GONADAL STEROID REGULATION OF CONNEXIN
EXPRESSION IN THE RAT BRAIN

by

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A thesis submitted with the requirements
for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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ABSTRACT

Intercellular gap junctions are formed by specialized proteins, termed connexins (Cx). In addition to effects on neuronal electrical excitability, gonadal hormones have been implicated in the modulation of connexins in the brain. The objective of this thesis was to document the expression patterns of two principal connexins in the brain, Cx43 and Cx32, in cycling female rats, castrate or hormone-treated adult female and male rats, and in neonatal rats. Androgen and/or estrogen exposure do not induce large-scale changes in brain connexin mRNA expression. In situ hybridization demonstrates that estradiol up-regulates Cx43 expression only slightly in a few, restricted regions of the brain, including the bed nucleus of the stria terminalis, the dorso-lateral septum, and the periventricular hypothalamus. A possible explanation for the restricted nature of the few significant connexin responses observed is that effects of gonadal steroids may be dependent on coincident or sequential electrical activation of the pathways involved.
Several people have been cited for having made contributions to this thesis: Grace Erb was responsible for the sequencing of the connexin43 in situ hybridization probe; Deborah Bowlby measured the serum hormone profiles reported in Figure 5; Libertad Puy performed in situ hybridization of pup brains as shown in Figure 19; Heather Edwards has been cited for her unpublished data of the regulation of connexin expression in epileptically kindled rats.

In addition, there are a number of people who I wish to acknowledge for their invaluable contributions to this thesis. First and foremost, I owe a debt of gratitude to my supervisor, Dr. Neil J MacLusky, Ph.D., for his skillful teaching and constant guidance, without which this thesis could not have been accomplished. I would also like to give special thanks to Dr. Theodore J. Brown, Ph.D. for his generosity of time, support, and mostly for his unparalleled sense of humour. I also thank Dr. Steven J. Lye, Ph. D. for helpful advice and suggestions throughout my research, and the members his laboratory, with special thanks to Angela Orsino and Grace Erb. To Julie Kim and Andreas Evangelou, I owe thanks for their lab assistance, support, and friendship. In particular, I must thank Deboeleena Roy for her 3-dimensional insight into science, life and my own insanity. A special thanks is also due to Robert “B.” Bradizza, who has helped me through the final stretch of this thesis, and has given me all the support, laughter and De Cecco pasta necessary to complete it. Furthermore, I thank my parents, Olwen and Ross Therrien, for their unflagging support and encouragement, and for having instilled in me the drive to achieve. A final thank you is extended to Margaret Wise Brown for her practical wisdom and unfailing guidance, that only she can give.
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<tr>
<td>Å</td>
<td>Ångström unit</td>
</tr>
<tr>
<td>AFP</td>
<td>α–fetoprotein</td>
</tr>
<tr>
<td>AMY</td>
<td>amygdala</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>AVPN-POA</td>
<td>anteroventral periventricular nucleus of the preoptic area</td>
</tr>
<tr>
<td>BC</td>
<td>bulbocavernosus muscles</td>
</tr>
<tr>
<td>bps</td>
<td>base pairs</td>
</tr>
<tr>
<td>BST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMTX</td>
<td>Charcot-Marie-Tooth disease</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTX</td>
<td>cortex</td>
</tr>
<tr>
<td>Cx</td>
<td>connexin</td>
</tr>
<tr>
<td>Da, kDa</td>
<td>dalton, kilodalton</td>
</tr>
<tr>
<td>5α–DHT</td>
<td>5α–dihydrotestosterone</td>
</tr>
<tr>
<td>E1, E2</td>
<td>extracellular domains of connexin protein</td>
</tr>
<tr>
<td>E2</td>
<td>estradiol</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response element</td>
</tr>
<tr>
<td>GDX</td>
<td>gonadectomized</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
</tr>
<tr>
<td>HIP</td>
<td>hippocampus</td>
</tr>
<tr>
<td>HPO</td>
<td>hypothalamus + preoptic area</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LA</td>
<td>levator ani muscles</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>LHHRH</td>
<td>luteinizing hormone releasing hormone</td>
</tr>
<tr>
<td>LSD</td>
<td>dorso-lateral septum</td>
</tr>
<tr>
<td>M1-M4</td>
<td>transmembrane domains of connexin protein</td>
</tr>
<tr>
<td>MBH</td>
<td>mediobasal hypothalamus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>OVX</td>
<td>ovariectomized</td>
</tr>
<tr>
<td>Pe</td>
<td>periventricular hypothalamic nucleus</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>PRE</td>
<td>progesterone response element</td>
</tr>
<tr>
<td>PVCP</td>
<td>periventricular caudate putamen</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>rER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>SDN-POA</td>
<td>sexually dimorphic nucleus of the preoptic area</td>
</tr>
<tr>
<td>SNB</td>
<td>spinal nucleus of bulbocavernosus</td>
</tr>
<tr>
<td>SON</td>
<td>supraoptic nucleus</td>
</tr>
<tr>
<td>TPA</td>
<td>phorbol ester tumour promoter</td>
</tr>
<tr>
<td>VAH</td>
<td>visceroatrial heterotaxia syndrome</td>
</tr>
<tr>
<td>VMN</td>
<td>ventromedial nucleus of the hypothalamus</td>
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</table>
CHAPTER 1 INTRODUCTION

Gap junctions are intercellular communication channels that are formed by specialized proteins, termed connexins. Gap junctional coupling is dynamically regulated by a variety of factors in both neural and non-neural tissues $^{16,186,225}$. Despite the extensive literature on hormonal regulation of gap junctions in the uterus $^{210,218}$, relatively little is known about how gonadal steroids affect connexins in the central nervous system (CNS). What information there is, however, suggests that CNS gap junctions may also be hormonally regulated $^{159}$. Supporting this view are recent studies that propose a possible role for electrical gap junctional coupling in the etiology of epileptic seizures $^{10,192}$, and further clinical observations of changes in epileptic seizure activity with alterations in gonadal steroid levels $^9$. The influence of gonadal steroids on epileptiform activity in both humans and animals is well documented, revealing the epileptogenic potential of estrogen, while progesterone appears to produce opposing, seizure-protective effects $^5,34,296$. The objective of this thesis was to investigate whether gonadal steroids could potentially affect neuronal activity by altering the expression of the two principal connexins found in the brain, connexin43 and connexin32. The following introduction is composed of three sections, summarizing the role of gonadal steroids in the central nervous system, the structure and regulation of gap junctions, and finally the gonadal steroid regulation of gap junctions.

ROLE OF GONADAL STEROIDS IN THE CNS

Gonadal steroid hormones play important roles in the organization of the CNS during fetal development, postnatal maturation, and adulthood. In addition to the reproductively oriented effects on the CNS, such as the control of sex behaviour and gonadotropin release, these hormones also demonstrate extensive actions on non-
Estrogens, progestins and androgens exert widespread effects on the brain at the cellular and molecular levels, which are believed to be involved in mediating the behavioural and neuroendocrine effects of these steroids.

Hormonal effects on sex-steroid sensitive neuronal structures have traditionally been categorized as either "organizational" or "activational". Organizational effects include estrogen and testosterone influences on neuronal development and neurocircuitry formation, which in the adult CNS, lead to permanent structural differences between the male and female. These sexual dimorphisms exist at many morphological levels, and include such hormone-dependent differences as nuclear volume, synaptic formation, and neuronal connectivity. Activational effects are neuronal responses which are impermanent, reversible, and dependent on the presence or absence of gonadal steroids in the animal. Traditionally, activational effects have been associated with adulthood, in which hormones exert effects on previously established neural circuits. However, it has now been established that the rat can show at least some activational responses to gonadal steroids early in life, while it remains sensitive to the organizational effects of those steroids. Activational effects in the CNS encompass a wide range of morphological as well as neurochemical parameters, including nuclear volume, neuro-glial connections and synaptic remodeling. The key difference between activational and organizational effects on neural architecture is that changes associated with activational effects are relatively short-term and dependent on the presence or absence of circulating hormones.

LOCALIZATION OF GONADAL STEROID RECEPTORS IN THE CNS

**Estrogen receptors**

Immunohistochemical and *in vitro* autoradiography studies have identified estrogen-containing neurons in specific brain regions which are known to regulate gonadotropin secretion and reproductive behaviour, namely the preoptic area (POA), hypothalamus and midbrain. In addition, *in situ* hybridization analyses have demonstrated the wide-spread distribution of neurons containing estrogen receptor (ER)
mRNA in the adult rat brain, and neonatal rat CNS. The distribution pattern of ER mRNA in the adult brain is consistent with the distribution of estrogen-concentrating cells and ER immunoreactive cells. No differences were observed in the overall pattern of estrogen binding in fetal and neonatal brains as revealed by autoradiography, but in situ hybridization profiles showed that on post-natal day 2 in rats, ER mRNA is increased in the female POA, whereas the ER mRNA in males does not change with age\textsuperscript{53}. There are several reports\textsuperscript{53,117,118} that suggest that sex differences in both preoptic area and hypothalamic ER density are at least in part the result of early androgen secretion in the male.

**Androgen receptors**

Autoradiographic studies and in situ hybridization analysis have demonstrated an extensive distribution of androgen-concentrating cells and androgen receptor (AR) mRNA-containing cells, respectively, within the mammalian nervous system\textsuperscript{230,231,237}. Many areas of the CNS express high levels of AR mRNA, including the bed nucleus of the stria terminalis, the amygdaloid complex, the hypothalamic anterior periventricular and arcuate nuclei, and the medial POA\textsuperscript{237}. Certain regions of the CNS show parallel expression patterns of both AR and ER, particularly in the hippocampus, amygdala, bed nucleus of the stria terminalis, medial POA, periventricular nucleus, arcuate nucleus, ventromedial nucleus, and periaqueductal grey matter.

The intracellular cytochrome P\textsubscript{450} enzyme aromatase converts testosterone to 17β-estradiol, the metabolite which mediates the majority of testosterone's effects within the male CNS. Testosterone-derived estradiol is crucial for the sexual differentiation of the central nervous system. Areas with the highest density of neurons containing both AR mRNA and ER mRNA also express aromatase\textsuperscript{221}. Immunohistochemical studies reveal aromatase-containing neurons in the medial and periventricular preoptic nuclei, bed nucleus of the stria terminalis, and amygdaloid nucleus during the late prenatal and early neonatal phase\textsuperscript{235,236}. Maturation of the rat is paralleled by increased aromatase activity in the lateral septum, bed nucleus of the stria terminalis and amygdaloid nucleus\textsuperscript{101}. Studies carried out by Beyer and Hutchison\textsuperscript{94,95} have demonstrated that dispersed cell
cultures generated from the brains of embryonic mice taken on the 15th day of gestation, before the presumed critical period for sexual differentiation, show a dramatic sex difference in their subsequent development of aromatase activity. Hypothalamic cultures derived from males show significantly higher levels of aromatase activity, as compared to similar cultures from the female brain. This sex difference was not observed in cultures derived from the cerebral cortex.

**THE ROLE OF TESTOSTERONE AND ESTROGEN IN THE CNS**

*Androgen and estrogen action on the developing CNS*

In the developing rat, the central nervous system is highly sensitive to the organizational effects of estrogen and testosterone. Organizational effects are primarily exerted during what is commonly referred to as the “critical period” for CNS sexual differentiation. The “critical period” is broadly defined as the species-specific developmental period during which the CNS is most sensitive to organizational effects of testosterone exposure. Exposure to the hormone either earlier or later may require supraphysiological sex steroid levels, or may be ineffective in terms of eliciting a permanent response.  

The second key concept for the current understanding of CNS sexual differentiation is the mechanism of androgen action within the brain. Testosterone is the principal sex steroid in males, and exerts cellular effects through three pathways: direct binding to AR; the enzymatic reduction of testosterone by 5α-reductase, to form 5α-dihydrotestosterone (5α-DHT) which binds to AR; and aromatization of the testosterone A-ring, by aromatase, to yield 17β-estradiol which can bind to ER. A critical component of testosterone action on the brain is mediated through testosterone-derived estrogen biosynthesis within neurons. Blockade of either estrogen biosynthesis or estrogen action interferes with normal defeminization of the brain in the male rat. However, the role of AR-mediated responses in the sexual differentiation of the brain cannot be discounted. Despite the importance of locally-formed estrogen in rats and mice, in other species estrogen may not be absolutely required for sexual differentiation.
of the CNS. For example, in female guinea pigs and monkeys, the expression of masculine sex behaviour is mimicked by prenatal treatment with the non-aromatizable androgen 5α-DHT 76.

