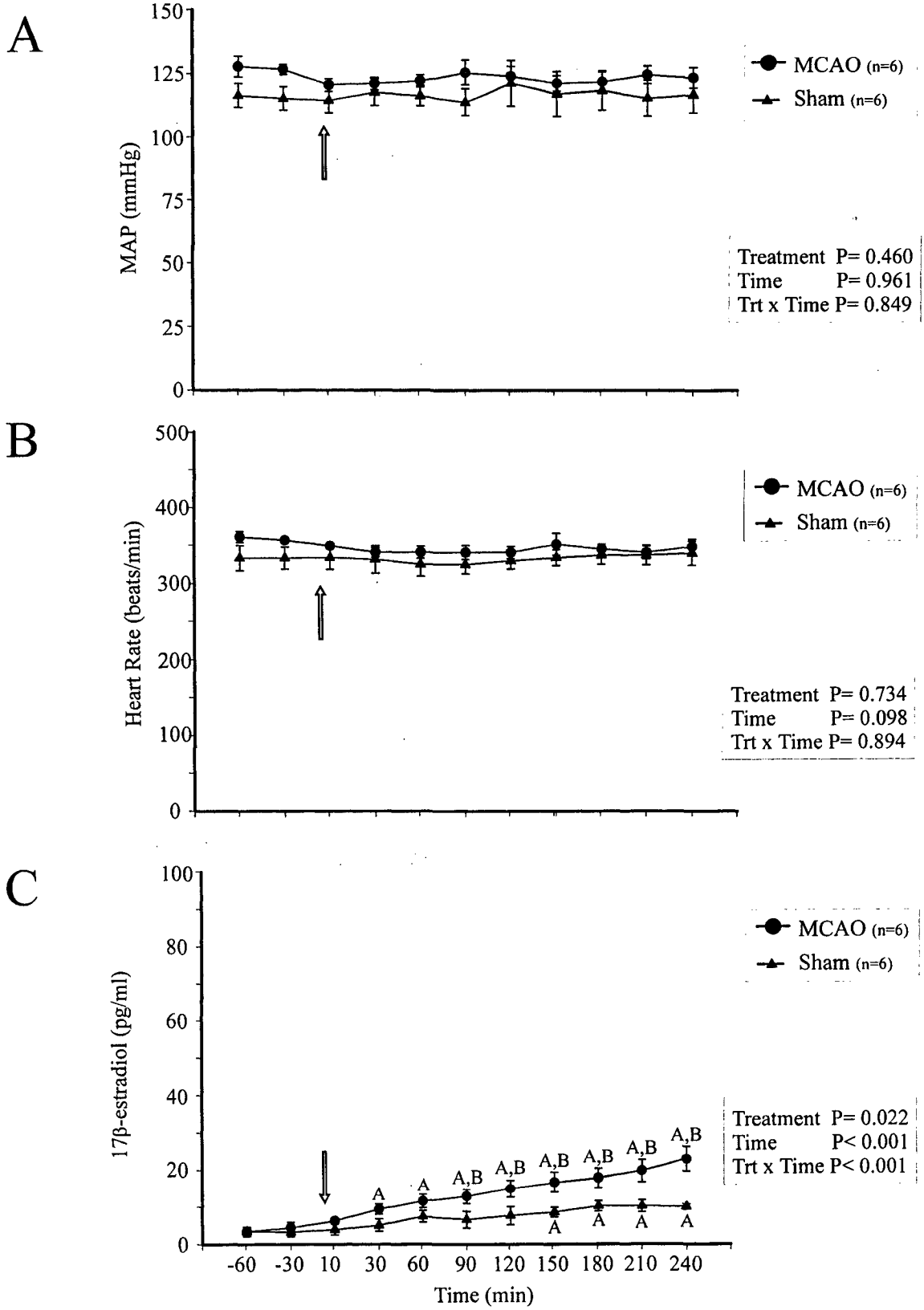


Figure 14. Dialysis in the IC peri-infarct. A: mean arterial blood pressure (MAP), B:mean heart rat, C: mean estrogen concetrations. The arrow indicates the time of MCAO and sham-MCAO.

and reaching a maximum of 23.0 ± 3.3 pg/ml at 240 minutes post MCAO (figure 14C). The mean estrogen concentrations from 30 minutes to 240 minutes post-MCAO were significantly elevated ($P < 0.05$; figure 14C) when compared to baseline values (less than 10.0 pg/ml).

In the sham group, there was a subtle increase in mean dialysate estrogen concentration after the time of sham-MCAO (figure 14C). The mean baseline estrogen values (less than 10.0 pg/ml) were significantly lower than mean estrogen concentrations detected at 150, 180, 210 and 240 minutes ($P < 0.05$; figure 14C). The differences between the MCAO and sham groups at each sampling time point were significant at 90, 120, 150, 180, 210, 240 minutes post-MCAO or - sham MCAO ($P < 0.05$; figure 14C).

3.2.3 PBN

3.2.3.1 Changes in blood pressure and heart rate

Prior to MCAO, there were no significant differences in the baseline MAP or baseline HR between the MCAO (n=7) and sham (n=6) groups ($P > 0.05$; figures 15A and B). However, the MAP decreased with time in the MCAO group but not in the sham group ($P < 0.001$ for treatment X time). The MAP measured at -30 minutes (121.7 ± 4.5 mmHg) was significantly higher than MAP values measured at 30, 60, 90, 120, 150, 180, 210, 240 minutes post-MCAO ($P < 0.05$; figure 15A). The MAP nadir (101.2 ± 7.3 mmHg) was reached at 180 minutes post-MCAO and remained at ~ 100 mmHg until 210 minutes post MCAO. In the sham group, the MAP measured following sham MCAO was not significantly different than baseline values ($P > 0.05$). There were no significant difference

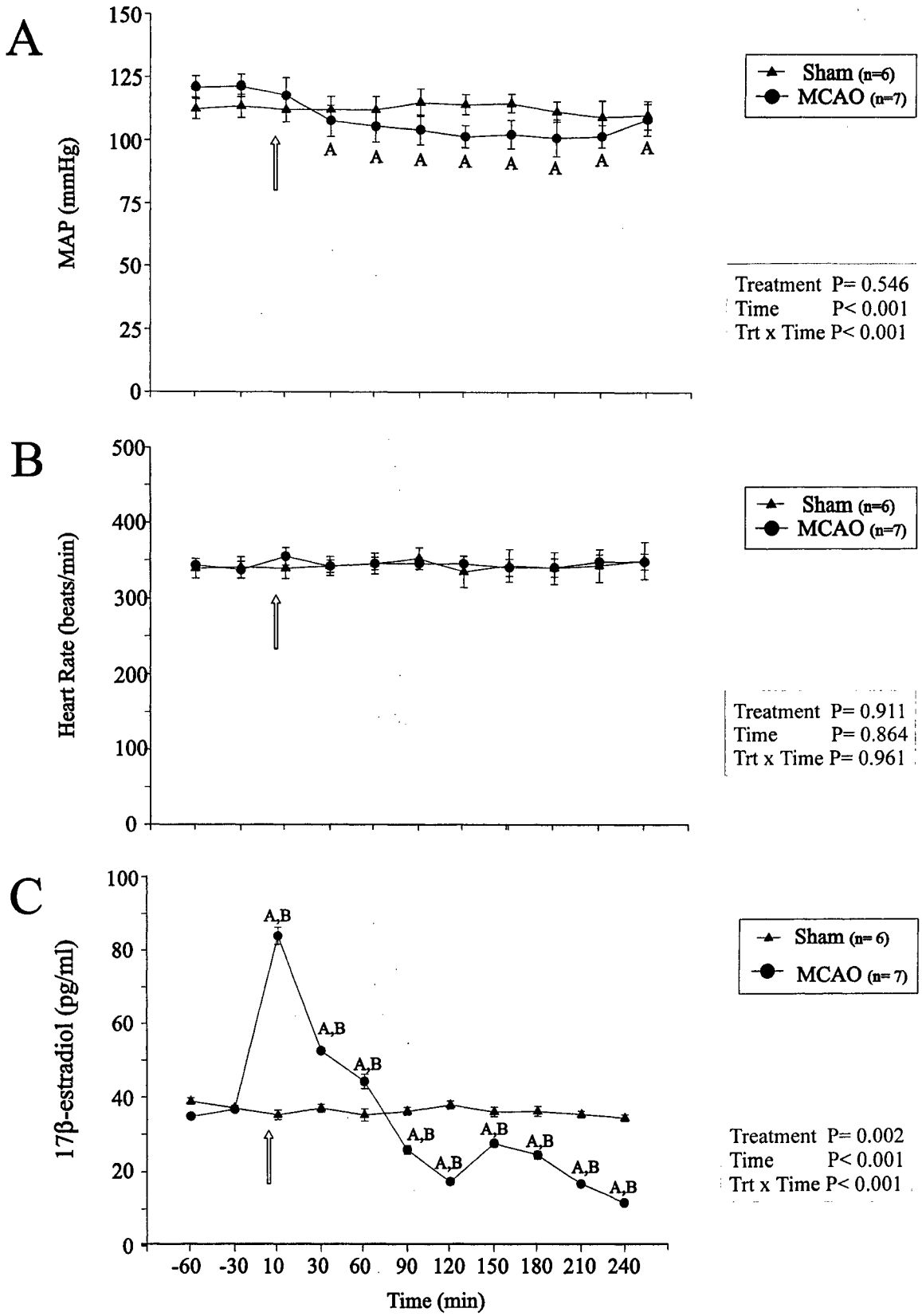


Figure 15. Dialysis in the PBN with no letrozole. A: mean arterial blood pressure (MAP), B: mean heart rate, C: mean estrogen concentrations. The arrow indicates the time of MCAO or sham-MCAO.

in MAP between the MCAO and sham groups when comparing each sampling time point ($P > 0.05$; figure 15A).

In the MCAO group (n=7), the mean HR remained around 344.6 ± 11.2 beats/minute over the course of the experiment with no significant changes ($P > 0.05$; figure 15B) when compared to baseline values established at -30 minutes (337.1 ±11.2 beats/minute). The sham group (n=6) remained at a mean HR of 342.7 ± 12.1 beats/minute, with no significant changes over the course of the experiment ($P > 0.05$; figure 15B). There were no significant differences in HR between MCAO or sham groups ($P > 0.05$; figure 15B).

3.2.3.2 Changes in dialysate estrogen concentrations

Prior to MCAO, dialysate estrogen concentrations in the PBN were not significantly different between the sham (n=6) and MCAO (n=7) groups ($P > 0.05$; figure 15C). Within the MCAO group, there was a two fold increase (83.7 ± 2.3 pg/ml) in the dialysate estrogen concentration at 10 minutes post MCAO ($P < 0.05$; figure 15C). By 30 minutes following MCAO, there was a rapid decrease in estrogen levels. This decrease continued gradually until 120 minutes after MCAO, reaching a low of 17.1 ± 0.9 pg/ml. Over the next 30 minutes a slight increase in estrogen concentration to 27.3 ± 1.0 pg/ml occurred at 150 minutes post MCAO (when compared with estrogen concentrations measured at 120 minutes post-MCAO; $P < 0.05$), which was significantly lower than baseline values ($P < 0.001$; figure 15C). From 150 minutes to 240 minutes post MCAO, there was another gradual decline in the dialysate estrogen concentration to 11.4 ± 0.4 pg/ml, which was well below baseline values.

Within the sham group, there was no significant change ($P > 0.05$; figure 15C) in mean estrogen concentrations over the course of the experiment when compared to baseline dialysate estrogen concentrations measured at -30 minutes (36.8 ± 0.7 pg/ml). When comparing the MCAO and sham groups, it was observed that the estrogen levels in the MCAO and sham groups were significantly different ($P < 0.05$; figure 15C) at all time points following MCAO/sham MCAO.

3.2.4 CNA

3.2.4.1 Changes in blood pressure and heart rate

Prior to MCAO or sham MCAO, there were no significant differences in the MAP and HR between the MCAO (n=6) and sham (n=6) groups ($P > 0.05$; figures 16A and B). Following MCAO or sham MCAO, there were no significant differences in the MAP over the course of the experiment between treatments ($P > 0.05$; figure 16A and B). There were no significant differences in mean HR between treatments at any time points, however there was a slight increase in mean HR over time ($P < 0.05$) reaching significance at 240 minutes post-MCAO.

3.2.4.2 Changes in dialysate estrogen concentrations

Prior to MCAO or sham MCAO, baseline dialysate estrogen concentrations in the CNA were not significantly different between the sham (n=6) and MCAO (n=6) groups ($P > 0.05$; figure 16C). In the MCAO group, there was a gradual increase in dialysate estrogen concentrations immediately following MCAO that remained over the course of the

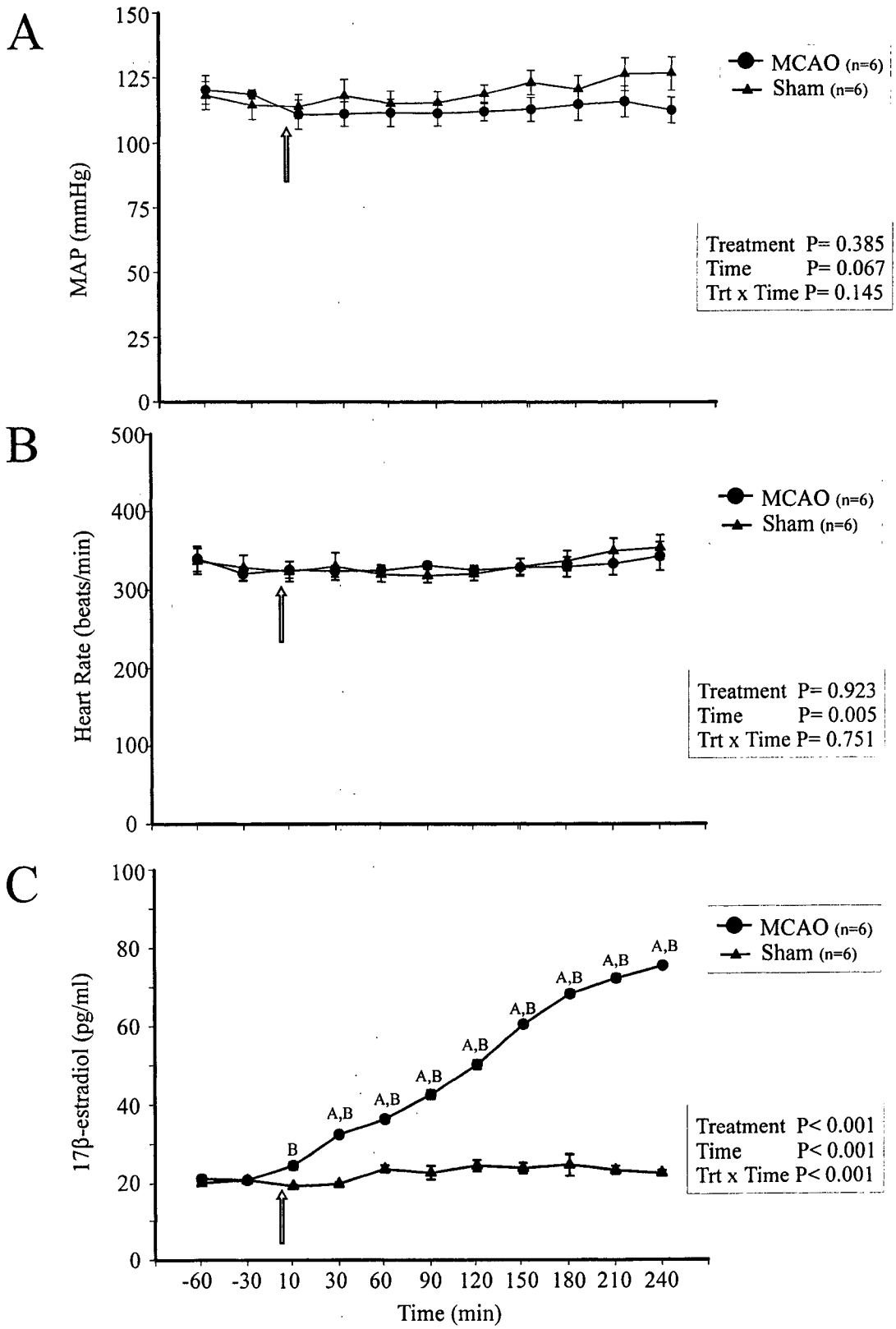


Figure 16. Dialysis in the CNA with no letrozole. A: mean arterial blood pressure (MAP), B:mean heart rate, C: mean estrogen concetrations. The arrow indicates the time of MCAO and sham-MCAO.

experiment, reaching a maximum of 76.7 ± 1.6 pg/ml by 240 minutes post-MCAO (figure 16C). The mean estrogen concentrations at 10 minutes post-MCAO (24.5 ± 0.9 pg/ml; figure 16C) were not significantly different ($P > 0.05$) from baseline values (20.8 ± 0.5 pg/ml; figure 16C) in the MCAO group. The mean dialysate estrogen concentrations from 30 minutes to 240 minutes post MCAO were significantly different from baseline values ($P < 0.001$, for all sampling time points; figure 16C).

In the sham group, the post-sham MCAO mean estrogen concentrations were not significantly different ($P > 0.05$; figure 16C) from baseline concentrations (20.8 ± 0.5 pg/ml). When comparing the sham and MCAO groups at each sampling time point, it was observed that the mean estrogen levels for the MCAO group were significantly different from the sham group at all post- MCAO or post-sham MCAO sampling time points ($P< 0.05$; figure 16C).

3.3 The effects of local inhibition of aromatase on changes in blood pressure, heart rate, and dialysate estrogen concentrations within cardiovascular regulatory nuclei in response to MCAO

To determine if estrogen released following MCAO was synthesized locally in the PBN and CNA, letrozole was administered to the CSF during dialysis 60 minutes prior to or immediately before MCAO. The effects of the letrozole administration in each nucleus will be described below.

3.3.1 PBN with letrozole administration at -60 minutes (pbnl-60)

3.3.1.1 Changes in blood pressure and heart rate

Prior to MCAO, there were no significant differences in the baseline MAP between the MCAO (n=7) and sham (n=6) groups ($P > 0.05$; figures 17A). However, there was a significant difference in HR at -30 minutes between the MCAO and sham groups ($P < 0.05$; figure 17B). The results of the two-way ANOVA indicated that there was a significant effect of treatment ($P < 0.05$, shown in figure 17), but there was no significant effect of time on MAP. Within the PBNl-60 MCAO group, the two-way ANOVA indicated a significant effect of time on changes in MAP ($P < 0.05$, shown in figure 17A). There was no significant change in MAP over time for the PBNl-60 MCAO group when compared to baseline values established at -30 minutes (102.6 ± 4.4 mmHg). Similarly, there were no significant changes in MAP within the PBNl-60 sham group ($P > 0.05$; figure 17A). When comparing the MAP of the PBNl-60 MCAO and the sham groups at each sampling time point following MCAO/sham-MCAO, some significant differences were observed at 10, 60, 90, 120, and 180 minutes post-MCAO ($P < 0.05$; figure 17A).

In the PBNl-60 MCAO group there was a gradual increase in mean HR over the course of the experiment. Significant differences were observed when comparing HR at -30 minutes (302.9 ± 8.6 beats/minute) with 90 (332.1 ± 7.3 beats/minute), 150 (335.7 ± 10.4 beats/minute), 180 (338.6 ± 13.7 beats/minute), 210 (339.3 ± 7.1 beats/minute), and 240 (345.0 ± 6.5 beats/min) minutes ($P < 0.05$; figure 17B). In the PBNl-60 sham group, there were no significant changes in HR over time when compared to baseline (331.7 ± 7.6 beats/minute). Through the comparison of both MCAO and sham groups for mean HR, it

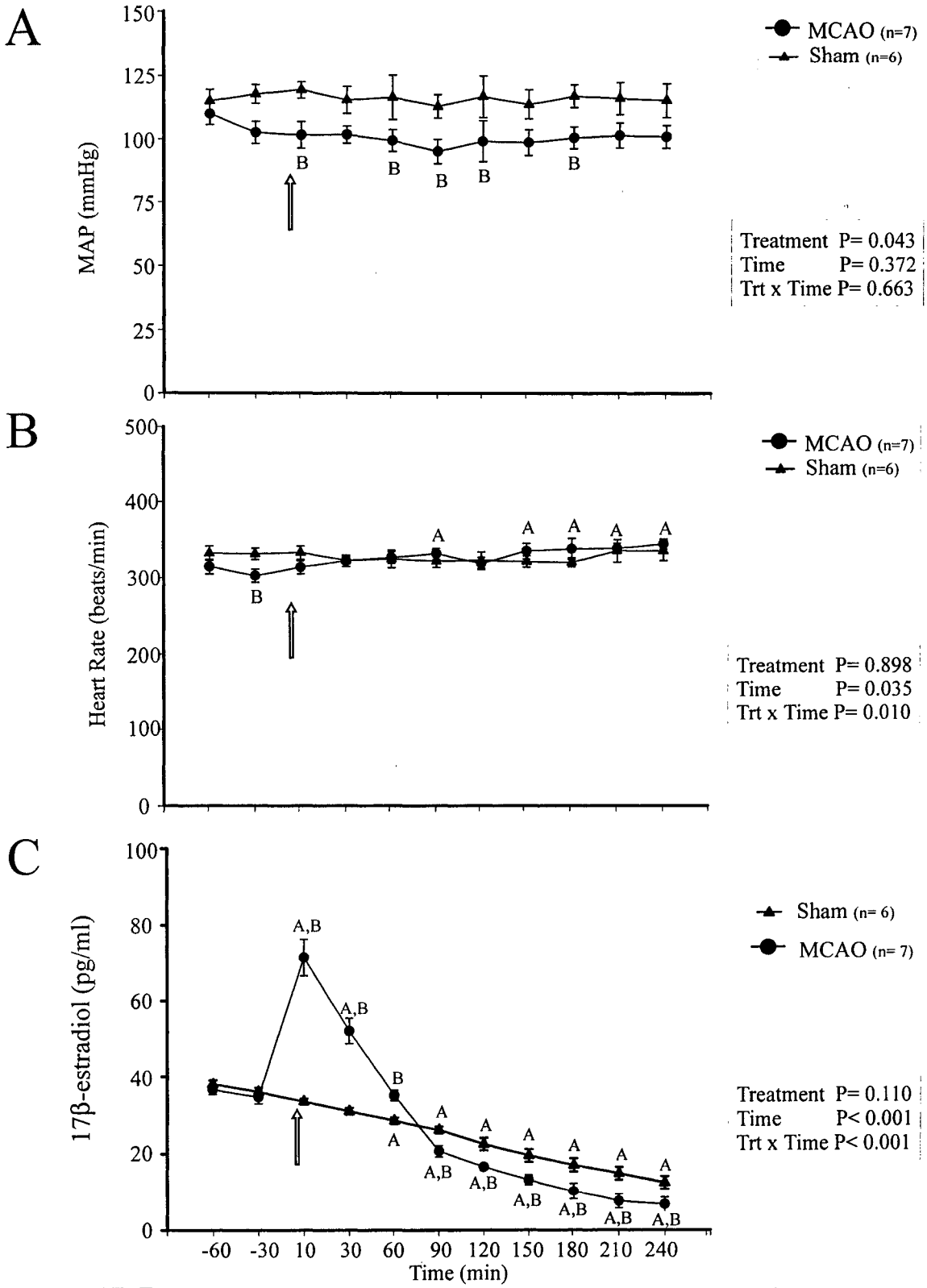


Figure 17. Dialysis in the PBN with administration of letrozole at -60 min. A: mean arterial blood pressure (MAP), B: mean heart rate, C: mean estrogen concentrations. The arrow indicates the time of MCAO or sham-MCAO.

was observed that the groups were significantly different ($P < 0.05$; figure 17B) at 30 minutes prior to MCAO/sham-MCAO only. At all other time points, there were no significant differences between groups.

3.3.1.2 Changes in dialysate estrogen concentrations

Prior to MCAO, there were no significant differences between baseline mean estrogen concentrations for the PBNl-60 MCAO (34.6 ± 1.7 pg/ml; n=7) and the sham (36.0 ± 1.9 pg/ml; n=6) groups. For the PBNl-60 MCAO group, a significant increase ($P < 0.05$; figure 17C) in mean estrogen concentration was observed at 10, and 30 minutes post-MCAO followed by a significant decrease ($P < 0.05$; figure 17C) at 90, 120, 150, 180, 210, 240 minutes post-MCAO when compared to baseline values established at -30 minutes (34.6 ± 1.8 pg/ml). Between 150 minutes and 240 minutes post-MCAO, the mean dialysate estrogen levels decreased below detectable levels (less than 10.0 pg/ml). Following sham-MCAO, there was a significant decrease in mean estrogen concentrations over the course of the experiment in the PBNl-60 group beginning at 30 minutes post-sham MCAO ($P < 0.05$; figure 17C). Baseline estrogen concentrations for PBNl-60 sham group were significantly higher than estrogen levels detected at 30, 60, 90, 120, 150 , 180 , 210 , and 240 minutes post- sham MCAO ($P < 0.05$; figure 17C). When comparing PBNl-60 MCAO and sham treatments, estrogen concentrations were significantly different at 10, 30, 60, 90, 120, 150, 180, 210, 240 minutes post-MCAO/post-sham MCAO sampling time points ($P < 0.05$; figure 17C).

3.3.2 PBN with letrozole administration at the time of MCAO (PBNlmcao)

3.3.2.1 Changes in blood pressure and heart rate

There was no significant difference in the baseline MAP between the MCAO (n=5) and sham (n=6) groups ($P > 0.05$; figure 18A). Following MCAO, there was no significant differences ($P > 0.05$; figure 18A) between the MCAO group and the sham groups in MAP. Before MCAO or sham-MCAO, there were no significant differences in mean baseline HR between MCAO (n=5) and sham (n=6) groups ($P > 0.05$; figure 18B). The results of the two-way ANOVA comparing MCAO and sham groups indicated that there was a significant effect of time on mean HR ($P < 0.05$, shown in figure 18B). Within the PBNlmcao MCAO group there was no significant change ($P > 0.05$; figure 18B) in mean HR over the course of the experiment. Similarly, the sham group did not show any significant change ($P > 0.05$; figure 18B) in mean HR over the course of the experiment as indicated by the post hoc analysis.

When comparing the MCAO and sham groups at each sampling time point, there were no significant differences in mean HR following MCAO/sham-MCAO.

3.3.2.2 Changes in dialysate estrogen concentrations

When comparing baseline mean estrogen concentrations, there were no significant differences between the PBNlmcao MCAO (n=4) and the sham (n=6) treatments ($P > 0.05$; figure 18C). The baseline estrogen concentration for the PBNlmcao MCAO group was 33.5 $\pm$ 2.6 pg/ml, and for the PBNlmcao sham group was 36.0 $\pm$ 1.9 pg/ml. When comparing the post-MCAO values with baseline estrogen concentrations, there was a significant increase

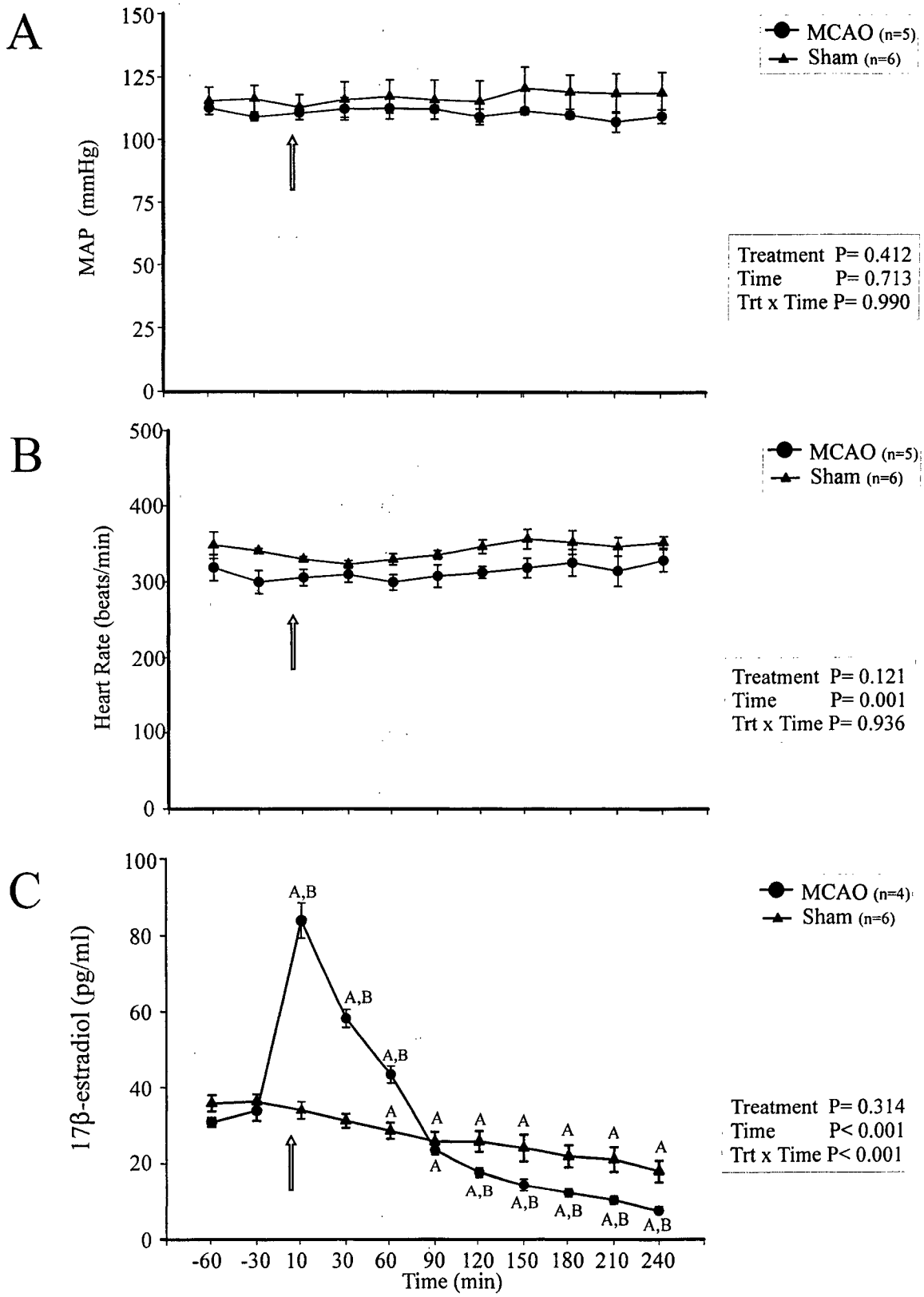


Figure 18. Dialysis in the PBN with letrozole administration at MCAO. A: mean arterial blood pressure (MAP), B: mean heart rate, C: mean estrogen concentrations. The arrow indicates the time of MCAO or sham-MCAO.