One apparent contradiction to this estrogenic theory arises from the female developmental pattern. Despite high levels of circulating estrogen, the female CNS does not experience the same estrogen-induced masculinizing effect that is seen in the male. In fact, CNS development in the female rat requires only relatively low levels of estrogens, which contrasts with the high levels of estrogens required in the male to induce masculine differentiation, gonadotropin secretion and sexual behaviour 76. In rats and mice, a specific mechanism exists that “protects” the female fetus from the effects of circulating estrogen. Circulating α-fetoprotein (AFP), an estrogen-binding protein, is believed to protect the female CNS from these masculinizing effects of estrogen. Although AFP exhibits a high affinity for estrogen, it does not associate with androgens, thereby selectively preventing the access of estradiol to the female brain.

**Sexual dimorphic nuclei in the brain**

Structural differences between adult male and female nervous systems are sex-steroid sensitive, and have been reported in many species including the rat, zebra finch and human (Table 1) 21,113,253. In particular, three sexually dimorphic nuclei have been the focus of much investigation: the sexually dimorphic nucleus of the POA; the anteroventral periventricular nucleus of the POA; and the spinal nucleus of bulbocavernosus.

Gorski et al. (1978) demonstrated a five-fold greater nuclear volume of the sexually dimorphic nucleus of the POA (SDN-POA) in the male compared to the female. Increased neuronal numbers in the male SDN-POA are attributed to the presence of testosterone in the rat, during perinatal development. The aromatization of testosterone to 17β-estradiol seems to mediate the majority of the morphological differentiation of the nucleus 262. Prolonged administration of testosterone to female rats (embryonic day 16 through postnatal day 10) increases the volume of the adult SDN-POA to levels of the
TABLE 1. Regions of sexual dimorphism in the central nervous system. All the indicated sex differences have been identified in mammals except those in the avian song related nuclei which are found in avian song-bird species, such as the zebra finch. (Adapted from Kawata, 1995)
male rat, and similarly, castration of neonatal males reduces the volume of the adult nucleus. The SDN-POA is first apparent on embryonic day 20, yet the sex difference in SDN-POA volume is only detected on postnatal day 1. Over the course of the next ten days, the nuclear volume of the male SDN-POA continues to increase, until reaching its adult size. Females undergo insignificant increases in nuclear volume.

The anteroventral periventricular nucleus of the POA (AVPN-POA) is one of the few sexually dimorphic structures that is larger in the female than in the male. The female AVPN-POA contains more cholecystokinin-, thyroid hormone releasing factor-, corticotropin-releasing factor-, and ER-containing neurons than males. Males, however, have more enkephalin neurons in the AVPN-POA as compared to females. Neurogenesis of the AVPN-POA extends from embryonic day 13 to 18 in both sexes, and testosterone-derived estradiol may be the driving force for an apoptotic demise of the AVPN-POA in males.

In adult male rats, the spinal nucleus of bulbocavernosus (SNB) is three to four fold larger than in the female. In the neonatal female rat, the bulbocavernosus (BC) and levator ani (LA) muscles that are innervated by motoneurons of the SNB undergo atrophy, thereby resulting in a significant loss of SNB motoneurons. The adult female rat is completely devoid of either BC or LA muscles. Breedlove and Arnold (1980) reported that androgen exposure during the critical period is important for normal SNB development, by acting directly on the muscles to prevent atrophy. In the SNB of mature rats, testosterone continues to exert activational effects on neuronal somata and dendrites of motoneurons, as well as the size and frequency of gap junctional plaques between motoneurons.

**Activational effects of gonadal steroids on the CNS**

In terms of sexually differentiated neuroendocrine functions and behaviours, the activational effects of circulating gonadal steroids have long been recognized. In the adult male rat, testosterone-derived estrogen is the primary activator of masculine sex behaviours and aggression. Locally aromatized androgens stimulate full
copulatory behaviour in the male, an effect which is diminished by inhibition of estrogen synthesis in the brain \(^{179}\), or treatment with anti-estrogens. In female rats, estrogens also exert a variety of effects on the CNS, including regulation of gonadotropin and prolactin secretion, activation of sex behaviour and the stimulation of neurogenesis and synaptogenesis \(^{149}\).

Accumulating evidence suggests that the synaptogenic effects of gonadal steroids are not restricted to the perinatal period, but also exist in adulthood \(^{22}\). These activational effects of testosterone and estrogen on neuronal connectivity and structure differ from the earlier organizational effects in that they are transient and dependent on the current hormonal status of the animal. The neural actions of steroids can be classified into two distinct mechanisms. In the non-genomic mechanism, steroids effect a rapid, short-term response, which lasts seconds to minutes, and may be mediated through direct interactions with neural membranes \(^{151,163}\). Administration of gonadal steroids to brain tissue \textit{in vitro} or \textit{in vivo}, can trigger rapid changes in electrical membrane properties, synaptic transmission, or ion channel activity \(^{151,181,294}\). Alternatively, gonadal steroids can act via the classical genome mechanism, to exert long-term effects, with a time course lasting hours to days. This hormonal mechanism is thought to involve steroid activation of intracellular receptors that regulate transcription and protein synthesis. For instance, synaptic remodeling in the hypothalamic arcuate nucleus has been shown to occur in parallel with the estrous cycle of the female rat \(^{189,201}\). Further studies using deafferented arcuate nuclei demonstrated an estrogenic induction of axonal sprouting and dendritic spine formation in the adult as well as the aged brain \(^{157,158}\). In the adult male rat, castration reduces the number of synaptic inputs on motoneurons of the spinal nucleus of bulbocavernosus (SNB), which is prevented with testosterone replacement \(^{156}\). Androgens have been reported to induce synaptic remodeling in the SNB \(^{136,160}\), as well as inducing growth of perikarya and dendrites of SNB neurons \(^{26,120}\).

In addition to their neuronal effects, gonadal steroids may exert part of their effect through astroglia. It is clear that glial cells are also influenced by changes in the sex steroid environment \(^{66}\). \textit{In vitro} studies have demonstrated the presence of receptors for gonadal hormones in glial cells \(^{105,106,127}\). In fact, astroglial sexual dimorphisms have also
been shown in several brain areas, with differences in glial morphology, immunoreactivity and gene expression in vitro and in vivo. Furthermore, glial cells participate in the synthesis of endogenous steroids by the nervous system. Due to the close interactions between neurons and glial cells, it is reasonable to expect a close communication between cell types via intercellular channels and/or secretion of soluble factors. Several steps during the development of sexually dimorphic neuronal networks may be regulated by glial cells, including proliferation, survival, migration and functional maturation of neurons. Glial cells, in turn, may be affected by hormonally-driven changes in neuronal activity.

In adult female rats, astroglia participate in the phasic synaptic remodelling that takes place during the estrous cycle in the arcuate nucleus of the hypothalamus under the influence of estradiol. In adult primates, there is a hormonally-induced astrocytic ensheathing of hypothalamic neurons associated with modification of the number of synaptic inputs to hypothalamic neurons. Furthermore, astroglia appear to be involved in the development of gender differences in synaptic connectivity. Sexual dimorphisms in astroglial organization in the rat arcuate nucleus in situ are evident by postnatal day 20, and occur following the exposure to perinatal androgens. By this point of development, males already show a higher astroglial coverage of neuronal surface than in females, a difference which may be related to the reduced number of synaptic contacts associated with neuronal soma.

It is abundantly clear that gonadal steroids exert extensive effects throughout the central nervous system from the embryonic period through to senescence. In addition to the regulation of morphology, connectivity and remodeling of neurons and glia, gonadal steroids can also modulate intercellular communication between these cells. Gap junctions are a potential target for gonadal steroids in certain sex-steroid sensitive neurons and glia of the CNS. Changes in cell-cell communication processes may represent an important component of the mechanisms by which gonadal steroids exert their effects within the nervous system. The next section considers the structure and function of gap junctions in greater detail. Subsequent sections will then return
GAP JUNCTIONS

Gap junctions are specialized membrane channels that serve as sites of intercellular communication, by forming an aqueous passage for small ions and molecules between neighbouring cells. Aside from a few terminally differentiated structures (e.g. erythrocytes, lymphocytes), gap junctions have been identified in virtually all cell types in both vertebrates and non-vertebrates. In fact, structures homologous to the mammalian gap junction have been observed in higher plant cells, which use a similar communication mechanism via plasmodesmata. Gap junction membrane channels possess a high degree of symmetry, and are structurally composed of two hemichannels, or “connexons”. As one connexon comes into contact with a connexon from an adjacent cell membrane, a continuous aqueous passage forms which enables small molecules and ions to move between cells. The connexon itself is formed by a hexamer of “connexin” proteins arranged around a central pore. Current nomenclature for specific connexins is designated by the abbreviation “Cx” followed by the molecular mass in kilodaltons (kDa) as predicted by analysis of the cloned genes (e.g. Cx43).

The accepted model of the connexon has been produced by a variety of imaging techniques including thin section, negative staining, low-dose cryo-electron microscopy, X-ray diffraction, and atomic force microscopy (Figure 1). All methods consistently show the connexon to be a cylindrical water-filled structure measuring 6-6.5 nm in diameter with a pore 1.5-2 nm in diameter, and a channel 1-1.7 nm in length with less than 1.0 nm protruding into the cytoplasmic space.

Since the first description more than 30 years ago, gap junctional intercellular channels have been implicated in numerous fundamental physiological processes, including tissue homeostasis and cell growth, embryonic neural organization, and the initiation of labour and parturition. As well, aberrations in connexin expression have been implicated in human pathological and disease states, such as cardiac...
FIGURE 1. Illustration of a gap junction plaque formed between two apposing cell membranes. Each plaque is composed of a cluster of intramembranous channels. The intercellular channel forms a continuous aqueous passage extending between adjacent cell cytoplasm. Each connexon is a hexameric structure composed of six connexin proteins. (Adapted from Beyer, E.C., 1993)
malformations, oncogenesis, a demyelinating disorder, and more recently, female infertility in mice. The following chapter serves as a general overview of the structure, regulation and potential roles of gap junctions and their connexin subunits.

STRUCTURE OF CONNEXINS

Connexin gene structure

The structure of the connexin gene is remarkably similar in all members of the connexin family, and is composed of one intron of variable size, flanked by an exon sequence on either side. Exon 1 comprises the 5' untranslated region and is usually a short sequence, approximately 100 base pairs (bps) long. The second exon encodes the uninterrupted open reading frame, with the translational start codon characteristically located within the first 100 bps of exon 2.

Until relatively recently, the wide diversity of connexin proteins (i.e. at least 13 rodent connexin isoforms, Table 2) was believed not to be associated with alternative splicing, but rather was attributed to each connexin being encoded by one gene. Recently however, O’Brien et al. (1996) reported the presence of an intron in the coding region of skate Cx35. In addition, the promoters of mouse Cx26, rat and mouse Cx32 and human Cx43 have been found to contain some consensus motifs characteristic of transcriptional control. Furthermore, the Cx32 gene appears to undergo alternative splicing and uses three promoters that are activated in a cell type-specific manner. In combination, these results suggest that the same connexin, expressed in different cell types, can be regulated differently.