($P < 0.05$; figure 18C) in mean dialysate estrogen concentrations at 10 minutes 83.8 pg/ml ± 4.6) post-MCAO within the PBNlmcao MCAO group. Following the 10 minute sample, there was a decline in estrogen concentrations (figure 18C). This decline continued gradually until 240 minutes post MCAO, dropping below detectable levels by 180 minute post MCAO (less than 10.0 pg/ml). All mean estrogen concentrations at each post-MCAO sampling time point were significantly different from baseline values ($P < 0.05$; figure 18C).

Within the PBNlmcao sham group there was a significant decline in mean dialysate estrogen concentrations ($P < 0.05$; figure 18C). When comparing sham baseline estrogen concentrations and post-sham MCAO values, there were significant decreases observed at 60, 90, 120, 150, 180, 210, and 240 minutes post-sham MCAO ($P < 0.05$; figure 18C).

Comparisons between the PBNlmcao MCAO and sham groups indicated significant differences ($P < 0.05$; figure 18C) at 10, 30, 60, 120, 150, 180, 210, and 240 minutes post-MCAO/post-sham MCAO.

3.3.3 CNA with letrozole administration at -60 minutes (CNAl-60)

3.3.3.1 Changes in blood pressure and heart rate

Prior to MCAO, there were no significant differences in the baseline MAP and baseline mean HR between the CNAl-60 MCAO (n=6) and sham (n=5) groups ($P > 0.05$; figures 19A and B). According to the two-way ANOVA there was a significant interaction between treatment and time ($P < 0.05$, figure 19A). Following the post hoc analysis, there were no significant changes in MAP over the course of the experiment ($P < 0.05$; figure

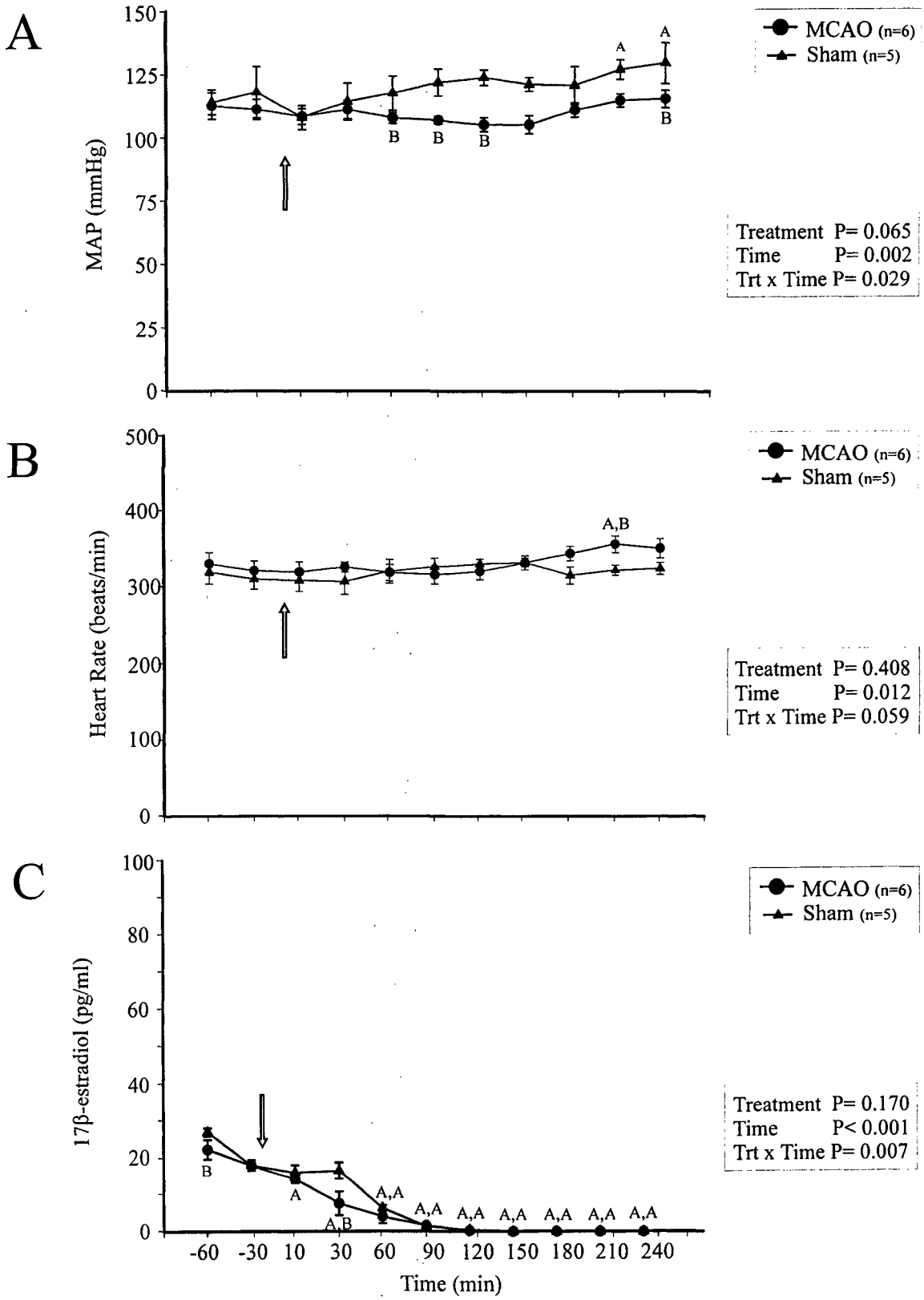


Figure 19. Dialysis in the CNA with letrozole administration at -60 minutes. A: mean arterial blood pressure (MAP) , B: mean heart rate, C: mean estrogen concentrations. The arrow indicates the time of MCAO or sham-MCAO.

19A) within the CNAl-60 MCAO group when compared to baseline (111.4 ± 4.0 mmHg). As for the sham group, there was a significant increases in MAP at 210 (127. 8 ± 3.9 mmHg) and 240 (130. 3 ± 8.3 mmHg) minutes post-sham MCAO ($P < 0.05$; figures 19A) when compared to baseline values. While comparing the CNAl-60 MCAO and sham groups for MAP at each sampling time point, significant differences were detected at 90, 120, 150, and 240 minutes post-MCAO ($P < 0.05$; figure 19A). During the examination of HR in the CNAl-60 MCAO group following MCAO, a significant increase ($P < 0.05$) was observed at 210 minutes post-MCAO (356.7 ± 11.2) when compared to baseline (320.8 ± 12.9). However, when compared differences between the CNAl-60 MCAO and sham groups of mean HR at each sampling time point a significant difference was observed at 210 minutes post-MCAO/post-sham MCAO ($P > 0.05$; figure 19B).

3.3.3.2 Changes in dialysate estrogen concentrations

When comparing baseline estrogen concentrations, there was a significant difference ($P < 0.05$) between the CNAl-60 MCAO (n=6) and sham (n=5) groups ($P > 0.05$; figure 19C). The two-way ANOVA revealed a significant interaction of treatment and time on changes in endogenous estrogen concentrations. Within the MCAO group, there was an immediate decline in mean dialysate estrogen concentrations following MCAO. The mean estrogen levels declined below detectable levels by 30 minutes post-MCAO (less than 10.0 pg/ml). Compared with baseline estrogen concentrations (18.2± 1.4 pg/ml), significant differences were observed in mean estrogen concentrations at 10, 30, 60, 90, 120, 150 , 180, 210, 240 minutes post MCAO ($P < 0.05$; figure 19C), in the CNAl-60 MCAO groups.

For the sham group, there were a significant changes in mean dialysate estrogen concentrations prior to MCAO. A significant decline in estrogen levels between -60 and -30 minutes (27.0 ± 1.1 pg/ml and 18.2 ± 1.5, respectively; $P < 0.05$; figure 19C). Following the sham MCAO, there was a slight, but not significant, increase between 10 minutes (16.2 ± 2.1 pg/ml) and 30 minutes (16.7 ± 2.3 pg/ml) post MCAO ($P > 0.05$; figure 19C). Compared with baseline estrogen concentrations (18.3 ± 1.5 pg/ml), significant differences were observed in mean estrogen concentrations at 60, 90, 120, 150, 180, 210, 240 minutes post-sham MCAO ($P < 0.05$, figure 19C). From 30 minutes to 60 minutes post-sham MCAO the mean estrogen concentrations decreased below detectable levels (less than 10.0 pg/ml). When comparing the CNAl-60 MCAO and sham groups at each sampling time point, significant differences were observed only at -60 and 30 minutes post-MCAO/post-sham MCAO ($P < 0.05$; figure 19C).

3.3.4 CNA with letrozole administration at MCAO (CNAlmcao)

3.3.4.1 Changes in blood pressure and heart rate

Prior to MCAO there were no significant differences in MAP or mean HR between the MCAO (n=6) and the sham (n=6) treatments ($P > 0.05$; figure 20A and B) in the CNA with letrozole administered at MCAO group (CNAlmcao). The two-way ANOVA revealed a significant effect of time on changes in both MAP and HR ($P < 0.05$, shown in figures 20A and B). Through the comparison of the CNAlmcao MCAO group with baseline MAP (108.9 ± 5.3 mmHg), revealed no significant changes ($P > 0.05$) in MAP following MCAO/sham

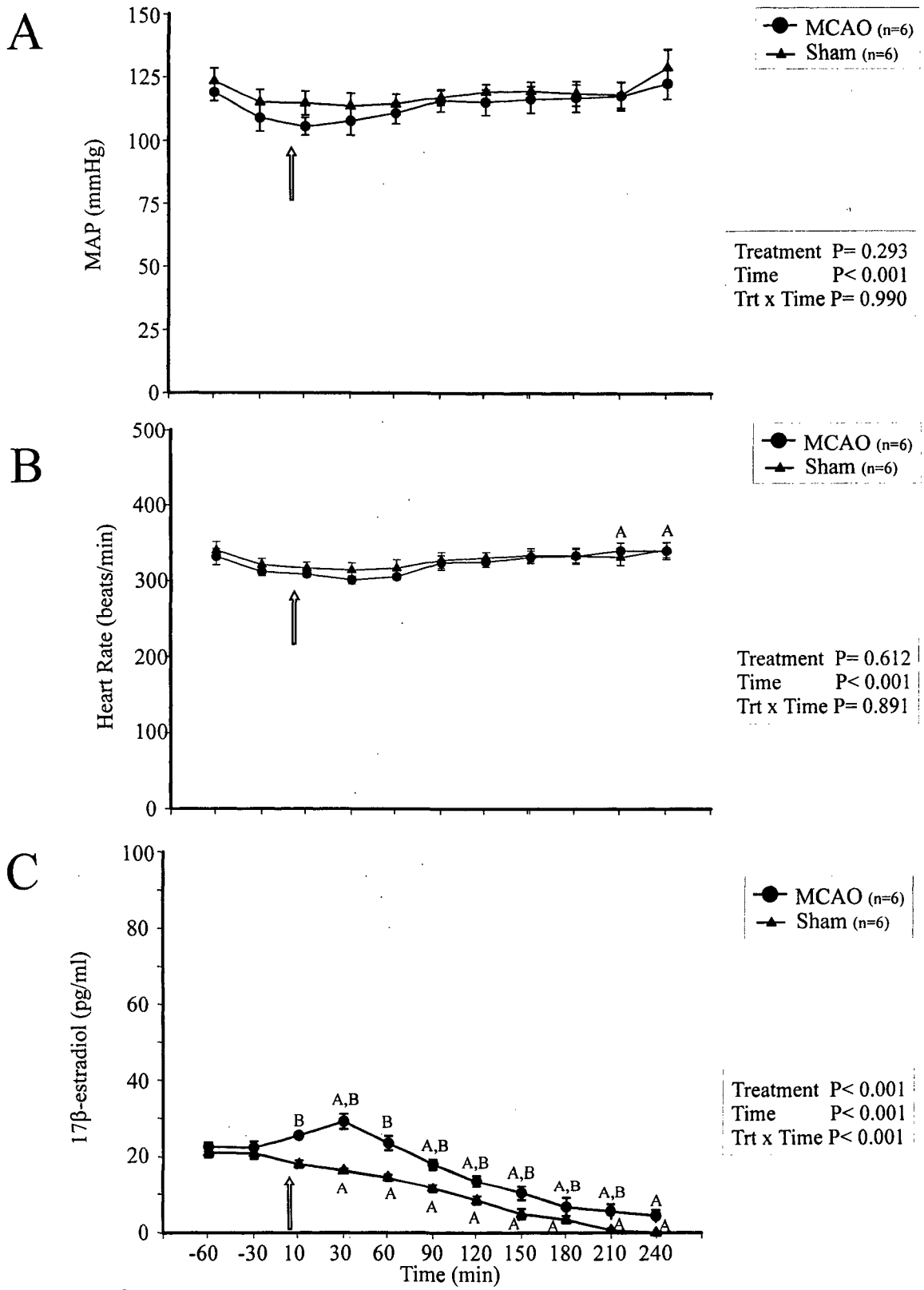


Figure 20. Dialysis in the CNA with letrozole administration at MCAO. A: mean arterial blood pressure (MAP), B: mean heart rate, C: mean estrogen concentrations. The arrow indicates the time of MCAO or sham-MCAO.

MCAO ($P > 0.05$; figure 20A). There were no significant differences ($P > 0.05$) in MAP between the MCAO and sham groups at any time point.

Within the CNAlmcao MCAO group there were some significant changes in HR over the course of the experiment. The mean HR measured at -60 minutes (330.0 ± 15.1 beats/minute) was significantly different ($P < 0.05$; figure 20B) from 210 (356.7 ± 11.2 beats/minute), and 240 minutes (350.8 ± 12.9 beats/minute) post-MCAO. Within the sham group, there were no significant ($P > 0.05$; figure 20B) changes in mean HR over the course of the experiment. When comparing the CNAlmcao MCAO and sham groups at each sampling time point, no significant differences ($P > 0.05$; figure 20B) in mean HR were observed.

3.3.4.2 Changes in dialysate estrogen concentrations

Prior to MCAO, no significant differences were observed in baseline values for mean dialysate estrogen concentrations between CNAlmcao MCAO and sham groups ($P > 0.05$; figures 20C). There was a significant interaction between the effects of treatment and time on changes in estrogen concentration ($P < 0.05$, shown in figure 20C). Within the MCAO group, the mean estrogen concentrations increased gradually immediately following MCAO. After MCAO, significant increases in mean dialysate estrogen concentrations were observed at 30 minutes post-MCAO (29.2 ± 2.0 pg/ml). Following this increase, significant decreases ($P < 0.05$; figure 20C) were observed at 90, 120, 150, 180, 210, 240 minutes post-MCAO when compared with baseline values (22.3 ± 1.7 pg/ml). This decrease continued, gradually, until 240 minutes post-MCAO, but declined below detectable levels (less than 10.0 pg/ml)

by 150 minutes post-MCAO (figure 20C). Within the CNAlmcao sham group, there was a gradual decrease in mean dialysate concentrations following the administration of letrozole. When compared to baseline values (20.8 ± 1.4 pg/ml), mean estrogen concentrations measured at 30, 60, 90, 120, 150, 180, 210, 240 minutes post-sham MCAO were significantly decreased ($P < 0.05$; figure 20C). By 90 minutes after administration of letrozole, mean estrogen levels declined below detectable levels (less than 10.0 pg/ml) in the sham group.

When comparing the CNAlmcao MCAO and sham treatments at each sampling time point, significant differences were observed at 10, 30, 60, 90, 120, 150, 180, 210 minutes post-MCAO/sham MCAO ($P < 0.05$; figure 20C).

3.4 Baseline comparisons between nuclei

Mean heart rate measured at -30 minutes show there were no significant differences in baseline mean heart rate between the PBN, CNA, IC infarct, and IC peri-infarct (table 4). There were no other significant differences observed when the baseline MAP was compared between nuclei (table 4).

Unlike the mean heart rate and MAP, all comparisons resulted in signifcant differences in mean estrogen concentrations (table 4).

Table 4. Baseline comparisons of mean HR, MAP, and dialysate estrogen concentrations for significant differences between nuclei.

	Heart Rate	MAP	Estrogen
	-30 min	**-30 min**	**-30 min**
PBN vs CNA	p=0.25	p=0.68	p<0.001
CNA vs IC infarct	p=0.65	p=0.24	p<0.001
CNA vs IC peri-infarct	p=0.06	p=0.19	p<0.001
IC infarct vs IC peri-infarct	p>0.05	p=0.04	p<0.001
PBN vs IC infarct	p>0.05	p>0.05	p<0.001
PBN vs IC peri-infarct	p>0.05	p>0.05	p<0.001

3.5 Immunohistochemistry

Mean number of cells for the PBN, its subnuclei, the CNA, and its subnuclei are shown in table 5. When comparing the mean number of positively stained cells between the sham and MCAO treatments, there were no significant differences observed (table 5; figure 21). Examples of positive staining for both sham and MCAO treatments in the PBN and CNA groups are shown in figures 22 and 23.

3.6 Probe sites

Representative dialysis probe tracts in the PBN and CNA are shown in figures 24 and 25. Dialysis probe sites were also mapped for all treatment groups.

3.7 Infarct sizes

Mean areas of right brain hemisphere, left brain hemisphere, infarct and percentage of brain hemisphere area infarcted are shown in table 6. When comparing the right and left brain hemispheres following MCAO within each nucleus and letrozole treatments, there were only two treatments showing significant differences (table 6). Those two groups were PBNl-60 and CNAl-60 with ($P < 0.05$). Within all other groups there were no difference in mean areas of right brain hemisphere verses left brain hemisphere ($P > 0.05$; table 6). Following the comparison of the percentage brain hemisphere area infarcted between brain nuclei and letrozole treatments, one significant difference was observed. It was determined that the infarct areas in the IC infarct group (1544.49 ± 86.10 mm^2; table 6)

Table 5. Quantification of cells stained positively for the aromatase enzyme in the PBN and CNA.

Nucleus	Treatment	Subnuclei	n	Mean Number of Cells ± SEM	Comparison Between Treatments	
					Subnuclei	P-value
PBN	MCAO	LPBV	3	8.78 ± 1.89	LPBV	P = 0.78
		MPB	3	5.25 ±0.95		
		Total	3	4.29 ± 2.82	MPB	P = 0.08
	Sham	LPBV	3	7.89 ± 1.89		
		MPB	3	2.61 ± 0.66	Total	P = 0.38
		Total	3	10.50 ± 2.66		
CNA	MCAO	CeC	3	7.11 ± 0.841	CeC	P = 0.97
		CeL	3	1.72 ± 0.54		
		CeM	3	5.67 ± 1.75	CeL	P = 0.8
		Total	3	14.5 ± 2.90		
	Sham	CeC	3	7.16 ± 0.98	CeM	P = 0.6
		CeL	3	1.50 ± 0.59		
		CeM	3	4.28 ± 1.68	Total	P = 0.75
		Total	3	13.10 ± 3.13		

LPBV- lateral parabrachial nucleus, ventral part
MPB - medial parabrachial nucleus
CeC - central amygdaloid nucleus, capsular part
CeL - central amygdaloid nulceus, lateral division
CeM - central amygdaloid nucleus, medial division
Total - whole nucleus, including subnuclei

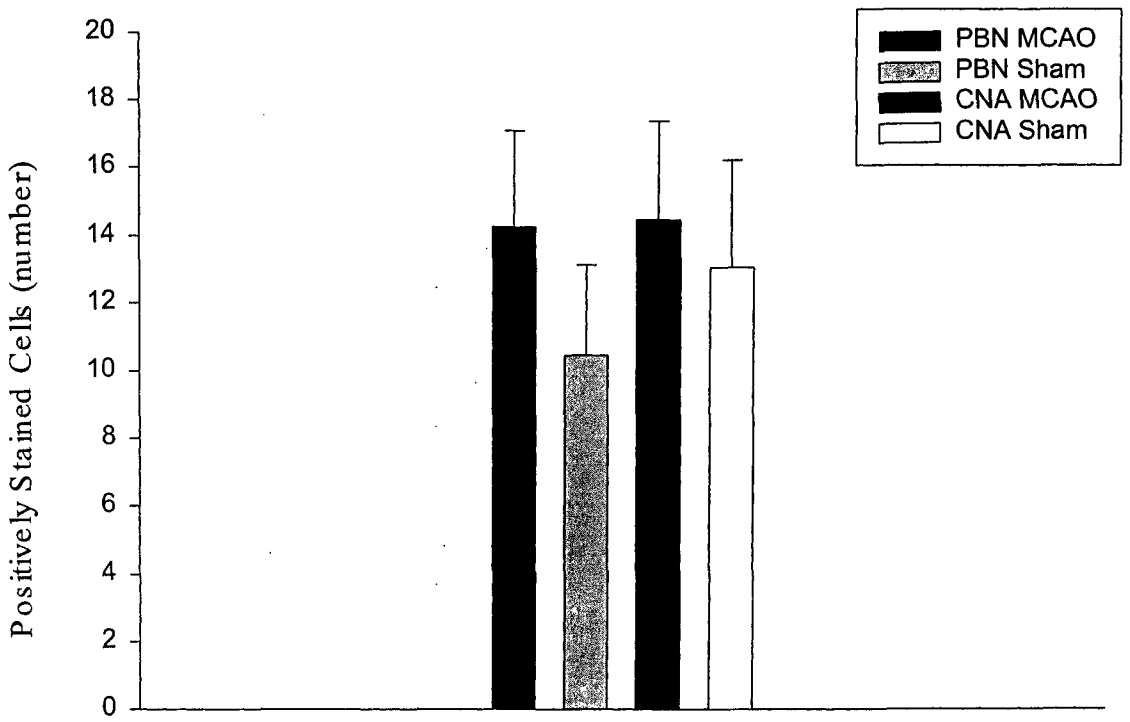


Figure 21. Cells stained positively for the aromatase enzyme within the PBN and CNA following sham and MCAO treatments. The immunohistochemistry data used is also shown in table 12.

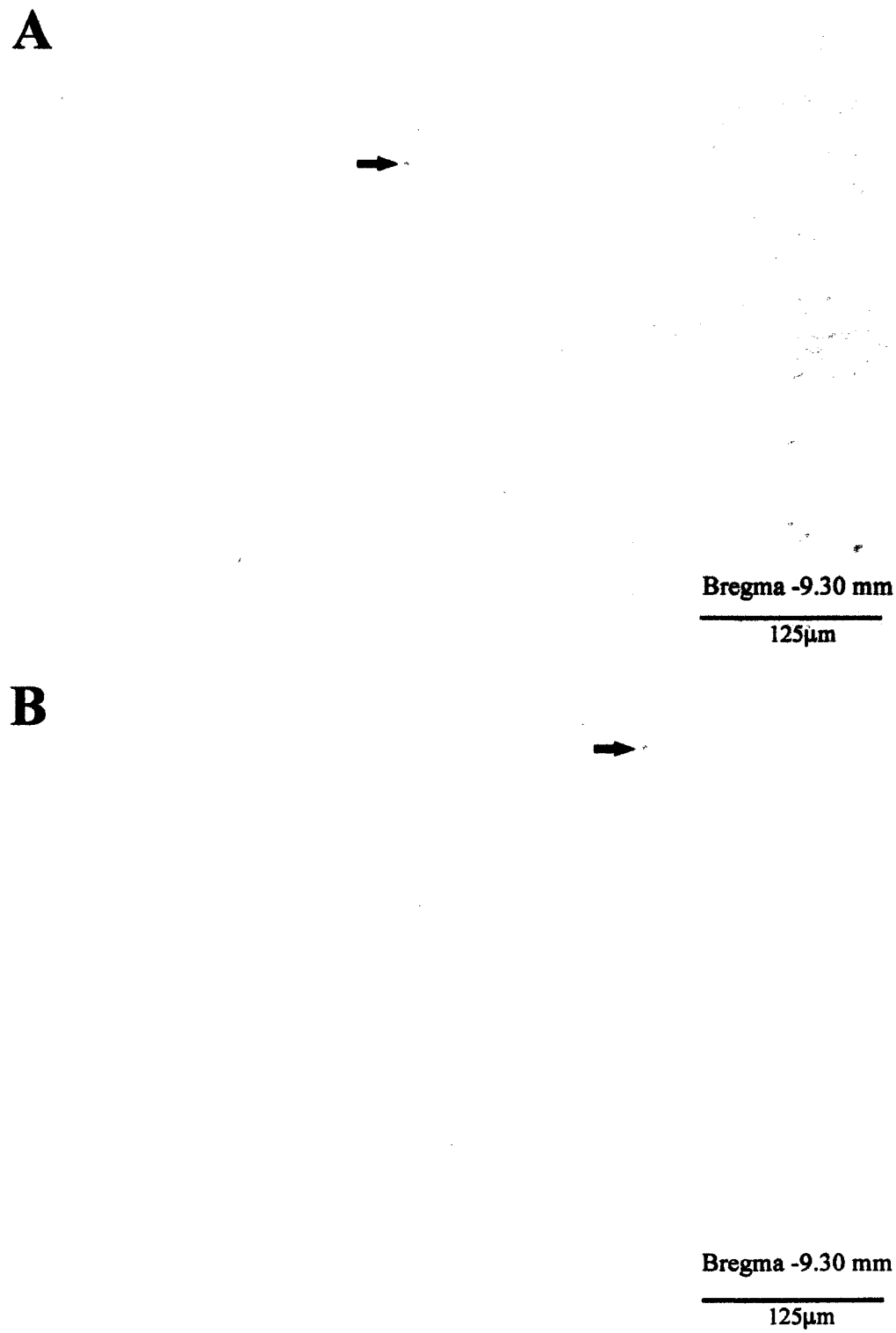


Figure 22. Immunohistochemistry staining for aromatase in the PBN. A: staining following MCAO, B: staining following sham MCAO. An example of positive staining is indicated by the arrow shown above.

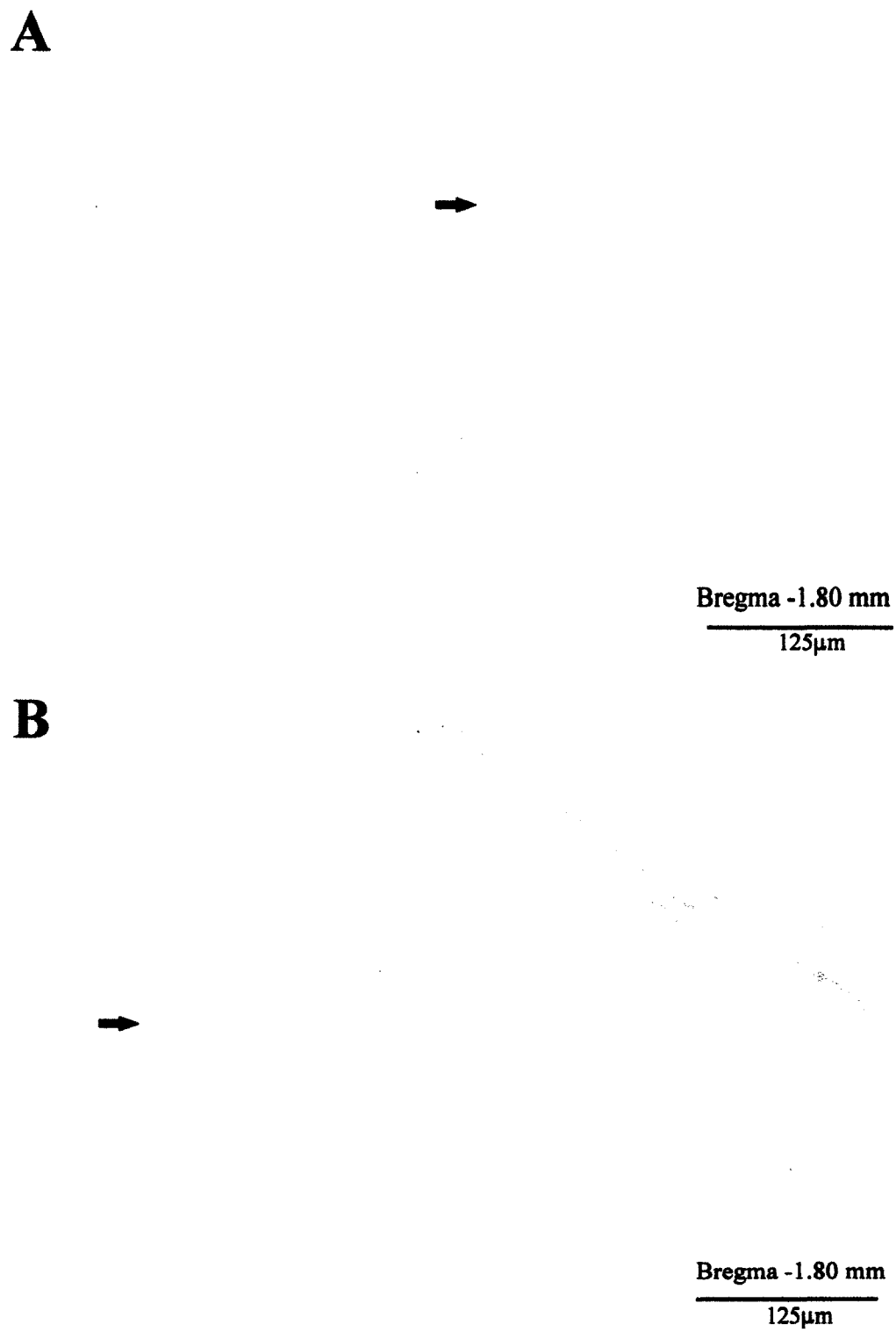


Figure 23. Immunohistochemistry staining for aromatase in the CNA. A: staining following MCAO, B: staining following sham MCAO. An example of positive staining is indicated by the arrow shown above.