Chromosomal mapping has been performed on several connexin genes, from which it was concluded that genes for Cx37 and Cx40 are co-localized on human chromosome 1, Cx26 and Cx46 on human chromosome 13, and Cx32 on chromosome X. The proximity of different connexin genes may suggest similarities in expression patterns and functions, however some co-localization experiments on co-expressed connexin genes have indicated the contrary. There are not yet consistent results.
<table>
<thead>
<tr>
<th>Connexin</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26</td>
<td>Hepatocytes, pancreatic acinar cells, mammary gland alveolar cells, keratinocytes, kidney, leptomeninges, pinealocytes, intestine</td>
</tr>
<tr>
<td>Cx30</td>
<td>Brain, skin</td>
</tr>
<tr>
<td>Cx30.3</td>
<td>Kidney, skin, preimplantation embryo</td>
</tr>
<tr>
<td>Cx31</td>
<td>Kidney, keratinocytes, trophoderm, preimplantation blastocyst</td>
</tr>
<tr>
<td>Cx31.1</td>
<td>Keratinocytes, preimplantation blastocyst, squamous epithelia</td>
</tr>
<tr>
<td>Cx32</td>
<td>Hepatocytes, pancreatic acinar cells, mammary gland alveolar cells, neurons, oligodendrocytes, Schwann cells, thyroid follicular cells, proximal tubules</td>
</tr>
<tr>
<td>Cx33</td>
<td>Testes</td>
</tr>
<tr>
<td>Cx37</td>
<td>Endothelium, keratinocytes, heart, stomach, testes, cortical neuroblasts</td>
</tr>
<tr>
<td>Cx40</td>
<td>Endothelium, conductive myocardium (His bundle, Purkinje fibers), preimplantation blastocysts</td>
</tr>
<tr>
<td>Cx43</td>
<td>Cardiac myocytes, smooth muscle, endothelial cells, mammary gland myoepithelium, testes, lens and corneal epithelium, pancreatic β–cells, keratinocytes, preimplantation blastocyst, thyroid follicular cells, astrocytes, granulosa, fibroblasts, osteocytes</td>
</tr>
<tr>
<td>Cx45</td>
<td>Heart, intestine, kidney, lung, skin, preimplantation blastocyst, embryonic brain</td>
</tr>
<tr>
<td>Cx46</td>
<td>Heart, kidney, lens fibers, Schwann cells</td>
</tr>
<tr>
<td>Cx50</td>
<td>Lens fibers, corneal epithelium, atrioventricular valves</td>
</tr>
</tbody>
</table>

**TABLE 2.** Distribution of connexins in various rodent tissues  (Adapted from White and Bruzzone, 1996; Bruzzone *et al.*, 1996)
Connexin protein structure

Membrane protection assays using protease and antibody labelling techniques have illustrated the topological similarities of connexin isoforms, forming the basis of the folding structure shown by all connexins. The connexin protein traverses the membrane four times and can be broadly divided into three functional domains: the hydrophobic transmembrane domains thought to form the intercellular channel; two extracellular loops that function as cell-cell recognition and docking sites; and the cytoplasmic domain that seems to modulate the physiological properties of the channel (Figure 2).

Within the connexin molecule, there are both highly variable and conserved regions. The divergent regions comprise a major part of the cytoplasmic loop and carboxy tail, both in terms of sequence and length. X-ray diffraction measurements of Cx32 and Cx26 show that this cytoplasmic surface extends out to about 90 Å from the middle of the gap. These variable regions may confer specific channel conductances and distinct regulatory properties to different connexins. For instance, the cytoplasmic loop ranges from 37 amino acids in Cx31.1 to 70 amino acids in Cx45, while the cytoplasmic tail ranges from 18 amino acids in Cx26 to 211 amino acids in Cx50. This variability in length of the carboxy terminus may result in part from point mutations inserting or removing a stop codon. Sequences near the end of the carboxy terminals contain serine and basic amino acids which are putative phosphorylation sites.

In contrast to the variable structure of cytoplasmic domains, transmembrane regions are highly conserved regions of the connexin molecule. Each of the four transmembrane domains (M1-M4) are organized in an α-helical fashion of approximately 20 amino acids in length, as suggested by their hydrophobicity profiles. In fact, it is likely that some of the α-helical structure extends past the bilayer into the extracellular domains. The current model based on connexin sequencing, suggests...
FIGURE 2. Structural topology of a connexin protein within the plasma membrane. Each connexin protein contains 4 transmembrane, 2 extracellular, and a short N-terminal domain which are highly conserved. The cytoplasmic loop (A) and C-terminal domain (B) are variable in sequence and size among different connexins. (Adapted from Beyer, E.C., 1993)
that the putative M3 amphipathic helices from each of the six connexin subunits contribute to form the wall of the channel pore.

The two extracellular loops (E1-E2) are the most highly conserved regions of the connexin molecule, each being approximately 40 amino acids in length. Within each extracellular domain, there is a strictly conserved set of three cysteines, which form disulfide bonds within individual connexins \(^ {57,103}\). Extracellular loops are postulated to play a key role in cell-cell recognition and docking of connexon hemichannels in neighbouring membranes \(^ {222}\). Specific conserved amino acid sequences in the extracellular domains have also been implicated in voltage sensitivity \(^ {277}\), and in the binding of calcium ions for connexon docking \(^ {208}\).

**STRUCTURE AND MODULATION OF GAP JUNCTIONS**

*Formation of gap junctions*

Despite over three decades of research, the regulation of gap junction formation, stability and removal is still far from clear. There are two features of gap junction proteins, however, that contrast with other integral membrane proteins. First, connexon assembly appears to occur within the *trans*-Golgi network \(^ {188}\), a process which for other integral membrane proteins, usually takes place within the endoplasmic reticulum \(^ {93}\). Second, gap junctions display a remarkably rapid rate of turnover \(^ {61,123}\).

The assembly of connexins into gap junctions involves a lengthy and complex sequence of events: connexin oligomerization into connexons, post-translational modification, and finally transport and insertion into the plasma membrane. Then, the connexon of one cell joins in mirror symmetry with a connexin in the adjacent cell membrane to form an intercellular channel, and finally the connexons aggregate into a gap junctional plaque. Although connexin oligomerization into connexons has been clarified as an event localized to the *trans*-Golgi network \(^ {188}\), most of the late events involving connexon trafficking, docking and the formation of gap junction plaques still remain obscure.

Integration into the rough endoplasmic reticulum (rER) membrane is commonly the first event in the biosynthesis of an integral membrane protein, such as a connexin
However, \textit{in vitro} studies using dog pancreatic microsomal membranes suggest that
the insertion of connexin into the rER is accompanied by additional processing by a signal
peptidase\textsuperscript{60}. This processing is believed to occur as a result of a cryptic signal peptidase
cleavage site within the connexin sequence that is not normally used in the cell. Another
key difference between gap junction proteins and other integral membrane proteins is the
occurrence of post-rER oligomerization in the \textit{trans}-Golgi network. In general, folding
and assembly of most integral proteins occurs in the rER\textsuperscript{93}. Connexins, however, seem
to accumulate in the Golgi apparatus at least \textit{in vitro} and possibly \textit{in vivo}\textsuperscript{188}. Late
assembly of gap junction proteins may be a mechanism to avoid accidental pairing of two
connexons that could lead to the formation of intra-reticular gap junction channels.

The process of connexon transport from the Golgi complex to the membrane is
still largely unclear. Post-translational modification of the connexon protein is believed
to occur en route to the membrane surface. Puranam \textit{et al.} (1993) used rat cardiomyocyte
cultures to investigate the sequence of Cx43 phosphorylation. The initial phosphorylation
of Cx43 occurs in the rER or at the \textit{cis}-medial Golgi from the 40-kDa to the 41-kDa form,
and eventually undergoes more extensive phosphorylation to 42-kDa and 44-kDa form at
locations distal to the \textit{trans}-Golgi cisternae. It is not clear whether phosphorylation of
Cx43 occurs this early in all cell types, nor whether the reaction is critical for trafficking
or correct assembly. Phosphorylation is not essential for all connexins as there are
instances of functional non-phosphorylated connexin isoforms (i.e. Cx26).

Both docking and aggregation of connexons require communication between
adjacent cells. In order for docking of connexons to occur, the membrane of each
interacting cell must be very close together before intercellular gap junction channels can
form. Furthermore, the docking and aggregation of connexons in one cell membrane
should be matched with comparable events occurring in the apposed cell membrane.
Thus, there must be some form of interaction across the intervening extracellular space.
Accumulating evidence indicates that cell adhesion molecules (e.g. cadherins) have an
important influence on gap junction formation and communication between cells. Kanno
\textit{et al.} (1984) reported that treatment of teratocarcinoma cells with antibodies against E-
cadherin inhibits dye transfer between the cells. Alternatively, transfection of a poorly
coupled cell line with E-cadherin greatly increased coupling \(^{185}\), as demonstrated by the appearance of phosphorylated connexons in gap junction plaques in regions of cell-to-cell contact \(^{185}\). In regenerating hepatocytes, an E-cadherin/α-catenin complex provides the close intercellular contact necessary for docking of connexon hemichannels, and can act to generate an intracellular signal to initiate and regulate the synthesis, oligomerization and translocation of connexins \(^{65}\).

Following connexon docking in the membrane, most connexons remain closed until they form a complete intercellular channel with a partnered connexon \(^{188}\). In fact, after arrival at the cell surface, there is a rapid formation of intercellular channels upon cell contact. There have been reports, however, of connexons that are functionally competent to switch between open and closed states without joining to a juxtaposed connexon \(^{204,285}\), though this is not a common occurrence. Stability of gap junction channels is achieved when several channels cluster to form a gap junction plaque, the size of which can be over 1 μm in diameter. Plaque formation has also been correlated with extensive phosphorylation of Cx43 \(^{187}\). Oh \textit{et al.} (1993) showed that incompletely phosphorylated species of Cx43 could also assemble into gap junction plaques although these channels were not functional.

The removal mechanisms of gap junctions from the cell surface are poorly understood. Several studies indicate that gap junctions are removed from the membrane by internalization of the entire junction within one of the coupled cells, thereby forming "annular" gap junctions \(^{128,130}\), which eventually degrade in lysosomes \(^{129,274}\). Fujimoto \textit{et al.} (1997) present further findings from regenerating mouse hepatocytes that suggest that during the disappearance of gap junctions, most of the actual plaques are also broken up into smaller aggregates, internalized and finally degraded. Alternatively, degradation may occur via a non-lysosomal mechanism, such as an endopeptidase \(^{12}\).

The other key feature that sets gap junction channels apart from other integral membrane proteins, is their remarkably high rate of turnover \(^{300}\). Gap junctions are not static long-lived structures but actually undergo a continual process of assembly and degradation, which serves as a regulatory mechanism of intercellular communication. \textit{In}
in vivo studies with [C\(^{14}\)] bicarbonate labelling revealed half-life times of five hours for mouse liver gap junctions\(^6\). Pulse-chase experiments revealed that Cx32 and Cx43 have half-lives as short as 1.5-3.5 hours\(^1\).\(^2\).\(^6\).

Compatibility of interactions between connexins

The multiplicity of connexins that exist within each species suggests that the number of physiologically distinct channel types that could theoretically exist is immense, in the magnitude of the hundreds. In fact, most cells express more than one type of connexin, giving rise to either heteromeric (composed of two or more connexins) or homomeric (single connexin species) connexons. Further, each gap junction channel can be classified as either heterotypic (joining of two homomeric connexons made from different connexins), homotypic (two homomeric connexons made from the same connexin), or heteromeric (heteromeric channel associated with either another heteromeric connexon or homomeric connexon). Selectivity of channel formation is displayed by all members of the connexin family, and may play a critical role in the establishment of communication boundaries.

Despite the enormous potential for heterogenous gap junctions, compatibility of connexins within a channel is a limiting factor. The *Xenopus* oocyte expression system is often a model of choice to elucidate the properties of individual connexins. Several studies show that juxtapositioned cells that express different connexins have little probability of assembling gap junctions\(^2\).\(^8\)\(^4\). Most connexins are able to form channels with approximately half of their tested partners, although some extreme examples of compatibility exist: Cx31 only establishes homotypic channels, while Cx46 interacts with five out of six partners tested. The pattern of compatibility of any given connexin, however, is as yet unpredictable.

The structural locus that controls connexin compatibility has been identified as the extracellular E2 domain\(^2\).\(^8\)\(^6\). For example, Cx43 functionally interacts with Cx46 but not Cx50. Swapping the E2 domains of Cx50 with that of Cx46 inverts the ability to form heterotypic channels with Cx43, such that the Cx50 chimera with the E2 region from Cx46 is able to interact with Cx43, and the opposite is true for the Cx46 chimera.
whether all selective connexins discriminate between different partners on the basis of their E2 sequences remains to be determined.

The ability of connexins to discriminate between one another may have profound biological consequences in normal physiological and pathological situations. Multiple connexin expression may provide a powerful mechanism to limit or segregate, rather than facilitate communication, such as that seen in “communication compartments” 29. These compartments are characterized by clusters of cells that communicate within, but not between, adjacent groups of cells, and are frequently observed during developmental differentiation. The expression of incompatible connexins could achieve the compartmentalization required without compromising cell-to-cell contacts. Gap junctions expressing different connexin components demonstrate unique properties of unitary conductance, ionic permeability, size cutoff and voltage gating27,247,275.