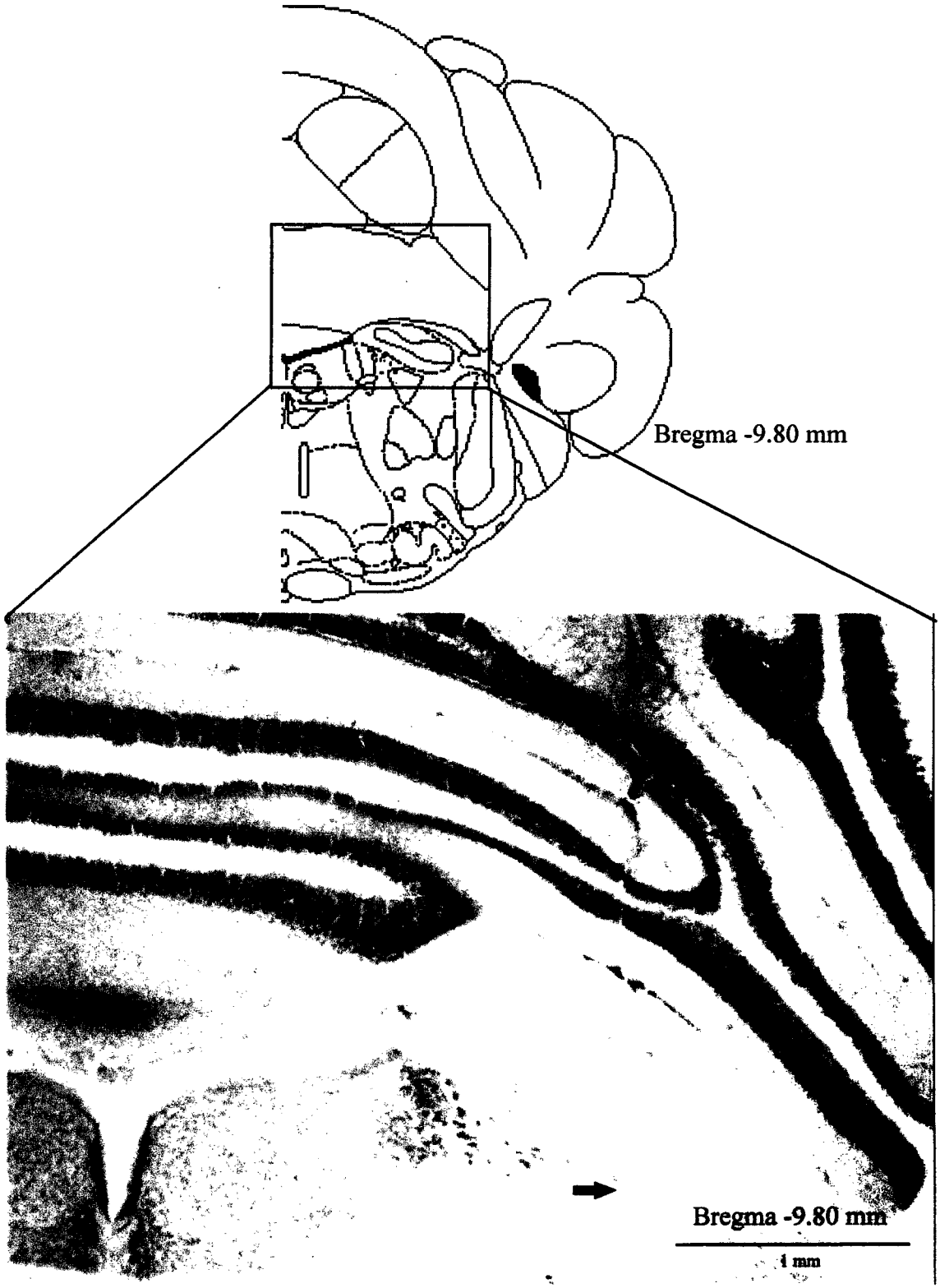


Figure 24. Dialysis probe tract in the PBN. The black arrow indicates the maximum depth reached by the dialysis probe. Unlabeled diagram of the left hemisphere shown at the top of the figure was modified from (154).

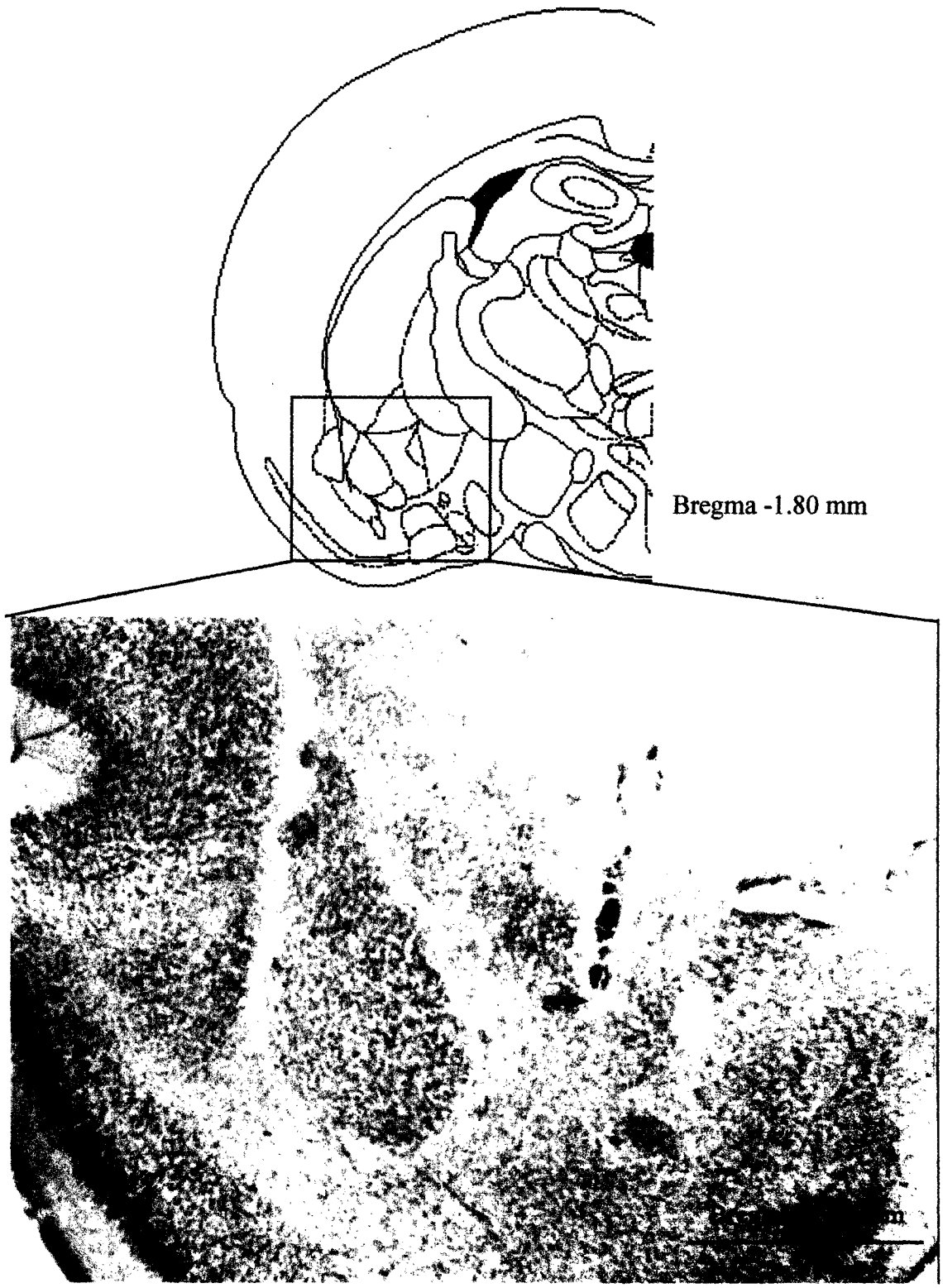


Figure 25. Dialysis probe tract in the CNA. The arrow indicates the maximum depth reached by the dialysis probe. Unlabeled diagram of the right hemisphere shown at the top of the figure was modified from (154).

Table 6. Brain hemisphere areas and infarct areas. Area of the left and right brain hemispheres were compared between each brain nucelus and letrozole treatment (paired t-test). Infarct area between brain nuclei and letrozole groups were compared using one-way ANOVA.

Dialysis probe site	Sample size (n)	Area in mm^2 of left hemisphere (Mean ± SEM)	Area in mm^2 of right hemisphere (Mean ± SEM)	P-value	Area in mm^2 of infarct (Mean ± SEM)	Infarct % of hemisphere (Mean ± SEM)	ANOVA p-value
PBN	7	4862 ± 291	5070 ± 335	P= 0.335	1143 ± 198	22.12 ± 3.42	P= 0.573
CNA	6	5227 ± 95	5380 ± 100	P= 0.156	1289 ± 362	23.87 ± 6.62	
IC infarct	5	4639 ± 228	4935 ± 187	P= 0.106	1544 ± 86	31.22 ± 0.70	
IC peri-infarct	6	4340 ± 135	4605 ± 109	**P= 0.014**	957 ± 171	20.85 ± 3.74	
PBN letrozole at -60 min	5	4485 ± 137	5057 ± 136	**P= 0.032**	1608 ± 425	31.36 ± 7.90	
PBN letrozole at MCAO	4	4529 ± 191	4643 ± 179	P= 0.633	1368 ± 271	29.63 ± 5.81	
CNA letrozole at -60 min	5	4985 ± 108	5676 ± 159	**P= 0.001**	1655 ± 270	29.32 ± 4.91	
CNA letrozole at MCAO	6	4592 ± 90	4818 ± 115	P= 0.057	1418 ± 228	29.87 ± 5.2	

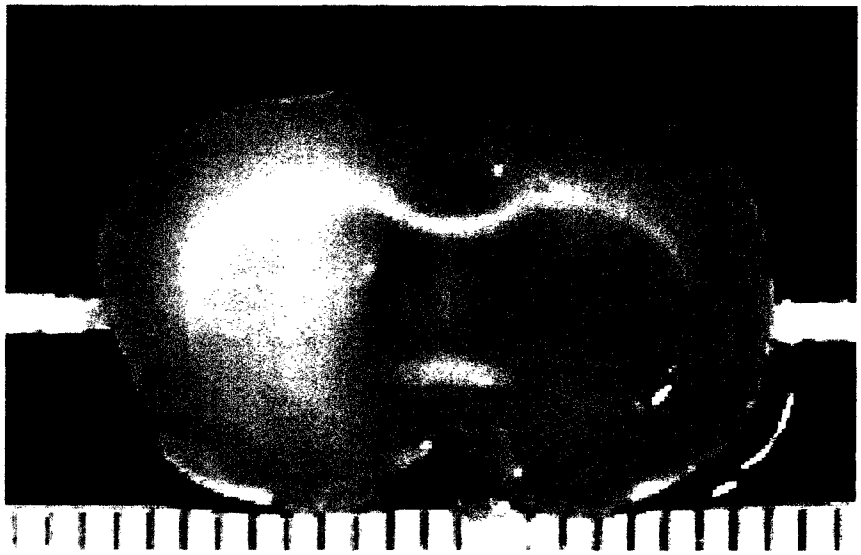
Significant differences indicated by bold text

made up a significantly ($P< 0.05$; table 6) larger percentage of the right brain hemisphere area than the IC peri-infarct (957.65 ± 171.00 mm^2; table 6) group. Similarly, the CNAl-60 MCAO group (1655.13 ± 270.14 mm^2; table 6) had significantly larger infarct areas than the IC infarct MCAO group ($P= 0.05$; table 6). Examples of a typical TTC-stained coronal section following sham MCAO and MCAO are shown in figure 26.

Chapter 4. Discussion

Previous work in our laboratory (171;172) as well as others using the rodent MCAO model of stroke (34;40;74;159) have provided evidence of a significant increase in sympathetic tone and significant decrease in both parasympathetic tone and BRS. The administration of estrogen, via intravenous injection, 30 minutes prior to MCAO entirely prevented the MCAO-induced changes in these autonomic and cardiovascular reflex from developing during the four-hour observation period (171). Local injection of estrogen into the IC reduced the size of the infarct that developed 4 hours post-MCAO when compared with saline treated controls, but did not prevent the stroke-induced cardiovascular dysfunction (172). This suggests that the stroke-induced cardiovascular dysfunction is not dependent on infarct size. It also indicates that, as discussed in the introduction, subcortical nuclei such as the PBN and CNA in the autonomic pathway may mediate the cardiovascular dysfunction observed following ischemia of the IC. Thus, it appears that estrogen plays two important roles: first, within the IC to prevent cell death and second, in subcortical autonomic nuclei to preserve autonomic function (164;165). This raised the question as to

A



B

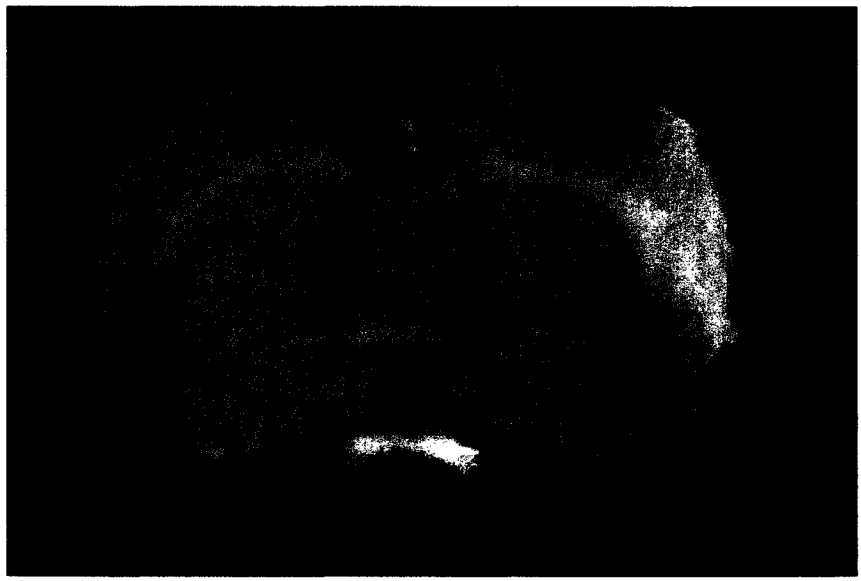


Figure 26. TTC stained coronal sections four hours following MCAO and sham MCAO. A: Representative coronal section following sham MCAO. B: Representative infarction four hours following MCAO. Infarcted area is indicated by the unstained white region shown above.

whether or not estrogen is produced and/or released locally within the IC, the PBN, and the CNA.

In order to demonstrate that any changes observed in estrogen concentrations in the brain were not the result of changes in systemic estrogen concentrations, we first measured plasma estrogen concentrations before and after MCAO and sham-MCAO in male rats. Although there was a statistical difference in estrogen concentrations with time in the MCAO group versus the sham-MCAO group, there was clearly no significant sustained increase in plasma estrogen concentrations following MCAO. If anything, there was a small drop in plasma estrogen concentrations following MCAO. The baseline circulating plasma estrogen concentrations for the intact male rats was ~ 20 ± 0.9 pg/ml, which is normal for a male rat and consistent with measurements from previous studies in our lab (175). The baseline estrogen concentrations in the MCAO-treated rats were significantly higher in the MCAO group, but as all rats were treated identically prior to the MCAO, the differences in baseline can not be attributed to the procedure. As the animals in this part of the study were the first animals who underwent the procedure, these minor differences may have resulted from some unidentified technical error in sample handling or collection. Nevertheless, these results clearly demonstrate that plasma estrogen concentrations do not increase following MCAO in male rats.

We next set out to determine if, in the absence of systemic changes in plasma estrogen concentrations, there were increases in estrogen concentrations in the IC or the subcortical nuclei (PBN and CNA). We used microdialysis to measure local changes in estrogen concentration. As mentioned previously, it must be noted that the estrogen

concentrations presented do not reflect a true quantification of extracellular estrogen concentrations. Rather, the values merely demonstrate changes in estrogen concentrations that occur during a set time period when compared to baseline concentrations established prior to MCAO or sham-MCAO using the same techniques.

In the IC infarct MCAO group, there was a significant increase in estrogen dialysate concentrations by 30 minutes post-MCAO followed by a decline to below detectable levels. This trend was similar to the pattern of change observed in the PBN. As previously described in the introduction section, within four to six minutes of onset of ischemia, the cells in the core region of the infarct are irreversibly damaged (211), and these cells may no longer be able to synthesize or release estrogen. This may explain the rapid decline of estrogen levels by 60 minutes following MCAO. In contrast to the MCAO group estrogen concentrations measured in the sham IC infarct group gradually increased over the course to the experiment. Perhaps removal of the skull (as shown in the sham group) and changing the internal pressure within the CNS, removal of the dura matter, placing of the probe into the region, or exposing the cortical tissue to the air may have caused some minor tissue injury, triggering a protective increase in local estrogen. The changes, however, suggest that the IC is capable of altering estrogen release or synthesis in response to disturbances.

A significant increase in estrogen dialysate concentrations within the IC peri-infarct zone following MCAO was also observed. In the MCAO group, there was a continuous increase in estrogen dialysate concentrations in the IC peri-infarct over the four hours. The sham IC peri-infarct group followed the same pattern of change over the course of the experiment but the increase was much less than the MCAO group and only reached

significance by 150 min and never reached the concentrations observed in the MCAO group 30 min after the MCAO. Similar to the IC infarct, perhaps removal of the skull and changing the internal pressure within the skull, removal of the dura mater, placing the probe into the region, or exposing the cortical tissue to the air may have stimulated increased estrogen synthesis and/or release in the IC peri- infarct. Like that of the IC infarct results, the changes suggest that the IC peri-infarct region also responds to disturbances by altering estrogen release or synthesis.

Results from a previous experiment in our lab provided evidence for a basal concentration of estrogen (38.0 ± 4.0 pg/ml) present in the dialysate fluid within the PBN, and showed that estrogen levels were significantly increased (64.0 ± 4.0 pg/ml; $P< 0.05$) by visceral afferent activation (175). Similar to the vagal stimulation study, baseline dialysate fluid concentrations measured in the PBN during this study were ~ 36.4 ± 0.6 pg/ml and increased to a maximum of 83.7 ± 2.3 pg/ml 10 minutes following MCAO. The PBN sham group demonstrated no changes in endogenous estrogen concentrations following sham-MCAO suggesting that the presence of the probe in this nucleus did not affect estrogen release.

There was a dramatic, continuous rise in dialysate estrogen concentrations in the CNA in the four hours following MCAO. As early as 10 minutes post-MCAO, there was a significant increase in local estrogen levels when compared to sham values. This increase continued over the course of the experiment reaching a four-fold increase by 240 minutes post-MCAO. Since the CNA sham group did not show any change in estrogen

concentrations over the course of the experiment, it can be assumed that the presence of the probe in this nucleus did not cause any changes in estrogen levels.

These results indicate that the PBN and CNA both respond to physiological disturbances by increasing synthesis or release of estrogen into the extacellular space. Together these results demonstrate that in the IC and in two important subcortical nuclei, there is a basal level of estrogen that increases in response to MCAO. One key difference, however, is that both the PBN and CNA release or synthesis considerably more estrogen in response to MCAO than the IC regions even though these areas are not directly damaged. The first experiment also demonstrates that these changes are not due to changes in peripherally circulating plasma estrogen levels since no change occurs following MCAO. Baseline estrogen dialysate concentrations in the IC infarct and peri-infarct regions were significantly lower than any other nuclei measured. Perhaps this suggests limited importance of estrogenic activity in this region under normal conditions. As indicated above, we observed very similar baseline estrogen dialysate concentrations to those previously observed. However, estrogen concentrations have not previously been measured in the CNA. It is interesting to note that the baseline concentrations of estrogen dialysate in the CNA was lower than that in the PBN (appendix C and F). Perhaps these differences in baseline concentrations reflect relative differences in the importance of estrogen as a neuromodulatory steroid under normal physiological conditions.

Regardless of the potential significance of differences in baseline dialysate concentrations of estrogen, the fact that estrogen dialysate concentrations change after MCAO in all the nuclei examined strongly suggests that estrogen plays a role in modulating

the response of brain tissue to this insult. If estrogen concentrations are changing, how are these changes being modulated? The pattern of change in extracellular estrogen concentration within the CNA is a gradual increase over time and may indicate increased synthesis of estrogen, since *de novo* estrogen synthesis requires a longer period of time. On the other hand, the rapid increase in the PBN with a subsequent decrease could be consistent with an increased release of estrogen. To assess the role of synthesis of estrogen by aromatase, we examined the effects of local administration of letrozole on the observed changes in estrogen concentrations. Letrozole is a non-steroidal aromatase inhibitor and acts by binding to a prosthetic heme group on the aromatase enzyme (91;99). A single 2.5 mg oral dose of letrozole decreased serum estrone and estradiol levels in healthy postmenopausal women by 77 and 79%, respectively; estrogen remained below baseline, even at 14 days after the single dose (91). According to Sioufi et al. (205) letrozole dissolved into a 10 ml solution and injected intravenously into healthy postmenopausal women has a half life of approximately 2 days, which is due to low intrinsic metabolic clearance. Through this mode of administration letrozole produced a plasma estrogen concentration of ~ 0.1 μM and maintained that concentration up to 8 hours following administration. It was felt that 0.1 μM concentration of letrozole dissolved in aCSF would be sufficient to attenuate estrogen synthesis in the region surrounding the dialysis probe.

The dialysis of letrozole at a concentration of 0.1 μM to the CNA resulted in inhibition of the MCAO-induced increase in estrogen concentrations at all time points and a significant decline in estrogen levels by the end of the experiment. Administration of letrozole one hour prior to MCAO prevented the significant increase in estrogen levels at 30

minutes post-MCAO (when compared to baseline) previously observed in either the no letrozole group or the letrozole administered at MCAO group. The sham groups that received letrozole, either at -60 minutes or at MCAO, resulted in a significant decline in estrogen levels over the course of the experiment. It appears that the earlier the letrozole was administered to the CNA, the more rapid the decline in estrogen synthesis. This indicates that estrogen is synthesized within the CNA and that estrogen is synthesized at a consistent rate, but MCAO results in an accelerated rate of estrogen synthesis. As a result of repeated ischemia-induced depolarizations originating in the IC and possibly terminating in the CNA following MCAO, estrogen maybe released from the terminals of the presynaptic neurons originating in the IC. This may in turn stimulate or inhibit the adjacent neurons or interneurons in the CNA. In the case of the CNA, perhaps repeated ischemia-induced depolarization in the CNA neurons may induce an up-regulation of estrogen synthesis.

The rapid increase in extracellular fluid concentrations of estrogen within the PBN, observed as early as 10 minutes post-MCAO, may be the result of increased estrogen release rather than increased synthesis. This may explain the decline observed in PBN extracellular estrogen levels following MCAO. The increase in estrogen levels at 150 minutes post-MCAO only reached 27.3 ± 1.0 pg/ml, and is perhaps due to consistent estrogen release and metabolism following MCAO. The slight increase in estrogen observed at 150 minutes post-MCAO may have been a result of an attempt to increase estrogen synthesis. Perhaps, the disruption in the autonomic balance following MCAO stimulated some estrogen synthesis and storage within the PBN. Like that of the CNA, letrozole was administered to the PBN

in order to determine if the increase in extracellular estrogen concentrations following MCAO was due to increased synthesis or storage and release of estrogen.

Letrozole did not attenuate initial increases in estrogen concentrations following MCAO. However, the letrozole inhibited the increase in estrogen concentration at 150 minutes post-MCAO following the administration of letrozole at -60 minutes and at MCAO. Following letrozole administration at -60 minutes, estrogen synthesis was likely inhibited for an hour before MCAO. This resulted in estrogen concentrations at 10 minutes post-MCAO that were significantly lower than the estrogen levels measured at the same time point in the groups receiving letrozole at MCAO. Similarly, letrozole administration to the PBN in the sham group induced a gradual decrease in basal estrogen release. This may be due to the inhibition of estrogen synthesis and the gradual depletion of estrogen stores as a result of normal estrogen turnover in the PBN. The presence of the aromatase enzyme in presynaptic boutons of neurons (15) suggest that it is possible for estrogen to be synthesized by neurons and/or astroglia cells and is then stored in vesicles within terminals of presynaptic neurons. Upon depolarization, estrogen maybe released from the terminals of the presynaptic neurons and in turn may stimulate or inhibit the adjacent neuron or interneuron. In the case of the PBN, due to repeated depolarizations originating in the infarct/penumbra region of the IC and terminating in the PBN, the vesicles within the terminals of neurons synapsing in the PBN become depleted of estrogen and perhaps other neurotransmitters/neuropeptides. If the inhibition of the aromatase enzyme prevented the increase in estrogen concentration at 150 minutes post-MCAO, this indicates estrogen synthesis within the PBN.

An increase in estrogen synthesis in the CNA and at later time points in the PBN could result from either increased substrate (testosterone) availability, removal of cofactor restrictions or other mechanisms leading to activation of enzyme activity, or increased synthesis of aromatase. To quantify the increased of aromatase enzyme immunohistochemistry was used. Based on our immunohistochemistry results, there were no significant differences between staining for the aromatase-positive cells between the sham and MCAO groups. Similarly, there were no significant changes in staining for aromatase in the PBN following MCAO. There was a slight, but not significant, increase in aromatase enzyme measured following MCAO when compared to the sham treatments. However, these results do indicate the presence of the aromatase enzyme within the PBN, and that PBN neurons are capable of *de novo* synthesis of estrogen. This has not been previously demonstrated for either the PBN or the CNA. A larger sample size may have been necessary to display any significant changes in aromatase enzyme concentrations.

Changes in the aromatase enzyme activity may not necessarily be linked to changes in aromatase enzyme concentrations within the CNA. One could speculate that the gradual increase in endogenous estrogen expression within the CNA following MCAO may be a result of increased aromatase enzyme activity rather than increased enzyme concentrations. There is generally a lag time between a signal to increase gene transcription or mRNA translation and increased the concentration of the protein, in these experiments there was not a substantial lag in the time from the insult to the increases in estrogen concentration in the CNA or PBN. Therefore, other mechanisms may be more important in the long-term

maintenance. This does not rule out increased aromatase expression as part of long-term response to stroke.

These results indicate the possible importance of estrogen in cortical and subcortical cardiovascular regulatory nuclei and suggests that some of these areas can synthesize and release estrogen. Estrogen may play an important role in regulation or protection of cardiovascular nuclei preventing cardiovascular dysfunction associated with stroke. The preceding discussion may provide some insight into estrogen's mechanisms of regulatory and/or protective effects.

The presence of ERα (4;191) and ERβ (191) subtypes in the CNA indicate potential estrogenic actions within the CNA. The results of a previous study demonstrated ERs in the CNA are required for the estrogen-mediated attenuation of the sympathoexcitation observed following MCAO (173). The introduction of an ER antagonist to the CNA prior to administration of systemic estrogen resulted in a significant attenuation (ranging from 79 to 94%) in the estrogen-induced recovery of autonomic function following MCAO (173). This suggests that estrogen-mediated recovery of autonomic function following MCAO is dependent of the availability of ERs in several forebrain and brainstem autonomic nuclei, including the CNA. Like the PBN, an ER antagonist administered to the CNA resulted in a significant decline in BRS at 60 minutes post-MCAO (173). 17β-estradiol may act in the CNA to inhibit neurotransmission in the PBN via an interaction with $GABA_A$ receptors on interneurons, which depolarize and release GABA and subsequently activate a $GABA_A$ postsynaptic receptor (174). Along with stimulating the release of GABA, estrogen has also demonstrated the ability to upregulate $GABA_A$ receptors (224). This could result in

hyperpolarization of PBN neurons (174) and subsequently decrease neuronal excitability (169), enhance parasympathetic tone and decrease sympathetic tone, thereby resulting in an enhanced BRS (164;171;174). Perhaps the increase in estrogen synthesis in the CNA following MCAO may be produced to increase or help maintain BRS for 3 to 4 hours or more. The sustained estrogen may result in hyperpolarization of PBN neurons for an extended period of time and subsequent enhancement of parasympathetic tone or a decreased sympathetic tone, thereby preventing stroke-induced pathological arrhythmias.