**Control of gap junction permeability**

Traditionally, gap junctions have been thought of as relatively non-selective channels, allowing the bidirectional transfer of most ions and molecules smaller than 1000-1200 Da. However, recent evidence indicates that size selectivity is a connexin-specific feature. The molecular basis of selective ionic permeabilities between connexins has been hypothesized to be the first extracellular loop which displays differences in net charge between connexins 13,31,287. Steinberg et al. (1994) compared intercellular communication in two cell lines of osteoblastic origin, and determined that despite equivalent levels of electrical coupling, the transfer of microinjected Lucifer Yellow (457 Da) differed between cells expressing Cx43 or Cx45. Another series of experiments 276 utilized a communication-deficient cell line transfected with Cx37, Cx40, Cx43 or Cx45, in order to restore communication. Differential permeabilities were observed between cells transfected with different connexins, in relation to both 6-carboxyfluorescein (376 Da) and to chloride ions. Together these results imply that intercellular channels may exhibit much greater selectivity for small molecules and ions than previously thought.

One consequence of permeability differences is that cells expressing heteromeric connexons may display the dominant phenotype imposed by one connexin. Thus,
dynamic changes in the levels of connexin expression in one group of cells may represent an additional means to modulate their communication. Gap junction permeability has also been shown to be reversibly altered by a variety of independent factors, including intracellular calcium, pH, cyclic AMP, and connexin phosphorylation.

**Intracellular Calcium:**

Calcium (Ca\(^{2+}\)) may play a dual role with respect to gap junctions, both in gating of the channels, as well as being transferred by diffusion through the channel. Junctional uncoupling occurs following elevations in intracellular calcium concentration ([Ca\(^{2+}\)]) from 10\(^{-7}\) to 10\(^{-5}\) M\(^{142,219}\). However, while an increase in [Ca\(^{2+}\)] has been correlated with reduced gap junctional communication, this occurs at high levels of [Ca\(^{2+}\)], that are probably observed only in some coupled cells, such as heart myocytes.

There have also been numerous reports that alterations in [Ca\(^{2+}\)] and intercellular propagating waves of free Ca\(^{2+}\) may play a role in a number of other important physiological activities. Several cell types can propagate Ca\(^{2+}\) waves across communicating cells, which appears to rely on intercellular permeability to inositol 1,4,5-trisphosphate (IP\(_3\)), the Ca\(^{2+}\)-releasing messenger\(^{224,228}\). These oscillatory changes in [Ca\(^{2+}\)] may serve to coordinate neuron-glial cell interactions. Nedergaard (1994) observed a direct gap junctional communication of Ca\(^{2+}\) between astrocytes and neurons, as demonstrated by the ability of altered levels of intracellular [Ca\(^{2+}\)] to be transferred between cells. One inconsistency however, is the apparent incompatibility between astrocytic Cx43 and neuronal Cx32, as illustrated in the *Xenopus* oocyte system\(^{287}\). The lack of interaction between Cx32 and Cx43 as reported by White and colleagues, contradicted earlier reports of heterotypic channel formation by these two connexins\(^{254,283}\). The major difference between these studies was the elimination of endogenous *Xenopus* Cx38 by White *et al.* (1995), a step which prevents coupling of Cx43-injected oocytes to uninjected cells. When *Xenopus* Cx38 is eliminated by injecting oocytes with antisense oligonucleotides, cells expressing Cx43 do not demonstrate electrical coupling with cells expressing Cx32\(^{242}\).
Gap junctional sensitivity to intracellular pH varies according to the system studied. Most connexons close in response to intracellular acidification, resulting in a reduction in gap junctional intercellular communication at pH 6.5-6.8.220 This sensitivity to intracellular pH is, however, connexin dependent. The length and sequence of the middle cytoplasmic loop and carboxy terminal tail play a critical role in this form of chemical gating. Embryonic cells are most sensitive in the region of pH 7, while liver gap junctions only respond when the pH falls below 6.5.245 Further, rat Cx32 is weakly sensitive to acidification, whereas Xenopus Cx38 rapidly closes with decreasing intracellular pH. The physiological significance of these responses to changes in pH is indeterminable however, since the intracellular pH is thought to be tightly regulated at pH 7.0-7.4.91

Intracellular Cyclic AMP (cAMP):

The intracellular second messenger, cAMP, plays an integral tissue-specific role in the regulation of cell coupling. Increased levels of [cAMP] can either result in an increase in junctional conductance, as in rat hepatocytes and heart myocytes,33,227 or else reduced conductance, as in rat myometrial cells, retinal horizontal cells, and Sertoli cells.225 The reason for this range of responses to cAMP in different tissues has yet to be elucidated. Junctional permeability may be modulated by cAMP via the phosphorylation of connexin proteins through a cAMP-dependent protein kinase.

Phosphorylation of Connexins:

Phosphorylation on serine, threonine and tyrosine residues is a common mechanism used by extracellular signals to gate ion channels. Phosphorylation represents a post-translational mechanism that specifically targets these residues in the cytoplasmic tail of connexin proteins, eventually resulting in a modification of intercellular communication. The mechanism by which this type of modification alters gap junctional intercellular communication is still unclear. Although phosphorylation-induced structural modifications of connexin may function to open or close gap junction channels,186 the
Evidence for a role of phosphorylation stems from studies demonstrating that agonists or antagonists of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), calmodulin/Ca$^{2+}$-dependent kinase, and tyrosine kinases modulate gap junctional communication. The activation of these protein kinases is kinase- and connexin-dependent. Several connexins have been identified as phosphoproteins (e.g. Cx32, Cx43, Cx46, Cx50). Phosphorylation-induced changes in permeability and unitary conductance are also connexin-specific, demonstrating a positive correlation between frequency of lower conductance states and decreased permeability.

**Serine threonine phosphorylation**

The phosphorylation of connexin was first demonstrated by the metabolic labelling of Cx32 with [P$^{32}$] orthophosphate in primary hepatocyte cultures. The addition of 8-bromo-cAMP increased gap junctional conductance, and enhanced the incorporation of [P$^{32}$] into Cx32 serine residues, though without a simultaneous increase in levels of Cx32 protein. Phosphorylation of serine residues in Cx32 is also modulated by cAMP-dependent protein kinase (PKA) in isolated rat liver gap junctions. Taken together, this evidence suggests that the phosphorylation of Cx32 by PKA may play a further role in gap junctional intercellular communication. In rat hepatocytes, Cx32 has also been shown to be phosphorylated by another serine/threonine kinase, protein kinase C (PKC). The significance of PKC-mediated phosphorylation of Cx32 is questionable, however, as the issue of cell-cell coupling was not addressed.

Cx43 is another ubiquitous connexin species that is post-translationally serine phosphorylated. The serine kinase(s) that may be responsible for Cx43 phosphorylation events has not yet been identified, although there are several candidates, including protein kinase C. A number of studies have demonstrated that the activation of PKC by phorbol esters tumour promoters (TPA) is correlated with a reduction of gap junctional communication as well as the serine phosphorylation of Cx43.
The importance of the post-translational conversion of Cx43 to its fully phosphorylated form lies partly in its ability to provide junctional competence. Musil et al. (1990) performed studies using a communication incompetent cell line that constitutively synthesized Cx43, yet failed to accumulate it on the cell surface or phosphorylate it to its mature form. Transfection of the cell line with the cell adhesion molecule, E-cadherin, led to phosphorylation of Cx43 to the mature form, expression of cell surface gap junctions, and renewed communicational abilities. Contradictory studies by Musil et al. (1991) later reported that non-phosphorylated Cx43 was, in fact, able to be exported to the cell surface.

Further evidence for a role of serine phosphorylation of Cx43 comes from genetic mutations in patients with visceroatrial heterotaxia syndrome (VAH), a disease associated with cardiac abnormalities. Analysis of the Cx43 gene from these patients indicate point mutations in serine and threonine residues believed to be involved in connexin phosphorylation.

Tyrosine phosphorylation

Cx43 is specifically phosphorylated on tyrosine residues in the presence of pp60\textsuperscript{src} (v-Src), a 60,000 MW plasma-membrane associated tyrosine protein kinase encoded by a viral oncogene. This method of tyrosine phosphorylation inhibits junctional communication. Overexpression of activated forms of the cellular Src protein in NIH 3T3 cells also results in reduced gap junctional communication, and is accompanied by the rapid accumulation of phosphotyrosine on Cx43.

Furthermore, this loss of gap junctional communication was neither due to a major loss of Cx43 gap junction plaques, nor to a reduction of Cx43 expression. In fact, elevated levels of Cx43 mRNA and protein were observed in v-Src-transformed cells. The inhibition of gap junctional communication in *Xenopus* oocytes, co-expressing v-Src and Cx43 mRNA was shown to be dependent upon the phosphorylation of Cx43 on tyrosine. Another member of the Src family of tyrosine kinases, v-Fps, also disrupts gap junctional communication and stimulates the phosphorylation of Cx32 on tyrosine. These studies suggest that the
correlation between V-Src phosphorylation of tyrosine residues on Cx43 and the disruption of junctional communication, may be due to altered intercellular gating 131.

GAP JUNCTIONS IN THE BRAIN

Evidence for gap junctional coupling between the diverse cellular compartments of brain tissues has been documented by a variety of different techniques 47, including structural mapping, functional analyses, and dye coupling. Results suggest that the degree of coupling is not a static phenomenon, but is subject to plasticity regulated either by developmental or functional factors, such as neurotransmitter effects. Gap junctions and dye coupling have been documented in many brain regions including the hippocampus 10,150, cerebral cortex 77, and retina 167. There are at least six different connexins expressed and widely distributed in the mammalian brain with Cx43 expressed abundantly in astrocytes 48,174,299, and Cx32 found at lower levels in neurons and oligodendrocytes 48. Gap junctions can be dynamically regulated in the CNS. Calcium ions can reduce intercellular coupling 216, as can arachidonic acid 178, diacylglycerol 14, phorbol esters 124, and acidosis 244. The neurotransmitter, dopamine, uncoupled electrotonic junctions in the retina 167. Astroglial gap junctional communication is increased by glutamate or high potassium ion concentration 59.

The physiological relevance of gap junctions within the CNS is poorly understood 47. Gap junctions between neuronal cells are believed to mediate synchrony among active cells or rapidly relaying signals from pre- to postsynaptic elements 17,54,141. Neuronal junctional communication may also be important in second-messenger exchange between coupled cells, as indicated by the diffusional ability of Ca2+, cAMP, and IP3. Yang et al. (1990) proposed that such a mechanism could modulate presynaptic neurotransmitter release by postsynaptic second messenger molecules. Astrocytic gap junctions probably provide a direct pathway to the site of potassium ion (K+) disposal into the perivascular compartments 68,116,195. However, astrocytic coupling may also increase the volume of the buffer sink, thereby allowing the astrocytic compartment to share the load of a highly active adjacent neuron 111,116. Further studies demonstrate both induced and spontaneous
Ca²⁺ waves spreading throughout the astrocytic network, indicative of a glial communication pathway that may play an active role in neuromodulation.³⁷²,³⁷³

Neural development is associated with increased electrotonic coupling,²⁰⁶ and exhibits temporal changes in the expression of neuronal connexins, starting with Cx26 prenatally and ending with Cx32 postnatally.⁴⁸ Also, neuropathological conditions such as ischemia,⁹⁰ and spreading depression, which is associated with epilepsy,⁸⁴, have been associated with changes in gap junctional protein levels. A direct intercellular communication pathway was identified in hippocampal CA3 neurons.¹⁵⁰ Baimbridge et al. (1991) demonstrated that epileptiform bursts from depolarizing currents in CA1 neurons were seen only in dye coupled neurons. As well as the presence of junctional communication in neurons, there is evidence of gap junctions in interneurons in the hippocampal CA1 region and the dentate gyrus.¹¹⁴ Electrotonic coupling of interneurons could be critical for epileptogenesis. Naus et al. (1991) demonstrated the increased expression of Cx43 mRNA in samples of temporal lobe neocortex obtained from patients with intractable seizure disorder. Significantly lower levels of Cx43 mRNA were present in the peritumoral tissue. Similar changes were observed in Cx32 mRNA levels, although the changes were not as dramatic.

FUNCTIONAL ROLES OF GAP JUNCTIONS

The wide distribution and modulation of connexins throughout the majority of cells and species, is suggestive of their fundamental and wide-spread importance for the basic physiology of the species. Gap junctions have important roles throughout the body, including: metabolic cooperation and tissue homeostasis;⁷³,²¹²,²²² electrical coupling;⁶⁹,²⁶⁸ embryogenesis;²⁷⁸ tissue response to hormones;¹³³,²²² growth;¹⁴²,¹⁷⁰,²²²; and labour initiation and parturition.⁳⁹,¹⁷⁷ Clearly, gap junctional intercellular communication is an essential element of most animals, and recently, interest has grown as to the possible implications of aberrations in the gap junctional system.

The first creation of a connexin-deficient mouse lacked the gene for Cx43.²¹⁷ Surprisingly, the mice embryos were found to survive to term but died shortly after birth. The animals exhibited anatomical defects in the heart, where the right ventricular outflow
tract was severely enlarged and the ventricular chamber was filled with septa that prevented the normal cardiac output to the pulmonary circulation. As a result, passage of blood from the right ventricle to the pulmonary arteries was impeded, thereby preventing postnatal oxygenation. This putative link between junctional communication and cardiac development is supported by certain human familial cardiac anomalies, which are also associated with connexin mutations. Cx43 mutations have been found in patients with viscerocarial heterotaxia syndrome (VAH)\(^{28}\), a disease characterized by defects of laterality, including atrial and bronchopulmonary isomerism, transposition of the great arteries, and often asymmetries of the abdominal viscera. Analysis of the Cx43 gene from patients with VAH identified point mutations in serine and threonine residues is believed to be involved in connexin phosphorylation\(^{28}\).

More recently, Simon \textit{et al.} (1997) were able to engineer a Cx37 knock-out mouse, which exhibits female infertility. Oocytes in Cx37\(^{-/-}\) animals were found to lack both recognizable gap junctions and intercellular communication with granulosa cells. The homozygous Cx37-deficient mice failed to develop mature (Graffian) follicles, were anovulatory, and showed an inappropriate formation of corpora lutea. Furthermore, lack of junctional communication caused an arrest of oocyte development before meiotic competence was achieved. Taken together, these observations suggest that gap junctional communication may be critical for successful oogenesis and ovulation.

Abnormalities of the Cx32 gene has been associated with the X-linked form of Charcot-Marie-Tooth disease (CMTX), which is a demyelinating disorder of the peripheral nervous system\(^{18}\). The Cx32 gene in CMTX patients exhibits a total of 42 mutations, and analysis using \textit{Xenopus} oocytes indicate that these mutations are responsible for channel inactivity\(^{32}\). Despite the wide expression pattern of Cx32\(^{203}\), the clinical manifestations of CMTX are restricted to the peripheral nervous system. It is possible that, except in myelinating Schwann cells, alternative connexins may compensate for the loss of function due to Cx32 mutations. Some of these mutations, however, act as selective dominant negative inhibitors, when coexpressed in paired \textit{Xenopus} oocytes with compatible connexins\(^{32}\). If this situation occurs \textit{in vivo}, the ability of Cx32-expressing
GONADAL STEROID REGULATION OF GAP JUNCTIONS

GAP JUNCTIONS IN THE UTERUS

Gap junctions in the uterus are generally undetectable with the exception of those that are up-regulated during late gestation and parturition. For this reason, most investigations of gap junction regulation in the uterus have focused on their role in the synchronization of muscular contractions during labour. Elevated levels of gap junctions accommodate the greater need for electric activity and synchronized uterine contractions for an effective expulsion of the fetus. Recently, Risek et al. (1995) examined gap junction regulation by estrogen and progesterone in the immature rat uterus, thereby enabling a separation of the regulatory actions of estrogen and progesterone. Risek reported an estradiol up-regulation of Cx43 expression in the myometrium and endometrial stroma, which was entirely suppressed by progesterone.

Gap junctions occur in the uterus during pregnancy in a number of species, including the rat, sheep, and human. During most of pregnancy, gap junctions are undetectable. However, approximately 24-48 hours prior to labour in the rodent, gap junction Cx43 mRNA and protein levels increase dramatically in the myometrium, with maximal levels occurring during delivery itself. In fact, parturition does not commence in rats until Cx43 protein is assembled into functional gap junctions in the myometrial plasma membrane. Further studies in sheep and humans demonstrated similar increases in Cx43 mRNA and protein with the onset of labour. In both pregnant and non-pregnant animals, there is also evidence that Cx43 synthesis and/or trafficking is regulated positively by estrogen and negatively by progesterone. Similarly, Cx43 protein levels are also modulated by estrogen and progesterone in rat myometrium. Western analysis suggests that estrogen treatment is correlated with a shift from the un- and mono-phosphorylated forms of Cx43 protein, to the di-phosphorylated form. The administration of progesterone in conjunction with estrogen reduces the
estrogen-induction of Cx43 phosphorylation, and switches the banding pattern back to the unphosphorylated state. The exact mechanism of gonadal steroid action on myometrial cells to induce increased contractility and excitability during labour is unknown. Estrogen is known to directly increase transcription of the Cx43 gene as a result of interactions between the dimers of the ligand bound receptor and the putative estrogen response element (ERE) within the promoter region of the Cx43 gene. However, estrogen could also act indirectly by inducing the synthesis of nuclear transcription factors (e.g. c-myc, c-fos and c-jun). These transcription factor protein products may in turn act through the putative activator protein-1 (AP-1) sites on the promoter region of the Cx43 gene, to increase transcription of Cx43. The c-fos and c-jun genes are of particular interest since Lefebvre et al. (1995) identified an AP-1 site within the murine Cx43 promoter. The c-fos protein has a leucine zipper motif that promotes dimerization with other oncogene products, most commonly with the jun family (c-jun, jun B, and jun D). The AP-1 factor is comprised of specific combinations of the Jun and Fos protein families. Binding of these protein dimers occurs at a specific DNA AP-1 binding site, also called the TPA response element (TRE) which is present in many genes, including the murine Cx43 gene.

As outlined previously, the ability of estradiol to enhance the expression of myometrial Cx43 and its importance in parturition has been well documented. While the 5' Cx43 promoter region displays several potential EREs and is responsive to estrogen, these response elements do not conform to consensus sequences, and comparisons between rat and human 5' regions illustrate that these EREs are not well conserved. In contrast, there is one AP-1 binding site within the Cx43 promoter within 200 base pairs of the transcription start site that is highly conserved among mouse, rat and human genes. This is of interest because estrogen has been reported to increase the expression of the mRNA- and protein-encoding fos and jun in the non-pregnant uterus. Petrocelli and Lye (1993) provide associative evidence of an estrogenic up-regulation of Cx43 gene expression through a protein cascade initiated by
estrogen, resulting in the synthesis of AP-1 factors which act through Cx43 AP-1 sites to stimulate transcription. Further data consistent with this hypothesis was provided by Piersanti and Lye (1995), who demonstrated that estradiol administration to ovariectomized rats resulted in a significant increase in Cx43 mRNA, which was preceded by elevated expression of c-fos and c-jun.

The expression of c-fos and c-jun may also be subject to regulation by progesterone. The presence of putative progesterone response elements (PRE) in the mouse gene 302 may be important since estrogen-induced expression of Cx43 and the normal prepartum increase in Cx43 mRNA can be blocked by progesterone 210. Progesterone blocked the estrogen-induced increase in c-fos mRNA in whole rat uteri 112, inhibited basal c-jun expression in the chick oviduct 250, but had no effect on c-jun expression in whole rat uteri 281. Thus, the ability of the Cx43 gene to respond to both estrogen and progesterone could be conferred through the AP-1 cis-acting element. Estrogen may also regulate Cx43 expression by enhancing the stability for its mRNA 163, as suggested by the repeated expression of a sequence on the 3’ region of Cx43 mRNA, which is known to increase mRNA instability. In addition to the possibility of a direct action via PREs, progesterone may also influence Cx43 gene transcription by decreasing the synthesis of estrogen receptors, or preventing the binding of stimulatory agents to the Cx43 promoter.

GAP JUNCTIONS IN THE CNS

By comparison with the extensive literature on hormonal regulation of connexin expression in the uterus, relatively little is known about how gonadal steroids affect gap junctions in the CNS. The first demonstration of hormonal regulation of connexin mRNA expression in the central nervous system was by Matsumoto et al. (1991), who examined the androgenic regulation of gap junction mRNA expression in the androgen-sensitive motoneurons of the spinal nucleus of the bulbocavernosus (SNB). Castration of male rats was found to reduce the density of signals for hybridizable Cx32 mRNA in the SNB motoneurons, a change that could be reversed by treatment with testosterone. This elevation of gap junction transcript levels was paralleled by an androgen-induced increase
in the number and size of junctional plaques between SNB motoneurons. More recently, Micevych et al. (1996) demonstrated a dramatic up-regulation of Cx32 mRNA expression in the rat supraoptic nucleus (SON) of the hypothalamic magnocellular nucleus, during late pregnancy and lactation, both periods characterized by increased neuronal membrane apposition and dye coupling. Immediately after birth, Cx32 transcript levels decreased and were similar to levels in non-pregnant rats. On postpartum day 13, when dye-coupling between magnocellular neurons is high, Cx32 transcript levels were again very high.

Further evidence for hormonal regulation of gap junctions in the brain was demonstrated by Pérez et al. (1990), who reported that estradiol replacement in ovariectomized rats resulted in an up-regulation of gap junctions in the arcuate nucleus. This observation suggests that estrogen-sensitive neurons of the arcuate nucleus may form gap junctions in response to estradiol in order to prepare for synchronous activity, which occurs in this nucleus under the influence of estrogen. Cobbett et al. (1987) demonstrated that castration profoundly lowers the incidence of dye coupling among magnocellular paraventricular nucleus neurons in male rats, and that testosterone replacement reverses these effects.

Possible role of gap junctions in the regulation of synchronous electrical activity

Changes in neuroendocrine function and behaviour ultimately reflect integrate alterations in the electrical activity of individual neurons. Not surprisingly, therefore, numerous studies have documented changes in neuronal activity associated with gonadal steroid exposure. Most of the rapid, short-term effects of steroids are due to direct interactions with neural membranes and in some cases have been shown to involve specific membrane receptors. For example, a progesterone metabolite has been shown to bind the GABA$_A$ receptor to potentiate the GABA-activated chloride current in CA1 neurons. Accumulating evidence demonstrates that naturally occurring fluctuations in estradiol during the estrous cycle affect the excitability of brain tissue. Administration of estradiol and testosterone has also been demonstrated to alter electrical activity in the limbic system as well as hippocampal pyramidal cells in vitro.
In addition to the reports supporting an association of normal neuronal excitability with gonadal steroids, there is considerable evidence that suggests that epileptic seizure activity may also be modulated by gonadal steroids. A variety of experimental models in several species support the epileptogenic potential of estrogen, especially in the kindling model of secondarily generalized seizures. The exogenous administration of estradiol in rabbits induces an increase in spontaneous electrical spike discharges, while estradiol replacement to female rats facilitates dorsal hippocampal kindled seizure acquisition. Conversely, progesterone reduces spontaneous and induced epileptiform discharges. Relatively little is known about the influence of testosterone on neuronal excitability, although the effect does not appear uniform.

The influence of gonadal steroids on epileptiform activity in humans is well documented, revealing an estrogen seizure-activating effect, while progesterone appears to produce opposing, seizure-protective effects. During catamenial epilepsy, seizure frequency varies within the menstrual cycle, showing a positive correlation with the serum estradiol:progesterone ratio. The ratio is highest during the days prior to ovulation and menstruation and is lowest during the early and midluteal phase. Some patients experience a midcycle exacerbation of seizures, which may be attributed to the preovulatory surge of estrogen, while the premenstrual exacerbation of seizures has been attributed to the withdrawal of the antiseizure effects of progesterone. Seizures are least common during the midluteal phase when progesterone levels are highest. In many patients with catamenial epilepsy, seizures can be significantly reduced or eliminated by administering gonadotropin releasing hormone (GnRH) agonists, which act to "shut down" ovarian function. Taken as a whole, the results provide experimental and clinical evidence of a role for estradiol in the catamenial exacerbation of seizures.
HYPOTHESIS

We hypothesize that the known effects of gonadal steroids, in particular estrogen, on physiological and pathological changes in electrical excitability within the brain may involve changes in gap junction formation. These changes may involve alterations in the biosynthesis of the principal neural gap junction proteins, Cx43 and Cx32.