As suggested above, the PBN has been shown to play a key role in mediating pathology-induced autonomic dysfunction. Blockade of transmission through this nucleus with lidocaine abolishes the sympathoexcitation and increased plasma catecholamines shown previously to result from vagal afferent stimulation (166). Interestingly, peripheral administration of estrogen in male rats prevented autonomic disturbances resulting from visceral afferent stimulation (165) while ovariectomized female rats supplemented with estrogen were completely immune to the deleterious effects of vagal stimulation on autonomic tone and baroreflex function (164). Local estrogen administration to the PBN resulted in a decline neuronal excitability (169), enhance parasympathetic tone and decrease sympathetic tone, thereby resulting in an enhance BRS (164;171;174). Estrogen may bind with an ER on a postsynaptic membrane of a neuron in the PBN (174). Then, the ligand-receptor complex may interact with a $GABA_A$ receptor (174), perhaps increasing GABA and decreasing glutamate released in the PBN (167).

The role of estrogen within the PBN in modulating neuronal function is still up for debate. Many ER positive cells have been detected in the lateral PBN in the sheep brainstem

(187), and mRNA for both ER subtypes were detected in the PBN of the rat (191). Some studies have demonstrated the presence of both ERα and ERβ subtypes within the lateral and medial subnuclei of the PBN (191). The results of a previous study demonstrated that ERs in several subcortical sites, the PBN, CNA, NTS, RVLM, and spinal cord, were required for the estrogen-mediated attenuation of the sympathoexcitation observed following MCAO (173). The presence of an ER antagonist in the intrathecal space of the spinal cord, Amb, NTS, RVLM, PBN or CNA prior to administration of estrogen resulted in a significant attenuation (ranging from 79 to 94%) of the MCAO-induced autonomic dysfunction (173). This suggests that estrogen-mediated recovery of autonomic function following MCAO is dependent of the available ERs in several forebrain and brainstem autonomic nuclei. Balthazart et al. (15) hypothesized that rapidly changing levels of locally produced estrogen may be available for non-genomic regulation of neuronal physiology in a manner more akin to the action of a neuropeptide. This may further explain the rapid increase in dialysate estrogen levels as early as 10 minutes following MCAO. Toran-Allerand and colleagues (216) suggests a novel ER (ER-X), which is intracellular and expressed throughout life, and acts as a functional, plasma membrane-associated receptor enriched in caveolar-like microdomains (216). Association with caveolar-like microdomain complexes positions ER-X uniquely to interact rapidly with kinases of the MAPK cascade and other signaling pathways, including stimulation of GABA release, providing a novel mechanism for mediation of the influences of estrogen on neuronal differentiation, survival, and plasticity (216). This indicates that estrogen may stimulate the brain's natural endogenous neuroprotective response through interactions with classical ERs, novel membrane bound

ERs, or through direct activation of the $GABA_A$ receptors. This suggests that the estrogen may be able to mediated rapid protective effects on neurotransmission within the PBN via a membrane bound ER. Estrogen may act in the CNA over an extended period of time to inhibit neurotransmission in the PBN through increased estrogen synthesis as demonstrated here. The locally synthesized estrogen could possibly interact with $GABA_A$ receptors resulting in hyperpolarization of PBN neurons (174) and enhanced BRS (164;171;174). Perhaps the increase in estrogen synthesis in the CNA following MCAO may be produced to increase or to maintain BRS for 3 to 4 hours or more. The sustained estrogen synthesis may result in hyperpolarization of PBN neurons for an extended period of time thereby preventing stroke-induced pathological arrhythmias.

Of further interest, and of potential clinical significance, is the decrease in the number of ERα immunoreactive cells and the increase in ERβ immunoreactive cells in OVX female rats as compared with their estrogen-replaced counterparts (175). Perhaps estrogen from a peripheral source (such as the ovaries) plays a role in regulating ER gene expression independent of extracellular estrogen concentrations within the nuclei (175). An in situ hybridization study demonstrated a correlation between ER mRNA in the hypothalamus and serum estrogen levels in the ovariectomized rat (190). Specifically, ER mRNA was found to be very low in the ovariectomized rat and significantly increased following exogenous estrogen administration (190). The down-regulation of ERs in response to decreased serum estrogen levels originate in the forebrain and proceed towards the brain stem (164). This may provide some insight into the decline in cardiovascular regulatory protection following menopause in women.

In this study, MAP and HR were measured to determine if there were changes in these variables in response to MCAO. If changes in MAP or HR are measured, those changes may correlated with changes in estrogen concentrations in some of the above mentioned autonomic regulatory nuclei. In the IC infarct and IC peri-infarct groups there were no differences in MAP and HR between baseline or sham groups. However , there was a significant decrease with time in the IC infarct group according to the two-way ANOVA effects ($P < 0.05$). Overall there were no significant differences in baseline MAP and HR between any of the above mentioned groups. However, we should not discount the possibility that the combination of a probe in these nuclei in addition to MCAO may have altered cardiovascular homeostasis to the point that these regulatory nuclei could not compensate.

During dialysis in the PBN, there was a significant decline in MAP 30 minutes following MCAO when compared to baseline. According to the two-way ANOVA time and treatment X time effects, there were significant changes in MAP over the course of the experiment. Following the post-hoc analysis, this decrease in MAP appears to level off at the same time estrogen levels begin to decline following the peak at 10 minutes post-MCAO. Perhaps the release of estrogen following MCAO resulted in a decrease in sympathetic tone and a subsequent decrease in MAP. Unfortunately, this decrease in MAP was not observed in either of the two other PBN letrozole groups, both of which display a similar increase in estrogen following MCAO. In contrast, the decline in endogenous estrogen concentrations observed in both sham groups receiving letrozole at -60 minutes would have caused an

increase in MAP or HR in these groups (appendix A,B and C) although this phenomenon was not observed when letrozole was administered at MCAO.

Similar to the findings in the PBN, the three CNA treatment groups (no letrozole, letrozole administered at -60 min, and letrozole administered at MCAO) showed some significant changes in MAP and HR but these changes could not be correlated with any particular event (Appendix D, E and F).

In previous studies, MCAO had no measurable effect on MAP or HR even though sympathetic tone was significantly increased and parasympathetic tone was decreased (175). This is not a unique finding since several other investigations utilizing a similar protocol, were also unable to demonstrate that permanent occlusion of the right MCA had any significant effect on these cardiovascular parameters (6;50;74;228). For example, in the study conducted by Hachinski and colleagues, renal sympathetic nerve activity and plasma norepinephrine levels were significantly increased independent of a change in MAP or HR (74). Further, no correlative evidence exists in clinical studies to suggest that sympathetic activity is significantly increased following stroke in humans (135) and it is not necessarily linked with changes in baseline cardiovascular parameters (175). Also, Oppenheimer and colleagues studied a patient population with left-sided stroke involving the IC and found no significant differences in their MAP compared to controls (149). However, in a study by Robinson and colleagues (159) they found that right-sided stroke in their patient population was associated with a significantly increased HR.

Even though strokes in both rats and humans have not been associated with changes in MAP or HR, it has been shown to be associated with altered autonomic tone and

cardiovascular reflex control as measured following activation of the cardiac baroreflex (53;159). Both clinical and experimental evidence suggest that stroke results in a depressed BRS (53;159). The results of a study conducted by Saleh and colleagues (171) indicate that the depressed BRS following MCAO is due to an increase in sympathetic tone and decreased vagal tone. Estrogen administered prior to stroke prevents the attenuation of BRS, while post-stroke estrogen treatment accelerated the recovery time of the BRS (171). Estrogen receptors in the NTS and ambiguus seem to be responsible for mediating estrogen's action in preventing MCAO-induced parasympathoinhibition (173). It was also demonstrated that the MCAO-induced increase in renal sympathetic tone was the major determinant for the depression of BRS (173). Taken together, it appears that cardiovascular changes following MCAO in rats and occlusive stroke in humans is not necessarily correlated to changes in autonomic tone of autonomic reflex function.

In this study, coronal sections (at the level -0.26 mm caudal to bregma) were measured for right and left hemisphere area, as well as the infarct area. No significant differences were noted when comparing the area of the right and left hemispheres following MCAO. When comparing the mean infarct areas between all treatment groups, it was found that both the IC infarct group and the CNAl-60 developed larger infarcts than the IC peri-infarct group. Perhaps the presence of the probe in IC infarct region resulted in increase damage to the IC region and therefore resulting in a larger infarct. In the case of the CNAl-60, letrozole may have diffused outward from the CNA region into the adjacent IC area. If letrozole reached the area of the IC region, then the letrozole may have reduced the amount of estrogen present in the IC resulting in an increased infarct size.

Chapter 5. Summary and conclusion

These results indicate that stroke-induced changes in local estrogen concentrations are not dependant on the systemic estrogen supply. They also suggest that estrogen is synthesized in the brain where extracellular concentrations are regulated by a combination of synthesis and release. These results also suggest that an increase in endogenous estrogen concentration within some autonomic regulatory nuclei may be mediated through an increase in aromatase enzyme activity rather than an increase in enzyme concentration. Overall, it can be concluded that some brain regions may implement a local neuroprotective response to ischemic damage through local release or increased synthesis of estrogen.

5.1 Future perspectives

In order to fully understand how the brain regulates estrogen synthesis or release much more work needs to be done. Measuring aromatase mRNA or enzyme activity in response to MCAO will help clarify how estrogen concentrations are increased in some autonomic regulatory nuclei. Understanding the mechanism of local estrogen synthesis and release could potentially led to the development of a treatment or preventative therapy for stroke victims or potential stroke victims (120).

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Appendices

Appendix A. Comparison of MAP between letrozole treatment groups in the PBN

Sample time	MCAO			Sham		
	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO
-60	121.2±4.3 vs 110.0±4.2	121.2±4.3 vs 112.7±2.8	110.0±4.2 vs 112.7±2.8	112.5±3.9 vs 115.0±4.4	112.5±3.9 vs 115.6±5.5	115.0±4.4 vs 115.6±5.5
-30	**121.7±4.5 vs 102.6±4.4**	121.7±4.5 vs 109.0±1.6	102.6±4.4 vs 109.0±1.6	113.6±4.5 vs 117.8±3.7	113.6±4.5 vs 116.4±5.3	117.8±3.7 vs 116.4±5.3
10	**117.8±6.9 vs 101.7±5.3**	117.8±6.9 vs 110.7±2.9	101.7±3.1 vs 110.7±2.9	112.2±4.9 vs 119.4±3.3	112.2±4.9 vs 113.1±5.1	119.4±3.3 vs 113.1±5.1
30	107.9±6.2 vs 101.9±3.4	107.9±6.2 vs 112.3±4.6	101.9±3.4 vs 112.3±4.6	112.2±5.2 vs 115.6±5.3	112.2±5.2 vs 116.1±7.2	115.6±5.3 vs 116.1±7.2
60	105.7±6.3 vs 99.5±4.3	105.7±6.3 vs 112.7±4.3	99.5±4.3 vs 112.7±4.3	112.2±5.2 vs 116.7±8.8	112.2±5.2 vs 117.5±6.7	118.0±3.5 vs 117.5±6.7
90	104.3±5.9 vs 95.2±4.8	104.3±5.9 vs 112.3±1.5	95.2±4.8 vs 112.3±1.5	115.3±5.6 vs 113.1±4.6	115.3±5.6 vs 116.1±8.0	113.1±4.6 vs 116.1±8.0
120	101.7±4.3 vs 99.3±8.2	101.7±4.3 vs 109.3±3.1	99.3±8.2 vs 109.3±3.1	114.5±4.0 vs 116.9±8.2	114.5±4.0 vs 115.6±8.2	116.9±8.2 vs 115.6±8.2
150	102.6±5.6 vs 98.8±5.0	102.6±5.6 vs 111.7±1.4	98.8±5.0 vs 111.7±1.4	115.0±3.7 vs 113.9±5.7	115.0±3.7 vs 120.8±8.6	113.9±5.7 vs 120.8±8.6
180	101.2±7.3 vs 100.7±4.4	101.2±7.3 vs 110.0±1.5	100.7±4.4 vs 110.0±1.5	111.7±4.0 vs 117.2±4.5	111.7±4.0 vs 119.4±6.8	117.2±4.5 vs 119.4±6.8
210	101.9±4.4 vs 101.7±5.0	101.9±4.4 vs 107.3±4.2	101.7±5.0 vs 107.3±4.2	109.4±6.6 vs 116.4±6.3	109.4±6.6 vs 118.9±7.9	116.4±6.3 vs 118.9±7.9
240	108.6±6.3 vs 101.2±4.6	108.6±6.3 vs 109.7±2.8	101.2±4.6 vs 109.7±2.8	110.3±5.6 vs 115.6±6.7	110.3±5.6 vs 119.2±8.3	115.6±6.7 vs 119.2±8.3

Comparisons in bold indicate significant differences ($P < 0.05$)

Appendix B. Comparison of mean HR between letrozole treatment groups in the PBN

Sample time	MCAO			Sham		
	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO
-60	**343.6±8.3 vs 315.0±9.6**	343.6±8.3 vs 319.0±17.4	315.0±9.6 vs 319.0±17.4	339.2±12.8 vs 332.5±9.5	339.2±12.8 vs 348.3±17.4	332.5±9.5 vs 348.3±17.4
-30	**337.1±11.2 vs 302.9±8.6**	**337.1±11.2 vs 300.0±15.2**	302.9±8.6 vs 300.0±15.2	340.8±13.8 vs 331.7±7.6	340.8±13.8 vs 340.8±3.3	331.7±7.6 vs 340.8±3.3
10	**355.0±11.6 vs 314.3±8.6**	**355.0±11.6 vs 306.0±10.9**	314.3±8.6 vs 306.0±10.9	339.2±13.2 vs 333.3±8.7	339.2±13.2 vs 330.0±3.7	333.3±8.7 vs 330.0±3.7
30	342.1±8.2 vs 322.9±7.5	342.1±8.2 vs 310.0±10.4	322.9±7.5 vs 310.0±10.4	342.5±12.6 vs 323.3±4.9	342.5±12.6 vs 323.3±5.1	323.3±4.9 vs 323.3±5.1
60	345.7±8.4 vs 327.1±7.8	**345.7±8.4 vs 310.0±10.4**	327.1±7.8 vs 310.0±10.4	345.8±13.8 vs 325.0±11.5	345.8±13.8 vs 330.0±7.3	325.0±11.5 vs 330.0±7.3
90	345.7±8.1 vs 332.1±7.3	**345.7±8.1 vs 308.0±14.9**	332.1±7.3 vs 308.0±14.9	352.5±14.2 vs 322.5±8.7	352.5±14.2 vs 335.8±5.5	322.5±8.7 vs 335.8±5.5
120	345.7±10.2 vs 319.3±8.0	345.7±10.2 vs 313.0±8.0	319.3±8.0 vs 313.0±8.0	335.0±20.5 vs 323.3±10.9	335.0±20.5 vs 346.7±8.8	323.3±10.9 vs 346.7±8.8
150	340.7±12.1 vs 335.7±10.4	340.7±12.1 vs 319.0±12.9	335.7±10.4 vs 319.0±12.9	343.3±21.4 vs 321.7±7.4	343.3±21.4 vs 356.7±12.9	321.7±7.4 vs 356.7±12.9
180	340.7±12.1 vs 338.6±13.6	340.7±12.1 vs 326.0±17.5	338.6±13.6 vs 326.0±17.5	340.0±21.1 vs 320.8±5.8	340.0±21.1 vs 352.5±15.4	320.8±5.8 vs 352.5±15.4
210	348.6±9.4 vs 339.3±7.1	348.6±9.4 vs 315.0±20.2	339.3±7.1 vs 315.0±20.2	343.3±21.7 vs 335.8±14.9	343.3±21.7 vs 346.7±12.2	335.8±14.9 vs 346.7±12.2
240	348.6±10.6 vs 345.0±6.5	348.6±10.6 vs 329.0±14.4	345.0±6.5 vs 329.0±14.4	350.0±24.5 vs 335.8±12.8	350.0±24.5 vs 352.5±7.5	335.8±12.8 vs 352.5±7.5