OBJECTIVE

The immediate objective of the work embodied in this thesis was to determine whether gonadal steroid exposure, either during development or in adulthood, might alter expression of the mRNAs encoding Cx43 and Cx32 in the brain.
As outlined above, gap junctional intercellular communication can be dynamically regulated by a variety of factors in both neural and non-neural tissues. For example, accumulating evidence suggests that the initiation of labour is closely associated with a marked increase in connexin expression in the myometrium. The up-regulation of functional gap junctions in the myometrium is thought to lead to the effective termination of pregnancy by allowing the propagation of electrical impulses throughout the uterus, thereby facilitating the synchronous muscle contractility of labour. Despite the extensive literature on hormonal regulation of gap junctions in the uterus, however, relatively little is known about how gonadal steroids affect connexins in the central nervous system. The current state of information suggests that gonadal hormones may also modulate electrical gap junctional coupling between cells in the brain, with clinical implications for epileptic seizure activity; but it remains unknown whether there are direct effects of gonadal steroids on neural connexin biosynthesis, comparable to the effects observed in the reproductive tract.

MATERIALS & METHODS

ANIMAL PREPARATION

Female and male Wistar rats (Charles River Co., St. Constance, PQ, Quebec; 250g) were housed under standard environmental conditions (14hr light/10 hr dark cycle, lights on at 0700 hrs) and fed Purina Rat Chow and water ad libitum. Rats were assigned to six different treatment groups, of which the latter three required surgical procedures: (1) cycling adult females; (2) male and female 18-hour postpartum rat pups; (3) intact adult males; (4) males one week post-gonadectomy (GDX); (5) ovariectomized (OVX) females; (6) OVX females after three days of 5% estradiol treatment. All animals were killed by decapitation, after which the uteri, heart and liver were removed and snap frozen in liquid nitrogen. The brain was quickly removed and either frozen over liquid nitrogen,
of these specific regions (cingulate cortex, hippocampus, hypothalamus + preoptic area, amygdala) were isolated and snap frozen in liquid nitrogen, depending on whether the tissue was to be used for *in situ* hybridization or Northern analysis, respectively. All tissues were stored at -80°C.

1) **Adult female cycling rats (tissues for Northern):** Adult female Wistar rats were obtained and maintained as previously described. The rats were followed with daily vaginal smears to determine the stage of the estrous cycle of the animal. After at least three consecutive 4-day cycles, the animals were sacrificed by decapitation at 10 am on the mornings of estrus, metestrus, diestrus and proestrus.

2) **Female and male rat pups (tissues for ISH):** Female and male rat pups delivered to female Wistar rats were sexed and decapitated, 18 hours post-delivery. The intact head was allowed to freeze over dry ice and was stored at -80°C. The pup heads were later cryo-sectioned for the purposes of *in situ* hybridization.

3) **Intact male rats (tissues for Northern and ISH):** Rats were not given any anaesthetic treatment or subjected to any surgery. On the morning that castrated males were sacrificed, the intact males were also killed, and tissues collected.

**Surgical Procedures** - Prior to surgery, each animal in the following three groups was anaesthetized by an i.p. injection (0.1ml/100g body weight) of a ketamine (100mg/ml, MTC Pharmaceuticals) and xylazine (20mg/ml, Miles Canada) mixture, in the ratio of 2:1.

4) **Ovariectomized female rats (tissues for Northern and ISH):** The ovaries were removed from rats through bilateral flank incisions. On the morning of the fifth day following ovariectomy, rats were lightly anaesthetized using carbon dioxide, as a sham control for estrogen capsule implantation, and promptly returned to their cages. After another three days, all animals were killed, and tissues harvested.
5) Ovariectomized, estradiol-treated female rats (tissues for Northerns and ISH): Ovaries were removed through bilateral flank incisions. On the morning of the fifth day following surgery, rats were anaesthetized using carbon dioxide and given a s.c. placed Silastic capsule (id, 1.47 mm; od, 1.96 mm; 1.0 cm length; Dow-Corning, Midland, MI) containing E2 diluted with 9 vol cholesterol. The rats were left another three days, after which the animals were sacrificed. OVX female rats treated with 5% E2 Silastic capsules for three days experience serum E2 levels of 80 pg/ml, which approximates hormonal levels observed in cycling female rats at 6pm on proestrus.

6) Castrated male rats (tissues for Northerns and ISH): Testes were removed through a midline, lower abdominal incision. After five days, rats were killed by decapitation and tissues collected.

NORTHERN ANALYSIS

Brain dissection

Brain regions (cingulate cortex, hippocampus, hypothalamus + preoptic area, amygdala) were dissected freehand using a sterile razor blade, as described by Luine et al. (1974).

Total RNA isolation

All tissues (brain regions, heart, liver and uteri) were homogenized in 1 ml of chilled TRIzol Reagent (Gibco BRL). Total RNA was extracted from tissues according to the procedure outlined in the product specifications. After homogenization of the tissue in Trizol solution, the mixture was left at RT for 5 min, after which 200μl of chloroform was added. The mixture was gently vortexed, and allowed to incubate at RT for 2-3 min. The RNA, DNA and protein phases were separated by centrifugation at 12,000xg at 4°C for 15 min, and the RNA phase was transferred to a new microfuge tube, along with 500μl of isopropanol, then incubated at RT for 15 min. The reaction mixture was centrifuged at 12,000xg at 4°C for 15 min, after which the pellet was reconstituted in 1ml of 75% ethanol, and stored at -80°C overnight. The following day, the mixture was
The RNA pellet was redissolved in DEPC-treated water, and stored at -80°C.

**RNA electrophoresis and Northern transfer**

Optical density readings were taken, in order to determine the concentration and purity of the RNA, immediately prior to loading the RNA samples onto an agarose gel. Total RNA samples (10μg) were combined with 5μl of 6X RNA tracking dye in a total volume of 25μl, and heated to 65°C for 5 min. Each gel was run with liver and heart samples which served as positive and negative controls for both Cx32 and Cx43 Northern blots. Total RNA was loaded onto a 1.2% agarose-0.66M formaldehyde denaturing gel and electrophoresed for 1-2 hours at 120-130 V. The RNA was transferred onto a Genescreen membrane (Dupont Canada Inc., Lachine, PQ) in 0.1M sodium phosphate for at least 16 hours. RNA was fixed onto the membrane by UV crosslinking (UV Stratalinker 1800, Stratagene), followed by baking at 80°C for 2 hours. The membrane was then stored in a heat-sealable bag at -20°C, prior to hybridization with labelled probe.

**Northern hybridization**

The membrane was incubated ≥ 1 hour in pre-hybridization solution (1% BSA (w/v), 0.35M sodium phosphate, 7% SDS and 30% deionized formamide) at 55°C to block non-specific binding. Hybridization was carried out using 1) Cx43 (1.3kb cDNA) probe (rat; a gift from Dr. David Paul, Department of Cell Biology, Harvard University, Boston, Ma.), 2) Cx32 (rat; 386 bp PCR product) and 3) 18S ribosomal RNA cDNA probe (a gift from Dr. D. Denhardt, Rutgers University, Piscataway, NJ). In between hybridizations with these different probes, each blot was stripped from the previous probe in 0.1% SDS, 0.1% SSC at 95°C for 30 mins.

Approximately 50ng of each probe was radio-labelled with [³²P]-dCTP by random priming (Multiprime DNA Labelling System, RPN16012, Amersham) to a specific activity of 10⁸ cpm/μg. Ammonium acetate precipitation of the radio-labelled probe was
carried out by adding 150μl STE (10mM Tris, 10mM EDTA, 0.5% SDS), 200μl 3 M ammonium acetate, 10μg tRNA and 1ml 95% ethanol to the labelled mixture. Following precipitation, the labelled probe was redissolved in the hybridization solution (final concentration of approximately 10^6 cpm/ml) and incubated with the separated RNA samples on Genescreen membranes at 55°C for 16-24 hours. Membranes were washed to a final stringency of 30mM sodium phosphate/0.1% SDS at 55°C and then exposed to X-ray film (Dupont Canada Inc., Lachine, PQ) with an intensifier screen at -80°C. Signals were visualized autoradiographically and quantified by computer-assisted densitometry.

Analysis of Cx43 and Cx32 expression was conducted by determining the relative signal intensity for these probes and that of 18S ribosomal RNA for the corresponding lane. The ratio of the optical density for the connexin and 18S signals (i.e. Cx43:18S, Cx32:18S), was calculated in order to control for possible differences in RNA loading.

Statistical analysis

Data are expressed as mean ± SEM. Data for Cx32 and Cx43 transcripts were subjected to a two-way analysis of variance (ANOVA) followed by a multiple comparison test (Student-Newman-Keuls Method) to determine between group differences. Natural log transformations of the data were performed when necessary, to correct for inhomogeneity of variance. Data for Cx32 mRNA and Cx43 mRNA transcripts in cycling female brains were subjected to a one-way ANOVA followed by the Student-Newman-Keuls test to determine differences between days of the estrous cycle. Data that could not be subjected to parametric analysis, because of residual inhomogeneity of variance that was not correctable by mathematical transformation, were analysed non-parametrically using Kruskal-Wallis One-Way ANOVA.

IN SITU HYBRIDIZATION

Glass slide subbing protocol

Frosted end, pre-cleaned glass slides (VWR Canlab) were dipped in ethanol and placed in a sterile metal staining rack. Slides were baked overnight at 280°C, cooled to
room temperature, and dipped in 0.02mg/ml poly-L-lysine (Sigma, High MW) in autoclaved 0.1% diethyl pyrocarbonate (DEPC) water. The subbed slides were dried overnight at 37°C, stored at 4°C and used within one week.

**Tissue preparation and fixation**

Tissue was removed from -80°C and allowed to equilibrate to the cryostat temperature of -26°C for at least 30 minutes. Each brain or pup head was sectioned at a thickness of 14μm in a cryostat (Reichert Jung Frigocut 2800E), and sections were freeze-thaw mounted onto poly-L-lysine coated slides. Rat heart and liver were also cryosectioned as a positive and negative control, respectively, for the Cx43 probe. All solutions for tissue fixation were made up with autoclaved 0.1% DEPC water to minimize RNA degradation. After sections were collected, slides were immersed in a Wheaton staining dish filled with 4% paraformaldehyde in 1XPBS, pH 7.4 (0.14M NaCl, 0.003M KCl, 0.01M Na₂HPO₄, 0.0018M KH₂PO₄), for 15 min at 4°C. The slide rack was transferred to a new dish filled with 3XPBS, pH 7.4 (0.41M NaCl, 0.008M KCl, 0.03M Na₂HPO₄, 0.005M KH₂PO₄) and incubated at 4°C for 5 min. This step is necessary to stop the paraformaldehyde fixation. Slides were transferred to a new dish containing 1XPBS, pH 7.4 at RT for 5 min. Sections were dehydrated through a series of 3 min incubations in 50% ethanol, 70% ethanol, 95% ethanol and 100% ethanol twice. Sections were allowed to air dry for 10-15 min, after which they were kept at RT for 2 hours-overnight in a vacuum desiccator. Slides were stored in the desiccator at RT for up to 2 days; for longer periods of time, they were stored at -80°C.

**Generation of connexin43 polymerase chain reaction product**

Complementary DNA (cDNA) from rat myometrium was generated in a 20μl reaction mixture containing 1μg of total RNA, 5ng/μl random hexamers (Pharmacia), 5U/μl MMLV reverse transcriptase (Gibco BRL), 5mM MgCl₂ (Perkin Elmer, Cetus), 1X PCR buffer (10mM Tris-HCl, 50mM KCl, Perkin Elmer, Cetus), 1mM each dNTP (dATP, dCTP, dGTP, dTTP, Pharmacia), 1U/μl RNase inhibitor (Boehringer Mannheim),
and 10mM DTT (Gibco BRL). This reverse transcription (RT) mixture was incubated at 25°C for 10 min, followed by 42°C for 30 mins, and finally heated to 99°C for 5 min.

Primers were created to specifically amplify a 294bp segment of connexin43 cDNA from rat myometrium (Primer 1: 5'-TAC CAC GCC ACC ACC GGC CCA-3', Primer 2: 3'-AAC GGA CTG CTG TCG GTT TTA CGG-5', obtained from ACGT Corporation, Toronto, Ontario). A polymerase chain reaction was performed using the total RT mixture (20µl) in a total volume of 100µl including 200µM of each deoxynucleoside triphosphate, 0.5µM of each primer, 2.5U Taq polymerase (Boehringer Mannheim) in PCR buffer (2mM MgCl₂, 50mM KCl, 10mM Tris-HCl, pH 8.3). Preincubation proceeded at 95°C for 5 min, denaturation at 95°C for 1 min, primer annealing at 60°C for 90 sec, and extension at 72°C for 1 min for 40 cycles. Cycling was completed by 7 min at 72°C to fully extend the DNA. The PCR product was electrophoresed in a 1.5% agarose gel in order to visualize amplification products. The Cx43 PCR product was sequenced by another member of the laboratory (G. Erb) in order to confirm the identity of the Cx43 PCR product. To further validate the specificity of the Cx43 probe, the Cx43 PCR product was used to probe a Northern blot of rat heart and liver tissue. The presence of a single transcript Cx43 signal in heart tissue and a lack of signal in liver tissue, indicates the Cx43 in situ hybridization probe is highly specific.

**DNA preparation for riboprobe**

The 294bp connexin43 PCR product was subcloned into the phagemid vector, pBluescript II SK (+/-) (Stratagene) and transformed into competent DH5α cells, as follows. Linearized vector product was generated from the PCR product in a 20µl reaction mixture by adding 15µl pBluescript II, 1U/µl of EcoRI (Gibco BRL), and 1/10M of React III at 37°C for 2 hours, after which the enzyme was heat inactivated at 65°C for 15min. In order to prevent re-ligation of the linear vector, the linearized pBluescript vector was dephosphorylated with 0.005U/µl alkaline phosphatase (Pharmacia) at 37°C for 30 min. The alkaline phosphatase was heat inactivated at 85°C for 15 min. Following this treatment, <1% of the vector DNA was presumed to retain terminal phosphate.
A product was electrophoresed on a 1% agarose DNA gel. The connexin43 PCR product (294 bp) was subsequently ligated into the purified, linearized vector. Equal amounts of Cx43 insert and vector DNA (0.1μg) were combined in a final volume of 7.5μl, which was heated to 45°C for 5 min, and promptly chilled to 0°C. For ligation of cohesive ends of the vector, the final reaction mix contained 1X bacteriophage T4 DNA ligase buffer (20mM Tris-Cl, pH 7.6; 5mM MgCl₂; 5mM DTT; 50μg/ml BSA), 0.1 Weiss unit of bacteriophage T4 DNA ligase, and 0.5mM ATP. The reaction was incubated at 12°C for 15 hours.

The ligation product was transformed into DH5α bacterial cells. Competent DH5α bacteria (100μl) were added to the total volume of Cx43/pBluescript ligation reaction volume (10μl), and left on ice for 30 min. The solution was heat shocked at 42°C for 2 min. Sterile LB media (400μl) was immediately added to the transformation mixture, and allowed to incubate for 1 hour at 37°C. On a premade LB-Amp plate treated with Xgal and IPTG, 100μl of the transformation reaction was spread and allowed to incubate at 37°C overnight. The following morning, white colonies were selected and grown overnight in LB media treated with 0.125mg/ml of ampicillin at 37°C.

DNA was isolated from the bacteria using a mini-prep procedure. 1.5 ml of the culture was microfuged for 20 sec at 10,000rpm. The pellet was resuspended in 350μl of sucrose solution (8% sucrose, 5% Triton X-100, 10mM Tris-HCl, pH 8, 50mM EDTA), and 25μl of 10mg/ml of lysozyme (in 10mM Tris HCl, pH 8), which was incubated at RT for 5 min. The sample was boiled for 40 sec, and spun immediately for 15 min at RT. An equal volume of isopropanol was added to the supernatant, and allowed to precipitate for at least 10 min at -20°C. The sample was centrifuged for 15 min at 4°C and the pellet was resuspended in sterile water.

An endonuclease digest using EcoRI to excise the Cx43 insert was performed on the mini-prep product to confirm if colonies containing DNA insert had been selected. The digest product was run on a 2% agarose gel. Subject to confirmation, more colonies were cultured, and a maxi-prep procedure was performed using the Qiagen protocol.
optical density measurements were performed on the DNA.

Directional antisense and sense Cx43 probes were prepared, the orientation of which was based on previous sequencing reactions. The Cx43 probe was linearized for later use as the antisense probe using SpeI, while linearization for a sense probe was generated using EcoRV. A reaction volume of 20μl included 5μg of maxi-prepped Cx43 DNA, 1U/μl restriction endonuclease enzyme and 1/10M Reaction buffer. The reaction mixture was incubated at 37°C for 1.5 hours, after which the contents were treated with 10μg of proteinase K in order to destroy any contaminating proteins. Samples were electrophoresed on a 2% agarose gel in order to confirm completion of linearization, and purified using Geneclean.

cRNA transcription reaction

Transcription of Cx43 DNA into a [S\textsuperscript{35}] radiolabelled riboprobe (RNA Transcription Kit, Stratagene) was performed using corresponding RNA primer polymerase sites on either side of the Cx43 insert according to the Bluescript vector map. A reaction volume of 25μl included final concentrations of 1X transcription buffer, 1μg restricted, proteinase-K treated Cx43 DNA template, 0.4mM each of rNTPs (rATP, rCTP, rGTP), 0.003M DTT, 4μCi/μl [S\textsuperscript{35}]-rUTP (Amersham), and 0.4U/μl of RNA polymerase (T3 polymerase for sense and T7 polymerase for antisense). After incubating the reaction mixture at 37°C for 1 hour, unincorporated nucleotides were removed using a Sephadex G-50 Quick Spin Column (Boehringer Mannheim).

Pretreatment of in situ hybridization slides

Sections that had been stored at -80°C after fixation, were removed from the freezer and kept in a vacuum desiccator at 4°C until completely thawed and dry (1 hour). Slides were immersed in a solution of 0.1% Triton and 2XSSC (0.3M NaCl, 0.003M sodium citrate) at RT for 30 min. Slides were washed twice in 2XSSC, for 5 min and 3 min respectively, followed by washes in 4% PFA in 1XPBS (pH 7.4) for 5 min, and
1XPBS for 5 min. Acetylation was performed using 0.1M triethanolamine + 0.25% acetic anhydride solution for 10 min to reduce non-specific binding of probe, followed by two washes for 2 min in 2XSSC. The tissue was dehydrated in ascending concentrations of fresh ethanol for 3 min each of 50% ethanol, 70% ethanol, 95% ethanol and 100% ethanol twice. Slides were allowed to air dry for 10 min and stored in a vacuum desiccator for 2-16 hours.

**Probe application and hybridization**

Hybridization solution was prepared by combining the riboprobe mixture and hybridization buffer in the ratio of 1:4 respectively. The riboprobe mixture contained 0.5 mg/ml tRNA, 10mM DTT, and [S\(^{35}\)]-labelled Cx43 riboprobe (adjusted to 10,000,000 cpm/ml of hybridization solution). The hybridization buffer contained 62.5% (w/v) redistilled formamide, 12.5% (w/v) Dextran sulfate, 0.375M NaCl, 1.25X Denhardt’s solution (50X stock: 1g each Ficoll, polyvinylpyrrolidone, BSA Fraction V, made up to 100ml), 0.0125M Tris, pH 8 and 0.0025M EDTA, pH 8. The hybridization solution mixture was heated to 65°C for 10 min to melt any annealed strands, and 75μl was applied directly to a 50 x 22 mm coverslip, onto which the slide bearing the tissue sections was gently applied. This method of application minimized air bubbles trapped over tissue sections. The slides were incubated overnight in a 58°C incubator, in a well humidified tray containing 5XSSC + 50% formamide.

**Post-hybridization treatment**

After 16-20 hours in the 58°C incubator, slides were removed and allowed to equilibrate to RT for 30 min. Slides were incubated in 4XSSC (0.6M NaCl, 0.06M sodium citrate) for 20-30 min with gentle agitation, in order to soak off the coverslips, then washed for 4 X 5 min in fresh 4XSSC. An RNAse digestion (RNAse A 20μg/ml, 0.5M NaCl, 0.01M Tris, 0.001M EDTA) at 37°C for 30 min removed most of the nonspecifically bound probe. Slides were then dipped in: 2XSSC/10mM DTT twice for 5 min; 1XSSC/10mM DTT for 10 min; 0.5XSSC/10mM DTT for 10 min; 0.1XSSC/10mM
Sections were dehydrated through a series of 3 min incubations in 50% ethanol, 70% ethanol, 95% ethanol and 100% ethanol twice, allowed to air dry 10 min and stored in a vacuum desiccator for at least 20 min.

The slides were exposed to Kodak Biomax MR High Resolution Autoradiography Film at RT for 2-3 days. Cx43 riboprobe signals were detected by autoradiography, and quantified by computer-assisted densitometry. Analysis of Cx43 mRNA expression was conducted by determining the relative optical density of various brain regions

**Emulsion Autoradiography**

For higher resolution analysis to distinguish individual cells, Kodak NTB-2 liquid autoradiography emulsion was used. Under a sodium vapour lamp, the emulsion was diluted with 600 mM ammonium acetate, and slides dipped while the emulsion was maintained at 42°C. Slides were dried at RT, and stored at 4°C in a light-proof container for 15 days. Development of the emulsion dipped slides was carried out for 3 min at 20°C in Kodak D-19 developer, diluted 1:3 with water. Slides were then washed for 30 sec in 2% acetic acid and fixed for 4 min in 5% sodium thiosulfate. Slides were washed in 20°C water for 20 min, and dehydrated by 2 min dips in 50%, 70%, 95%, and 100% ethanols. The slides were left in another 100% ethanol dip for 1 hour, followed by immersion in cresyl violet stain for 8 minutes, and a brief dip in 95% and 100% ethanol. The slides were then cleared in 3X xylene and coverslipped with Permount (Fisher).

**Statistical analysis**

All data are presented as mean ± SEM. Data were subjected to a two-way analysis of variance (ANOVA) followed by a multiple comparison test (Student-Newman-Keuls Method) to determine between group differences. Natural log transformations of the data were performed, where necessary, to correct for inhomogeneity of variance. Differences were considered significant at a level of p<0.05 (two-tailed).
CYCLING FEMALE RATS

Northern analysis of Cx43 and Cx32 transcript levels

To address the possibility of changes in connexin mRNA expression in the female rat during the four day estrous cycle, Cx32 and Cx43 transcript levels were quantified. Northern blots were performed on the cingulate cortex, hippocampus, amygdala, and hypothalamus/preoptic area dissected from female rats during estrus, metestrus, diestrus and proestrus. Data are shown in Figures 3 and 4. To facilitate comparison across different stages of the cycle, data are expressed relative to the values for the Cx43:18S ratio at metestrus. Cx32 transcript levels demonstrated no significant changes in any of the brain regions (hippocampus: $F_{(3,15)} = 1.28; p=0.318$ amygdala: $F_{(3,20)} =3.27; p=0.043$ hypothalamus/preoptic area: $F_{(3,17)} =0.173; p=0.913$). However, Northern analysis of Cx43 transcript levels demonstrated small, but statistically significant differences in the amygdala of the cycling female rat (Student-Newman-Keuls, $p<0.05$). There was a significant reduction of Cx43 mRNA expression during estrus, diestrus and proestrus, with respect to metestrus. There were no other significant differences observed in other tissues during the estrous cycle (cortex: $F_{(3,17)} =0.871; p=0.475$ hippocampus: $F_{(3,18)} =1.2; p=0.339$ hypothalamus/preoptic area: $F_{(3,2)} =0.655; p=0.589$)