Comparisons in bold indicate significant differences ($P < 0.05$)

Appendix C. Comparison of mean extracellular estrogen concentrations between letrozole treatment groups in the PBN

Sample time	MCAO			Sham		
	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO
-60	34.6±0.5 vs 36.4±1.2	34.6±0.5 vs 30.5±1.2	36.4±1.2 vs 30.5±1.2	38.7±0.9 vs 38.0±1.0	38.7±0.9 vs 35.5±2.2	38.0±1.0 vs 35.5±2.2
-30	36.4±0.6 vs 34.6±1.8	36.4±0.6 vs 33.5±2.6	34.6±1.8 vs 33.5±2.6	36.8±0.7 vs 36.0±1.0	36.8±0.7 vs 36.0±1.9	36.0±1.0 vs 36.0±1.9
10	**83.7±2.3 vs 71.3±4.8**	83.7±2.3 vs 83.8±4.6	**71.3±4.8 vs 83.8±4.6**	**35.0±1.3 vs 33.5±0.8**	35.0±1.3 vs 33.7±2.3	33.5±0.8 vs 33.7±2.3
30	52.3±0.7 vs 51.9±3.4	**52.3±0.7 vs 58.0±2.4**	51.9±3.4 vs 58.0±2.4	**36.8±1.1 vs 31.0±0.9**	36.8±1.1 vs 30.8±1.8	31.0±0.9 vs 30.8±1.8
60	**44.0±2.0 vs 35.0±1.3**	44.0±2.0 vs 43.3±2.3	**35.0±1.3 vs 43.3±2.3**	**35.0±1.7 vs 28.5±0.8**	35.0±1.7 vs 28.3±2.1	28.5±0.8 vs 28.3±2.1
90	25.6±1.2 vs 20.6±1.5	25.6±1.2 vs 23.3±1.3	20.6±1.5 vs 23.3±1.3	**36.0±1.0 vs 26.2±0.9**	36.0±1.0 vs 25.5±2.5	26.2±0.9 vs 25.5±2.5
120	17.1±0.9 vs 16.4±0.8	17.1±0.9 vs 17.5±1.2	16.4±0.8 vs 17.5±1.2	**37.7±1.1 vs 22.5±1.5**	**37.7±1.1 vs 25.5±2.7**	22.5±1.5 vs 25.5±2.7
150	**27.3±1.0 vs 13.1±1.2**	**27.3±1.0 vs 14.3±1.7**	13.1±1.2 vs 14.3±1.7	**35.8±1.3 vs 19.5±1.7**	35.8±1.3 vs 23.8±3.5	19.5±1.7 vs 23.8±3.5
180	**24.2±1.1 vs 10.1±2.1**	**24.2±1.1 vs 12.0±1.2**	10.1±2.1 vs 12.0±1.2	**36.0±1.3 vs 17.0±1.7**	36.0±1.3 vs 21.7±2.9	17.0±1.7 vs 21.7±2.9
210	**16.6±0.7 vs 7.4±1.8**	**16.6±0.7 vs 10.0±1.1**	7.4±1.8 vs 10.0±1.1	**35.2±0.9 v 14.8±1.6**	35.2±0.9 vs 20.8±3.3	14.8±1.6 vs 20.8±3.3
240	11.4±0.4 vs 6.6±1.8	11.4±0.4 vs 7.3±1.1	6.6±1.8 vs 7.3±1.1	**34.2±0.9 vs 12.5±1.6**	**34.2±0.9 vs 17.8±2.7**	12.5±1.6 vs 17.8±2.7

Comparisons in bold indicate significant differences ($P < 0.05$)

Appendix D. Comparison of MAP between letrozole treatment groups in the CNA.

Sample time	MCAO			Sham		
	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO
-60	120.5±5.4 vs 112.8±5.2	120.5±5.4 vs 118.9±3.4	112.8±5.2 vs 118.9±3.4	118.3±5.4 vs 114.3±5.0	118.3±5.4 vs 123.4±5.2	114.3±5.0 vs 123.4±5.2
-30	118.9±1.8 vs 111.4±4.0	118.9±1.8 vs 108.9±5.3	111.4±4.0 vs 108.9±5.3	114.7±5.5 vs 118.3±10.2	114.7±5.5 vs 115.0±5.1	118.3±10.2 vs 115.0±5.1
10	111.1±5.6 vs 108.6±3.1	111.1±5.6 vs 105.6±3.5	108.6±3.1 vs 105.6±3.5	114.2±4.7 vs 108.4±4.8	114.2±4.7 vs 114.7±4.7	108.4±4.8 vs 114.7±4.7
30	111.4±4.7 vs 111.4±3.6	111.4±4.7 vs 107.8±5.6	111.4±3.6 vs 107.8±5.6	118.3±6.2 vs 114.7±7.3	118.3±6.2 vs 113.6±5.1	114.7±7.3 vs 113.6±5.1
60	111.9±5.2 vs 108.1±2.2	111.9±5.2 vs 110.8±4.1	108.1±2.2 vs 110.8±4.1	115.5±4.7 vs 118.0±6.8	115.5±34.7 vs 114.5±4.0	118.0±6.8 vs 114.5±4.0
90	111.7±4.8 vs 107.2±3.7	111.7±4.8 vs 115.8±4.2	107.2±3.7 vs 115.8±4.2	115.8±4.2 vs 122.3±5.4	115.8±4.2 vs 117.2±3.1	122.3±5.4 vs 117.2±3.1
120	112.5±3.6 vs 105.6±2.7	112.5±3.6 vs 115.3±5.0	105.6±2.7 vs 115.3±5.0	119.2±3.6 vs 124.3±3.0	119.2±3.6 vs 119.5±3.0	124.3±3.0 vs 119.5±3.0
150	113.3±4.6 vs 105.6±3.5	113.3±4.6 vs 116.4±5.2	105.6±3.5 vs 116.4±5.2	123.7±4.8 vs 121.7±2.7	123.7±4.8 vs 119.7±3.6	121.7±2.7 vs 119.7±3.6
180	115.3±6.2 vs 111.4±2.9	115.3±6.2 vs 116.9±5.4	111.4±2.9 vs 116.9±5.4	121.1±5.1 vs 121.3±7.6	121.1±5.1 vs 118.9±4.9	121.3±7.6 vs 118.9±4.9
210	116.4±6.0 vs 115.3±2.6	116.4±6.0 vs 117.8±5.5	115.3±2.6 vs 117.8±5.5	126.9±6.4 vs 127.8±3.9	126.9±6.4 vs 118.3±5.2	127.8±3.9 vs 118.3±5.2
240	113.1±5.0 vs 115.9±3.5	113.1±5.0 vs 122.8±6.2	115.9±3.5 vs 122.8±6.2	127.2±6.5 vs 130.3±8.3	127.2±6.5 vs 129.2±7.6	130.3±8.3 vs 129.2±7.6

Comparisons in bold indicate significant differences ($P < 0.05$)

Appendix E. Comparison of mean HR between letrozole treatment groups in the CNA.

Sample time	MCAO			Sham		
	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO
-60	340.0±15.9 vs 330.0±15.1	340.0±15.9 vs 331.7±10.7	330.0±15.1 vs 331.7±10.7	336.7±16.3 vs 319.0±15.0	336.7±16.3 vs 340.0±11.8	319.0±15.0 vs 340.0±11.8
-30	320.8±9.3 vs 320.8±12.9	320.8±9.3 vs 311.7±5.6	320.8±12.9 vs 311.7±5.6	328.3±16.2 vs 310.0±13.0	328.3±16.2 vs 320.8±8.2	310.0±13.0 vs 320.8±8.2
10	325.8±10.7 vs 319.2±13.4	325.8±10.7 vs 308.3±4.6	319.2±13.4 vs 308.3±4.6	323.3±12.8 vs 308.0±13.9	323.3±12.8 vs 316.7±7.6	308.0±13.9 vs 316.7±7.6
30	324.2±7.4 vs 325.8±6.3	324.2±7.4 vs 300.8±5.2	325.8±6.3 vs 300.8±5.2	330.0±17.5 vs 307.0±16.7	330.0±17.5 vs 314.2±9.3	307.0±16.7 vs 314.2±9.3
60	325.0±7.3 vs 318.3±10.7	325.0±7.3 vs 305.0±4.1	318.3±10.7 vs 305.0±4.1	320.0±9.7 vs 320.0±15.5	320.0±9.7 vs 316.7±11.3	320.0±15.5 vs 316.7±11.3
90	331.7±4.8 vs 315.8±12.0	331.7±4.8 vs 324.2±9.6	315.8±12.0 vs 324.2±9.6	318.3±8.6 vs 326.0±11.7	318.3±8.6 vs 327.5±10.5	326.0±11.7 vs 327.5±10.5
120	325.8±6.1 vs 320.0±10.4	325.8±6.1 vs 325.0±6.5	320.0±10.4 vs 325.0±6.5	320.8±8.9 vs 330.0±6.3	320.8±8.9 vs 330.8±7.4	330.0±6.3 vs 330.8±7.4
150	329.2±11.1 vs 331.7±9.5	329.2±11.1 vs 331.7±8.5	331.7±9.5 vs 331.7±8.5	330.0±10.3 vs 332.0±5.8	330.0±10.3 vs 334.2±8.9	332.0±5.8 vs 334.2±8.9
180	330.0±12.6 vs 344.2±9.5	330.0±12.6 vs 333.3±9.1	344.2±9.5 vs 333.3±9.1	337.5±12.9 vs 315.0±11.4	337.5±12.9 vs 333.3±10.9	315.0±11.4 vs 333.3±10.9
210	334.2±14.4 vs 356.7±11.2	334.2±14.4 vs 340.0±11.0	356.7±11.2 vs 340.0±11.0	350.0±16.5 vs 322.0±6.6	350.0±16.5 vs 331.7±10.7	322.0±6.6 vs 331.7±10.7
240	343.3±18.2 vs 350.8±12.9	343.3±18.2 vs 340.0±11.0	350.8±12.9 vs 340.0±11.0	354.2±15.6 vs 324.0±8.1	354.2±15.6 vs 341.7±10.1	324.0±8.1 vs 341.7±10.1

Comparisons in bold indicate significant differences ($P < 0.05$)

Appendix F. Comparison of mean extracellular estrogen concentrations between letrozole treatment groups in the CNA.

Sample time	MCAO			Sham		
	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO	no letrozole vs. letrozole at -60	no letrozole vs. letrozole at MCAO	letrozole at -60 vs. letrozole at MCAO
-60	21.2±0.9 vs 22.4±2.6	21.2±0.9 vs 22.5±1.2	22.4±2.6 vs 22.5±1.2	20.2±0.7 vs 27.0±1.1	20.2±0.7 vs 21.0±1.2	27.0±1.1 vs 21.0±1.2
-30	20.8±0.5 vs 18.2±1.4	20.8±0.5 vs 22.3±1.7	18.2±1.4 vs 22.3±1.7	20.8±0.5 vs 18.2±1.5	20.8±0.5 vs 20.8±1.4	18.2±1.5 vs 20.8±1.4
10	24.5±0.9 vs 14.2±1.1	24.5±0.9 vs 25.5±0.6	**14.2±1.1 vs 25.5±0.6**	19.3±0.3 vs 16.2±2.1	19.3±0.3 vs 18.0±1.0	16.2±2.1 vs 18.0±1.0
30	**32.4±0.7 vs 7.6±3.2**	32.4±0.7 vs 29.2±2.0	**7.6±3.2 vs 29.2±2.0**	**19.8±0.4 vs 16.7±2.3**	**19.8±0.4 vs 16.4±0.5**	16.7±2.3 vs 16.4±0.5
60	**36.3±0.8 vs 4.0±1.9**	**36.3±0.8 vs 23.5±1.9**	**4.0±1.9 vs 23.5±1.9**	**23.7±0.9 vs 6.2±0.8**	**23.7±0.9 vs 14.6±0.8**	**6.2±0.8 vs 14.6±0.8**
90	**42.5±1.1 vs 1.6±1.0**	**42.5±1.1 vs 18.0±1.3**	**1.6±1.0 vs 18.0±1.3**	**22.7±1.7 vs 1.4±0.4**	**22.7±1.7 vs 11.6±1.0**	**1.4±0.4 vs 11.6±1.0**
120	**50.2±1.2 vs 0.2±0.2**	**50.2±1.2 vs 13.7±1.5**	**0.2±0.2 vs 13.7±1.5**	**24.5±1.4 vs 0.4±0.4**	**24.5±1.4 vs 8.4±1.0**	**0.4±0.4 vs 8.4±1.0**
150	**60.5±0.8 vs 0.0±0.0**	**60.5±0.8 vs 10.3±1.9**	**0.0±0.0 vs 10.3±1.9**	**23.8±1.3 vs 0.0±0.0**	**23.8±1.3 vs 4.8±1.3**	**0.0±0.0 vs 4.8±1.3**
180	**68.3±0.9 vs 0.0±0.0**	**68.3±0.9 vs 6.7±2.4**	**0.0±0.0 vs 6.7±2.4**	**24.7±2.8 vs 0.0±0.0**	**24.7±2.8 vs 3.4±0.9**	**0.0±0.0 vs 3.4±0.9**
210	**72.3±1.0 vs 0.0±0.0**	**72.3±1.0 vs 5.5±1.9**	**0.0±0.0 vs 5.5±1.9**	**23.3±1.1 vs 0.0±0.0**	**23.3±1.1 vs 0.6±0.6**	0.0±0.0 vs 0.6±0.6
240	**76.7±1.6 vs 0.0±0.0**	**76.7±1.6 vs 4.4±1.5**	0.0±0.0 vs 4.4±1.5	**22.5±0.7 vs 0.0±0.0**	**22.5±0.7 vs 0.0±0.0**	0.0±0.0 vs 0.0±0.0

Comparisons in bold indicate significant differences ($P < 0.05$)