These preliminary data suggested that there might be small regional changes in connexin43 expression in the brain, associated with the fluctuating levels of gonadal steroid hormones experienced during the female reproductive cycle. There are three principal hormonally-active steroids secreted by the ovary of the rat during the estrous cycle: estradiol, progesterone and testosterone. The concentrations of these steroids fluctuate during the estrous cycle in a highly predictable fashion. Figure 5 shows the serum levels of estradiol, progesterone and testosterone in this strain of rats, during the 4-day estrous cycle, measured previously by another member of this laboratory (D. Bowlby). Estradiol rises sharply over diestrus and proestrus, falling to undetectable levels at estrus. Progesterone rises at estrus, peaking at metestrus, while testosterone follows a pattern resembling that of estradiol. We hypothesized that the preliminary findings
obtained in cycling animals might reflect regional changes in connexin mRNA expression induced by one, or more, of the ovarian steroids. To investigate this hypothesis, further studies were performed using Northern analysis and *in situ* hybridization, in GDX rats treated with replacement doses of gonadal steroids. In addition, in view of the much higher circulating levels of testosterone experienced by males as compared to female rats, and the previously cited evidence for an effect of testosterone in males on spinal cord connexin expression \(^{159}\), we also compared intact and GDX males.
FIGURE 3. Composite graph showing mean (±) SEM of connexin32 transcript levels in different regions of cycling female rats, as determined by Northern analysis (n=4-6). Values are expressed as a percentage of the mean metestrus Cx32:18S. Cx32 transcript levels are not significantly different over the four day cycle.
FIGURE 4. Composite graph showing mean (±) SEM of connexin43 transcript levels in different regions of cycling female rats, as determined by Northern analysis (n=4-6). Values are expressed as a percentage of the mean metestrus Cx43:18S. * indicates significant difference versus metestrus (Kruskall-Wallis one way ANOVA; p<0.05).
FIGURE 5. Serum estradiol, testosterone and progesterone concentrations in cycling female rats sacrificed at 10 am on diestrus (D), proestrus (P), estrus (E), and metestrus (M). Results represent means ± SEM in 4-5 observations in each case. * indicates significant difference versus metestrus (Newman-Keuls Multiple Range test; p<0.05).
Northern analysis of Cx43 mRNA and Cx32 mRNA

In order to investigate gonadal hormonal influences on connexins in male and female rats, Northern analyses were performed on animals in the presence (OVX females with E₂ treatment; intact males) and absence of hormones (GDX males and females). Figure 6 demonstrates a typical Northern blot hybridized with Cx32 cDNA, Cx43 cDNA, and 18S ribosomal RNA, which is used to demonstrate equal loading of mRNA. Figures 7-10 demonstrate connexin transcript levels in the cingulate cortex, hippocampus, amygdala and hypothalamus/preoptic area in female and male rats. Comparison between treatment groups did not reveal any statistically significant differences in individual tissues, in either males or females. The ability to make reliable comparisons is limited to different hormonal treatments within each individual tissue, due to the fact that Northern blots were exposed to autoradiographic film for varying periods of time, in order to optimize visualization. However, control and treated samples from each tissue were run on the same RNA gel, thereby eliminating inter-gel variability for the treatment comparisons.
FIGURE 6. Northern blot hybridization of total RNA from hypothalamus/preoptic area of intact vs. gonadectomized (GDX) male rats (10 µg RNA/lane). Panel A: Blot probed with $^{32}$P-labelled Cx32, present in rat liver (positive control) and absent in rat heart (negative control). Panel B: Blot probed with $^{32}$P-labelled Cx43. Cx43 transcript is present in rat heart (positive control tissue) and absent from liver (negative control tissue). Panel C: The same blot reprobed with the house-keeping gene 18S ribosomal RNA to demonstrate equal loading of samples.
FIGURE 7. Individual graphs showing mean (± SEM) Cx32 transcript levels in different regions of the intact (empty bar) and gonadectomized (hatched bar) male rat brain. Northern analysis reveals high connexin mRNA expression in all brain regions from GDX male rats although levels were not significantly altered by steroid exposure.
FIGURE 8. Individual graphs showing mean (± SEM) Cx43 transcript levels in different regions of the intact (empty bar) and gonadectomized (hatched bar) male rat brain. Northern analysis reveals high connexin mRNA expression in all brain regions from GDX male rats although levels were not significantly altered by steroid exposure.
Female rats although levels were not significantly altered by steroid exposure. Northern analysis reveals high concentration mRNA expression in all brain regions from O VX different regions of the OVX (empty bar) and OVX + E2 (hatched bar) female rat brain. Individual graphs showing mean (±SEM) Cx32 transcript levels in

**Figure 9**
FIGURE 10. Individual graphs showing mean (± SEM) Cx43 transcript levels in different regions of the OVX (empty bar) and OVX + E2 (hatched bar) female rat brain. Northern analysis reveals high connexin mRNA expression in all brain regions from OVX female rats although levels were not significantly altered by steroid exposure.
Effects of gonadal hormones on brain Cx43 expression are shown in Figures 12-16. Figure 11 illustrates the location and orientation of distinct brain regions in which connexin expression was measured by densitometry. The densitometric data for each region were analyzed on a section-by-section basis, plotting the average regional densitometric data for all sections corresponding to similar hormonal treatments in the male and female rats. Figure 12 demonstrates \textit{in situ} hybridization for Cx43 mRNA in intact and gonadectomized male rat brains at different levels of the brain. Visual inspection suggests no significant differences in connexin mRNA expression between treatment groups. Figure 13 demonstrated no significant differences in Cx43 transcript levels based on hormonal status of distinct regions of intact and gonadectomized male rats. However, the \textit{in situ} hybridization assay demonstrated estradiol-induced increases in Cx43 transcript levels in the dorso-lateral septum, bed nucleus of the stria terminalis and periventricular hypothalamic nucleus of the female rat (Figure 14). Dark- and bright-field photomicrographs (Figure 15 and 16) show \textit{in situ} hybridization results for Cx43 mRNA, manifest in grains over cells, in the dorso-lateral septum and dorsal preoptic area of the control and estradiol-treated female rat. There is a small increase in Cx43 mRNA expression in sections from hormone replaced rats.
FIGURE 11. Atlas diagram illustrating the location and orientation of regions in which connexin transcript levels were analysed densitometrically (Figure 12 and 13). PVCP: periventricular caudate putamen; LSD: dorso-lateral septal nucleus; BST: bed nucleus of stria terminalis; Pe: periventricular hypothalamic nucleus; CTX: cingulate cortex; HIP: hippocampus; MBH: mediobasal hypothalamic nucleus; AMY: amygdaloid nucleus (Adapted from Paxinos and Watson, 1986)
FIGURE 12. In situ hybridization of Cx43 mRNA in intact (A, C) and gonadectomized (B, D) male rats at different levels of the brain reveals no regional labelling differences between treatment groups. Antisense \[^{35}\text{S}]\)-labelled riboprobe corresponding to a 294bp fragment specific Cx43 was used. A section hybridized with Cx43 sense riboprobe (E) serves as the negative control for specific probe interaction.
FIGURE 13. Composite graph showing mean (± SEM) Cx43 transcript levels, as measured by *in situ* hybridization, in different regions of the intact (empty bars) and gonadectomized (hatched bars) male rat. Histograms represent the average regional densitometric data for all sections corresponding to respective hormonal treatment groups (intact, n=4; GDX, n=4) ($F_{(1,480)} = 0.467; p=1.495$)
FIGURE 14. Composite graph showing mean Cx43 transcript levels, as measured by in situ hybridization, in different regions of the control (empty bars) and estradiol-treated (hatched bars) ovariectomized female rats. Histograms represent the average regional densitometric data for all sections corresponding to respective hormonal treatment groups. Vertical bars represent the SEM for the OVX E2 group ($F_{(1,330)} = 1.27$; $p=0.260$); filled circles represent average data points for each animal in the OVX group. (OVX, $n=2$; OVX + E2, $n=3$).
FIGURE 15. Photomicrographs of *in situ* hybridization for Cx43 mRNA in the dorso-lateral septum of OVX (a,b) and estradiol-treated OVX (c,d) rats. Photomicrographs were taken under dark-field illumination (a,c) to depict the distribution of reduced silver grains (white dots) resulting from exposure to the $[^{35}\text{S}]$-labelled cRNA probe. Bright-field illumination photomicrographs (b,d) demonstrate the location of cell nuclei and labelled Cx43 riboprobe (black dots). LV: lateral ventricle.
FIGURE 16. Photomicrographs of *in situ* hybridization for Cx43 mRNA in the dorsal preoptic area of OVX (a,b) and estradiol-treated OVX (c,d) rats. Photomicrographs were taken under dark-field illumination (a,c) to depict the distribution of reduced silver grains (white dots) resulting from exposure to the $^{35}$S-labelled cRNA probe. Bright-field illumination photomicrographs (b,d) demonstrate the location of cell nuclei and labelled Cx43 riboprobe (black dots). ac: anterior commissure; 3V: third ventricle
In Situ Hybridization of Cx43 transcript levels

No significant differences in connexin expression were observed between intact and GDX adult males. We were also interested in the possibility, however, that there might be effects of androgen on connexin expression at other stages of CNS development. The rationale for this hypothesis is based on the observation that there are significant sex differences in the migration patterns of neurons in the rostral hypothalamus and preoptic area, during perinatal development, resulting from androgen action in the male. In the female rat, the ovary is quiescent during early life and this lack of sex steroid secretion allows a different pattern of neuronal migration to occur. Since astrocytes, which express Cx43, are vitally important components of the mechanism controlling neuronal migration away from the neuroepithelial layers lining the ventricular walls, and the growth of astrocyte processes has been shown to be sensitive to gonadal steroids in vitro, we considered the possibility that neonatal androgen production in males might affect astrocytic Cx43 expression, as part of the mechanism of sexual differentiation.

To address the possibility of differences in connexin mRNA expression in neonatal female and male rat pups, levels of transcript were measured using in situ hybridization. Figure 17 depicts the sampling windows for the distinct brain regions of interest, including, cingulate cortex, hippocampus, hypothalamus/preoptic area, amygdala and periventricular hypothalamus. Cx43 transcript levels showed no significant differences between the female and male rat pups (Figure 18). However, despite this lack of large-scale change in connexin expression, examination of the sections under higher resolution revealed a smaller-scale regulatory effect of hormones on Cx43 mRNA levels, expressed in the form of regional differences in grain density around the apex of the third ventricle (Figure 19).
**FIGURE 17.** *In situ* hybridization of Cx43 mRNA in an 18-hours postnatal male pup at different levels of the brain. Cx43 labelling was analyzed on a section-by-section basis, using sampling windows as shown - A: cingulate cortex; B: hippocampus; C: amygdala; D: hypothalamus/preoptic area. Window D also corresponds to the periventricular hypothalamus in brain sections from a more frontal plane.
FIGURE 18. Composite graph showing mean (± SEM) Cx43 transcript levels, as measured by *in situ* hybridization, in 18-hour postnatal female and male rat pups. Histograms represent the average regional densitometric data for all sections corresponding to female or male pups (n=3) ($F_{(1,193)} = 1.03; p=0.312$)
FIGURE 19. Photomicrographs taken under bright-field illumination of female (A) and male (B) pup brain to depict the location of nuclei and labelled Cx43 riboprobe (black dots). Arrows indicate the neuroepithelial layer at the apex of the third ventricle. (L. Puy, unpublished observations)
The influence of gonadal steroids on the electrical activity of the brain of both animals and humans is well documented. In animals, changes in estrogen levels are associated with dramatic changes in the electrical and neurochemical excitability of neurons, in several regions of the brain \(^{181,294,298}\). Pathological changes in electrical activity associated with epileptic motor seizures are also highly sensitive to gonadal steroids. Estrogen potentiates seizures in the central nervous system \(^{34,83}\), while progesterone seems to produce opposing, seizure-protective effects \(^{125,196,243}\). Recent evidence suggesting that gonadal steroids might modulate connexin expression in the brain, coupled with studies indicating a possible role for electrical gap junctional coupling in the etiology of epileptic seizures \(^{84,192}\), led to our hypothesis that gonadal hormones could potentially affect neuronal activity by altering expression of the two principal connexins found in the brain, Cx43 and Cx32.

Gonadal steroids have been shown to strongly regulate connexin biosynthesis in non-neural target tissues, such as the uterus, in both the pregnant and non-pregnant state \(^{218}\). Elevated levels of uterine gap junctions accommodate the need for the synchronization of muscular contractions during the process of labour. Currently, it is thought that the onset of labour is driven by the dramatic up-regulation of Cx43 mRNA and protein, a process which is associated with an increase in the maternal plasma estrogen:progesterone ratio \(^{148}\). In the myometrium of both pregnant and non-pregnant rats, there is also evidence that Cx43 synthesis and/or trafficking is regulated positively by estrogen and negatively by progesterone. The mechanism through which gonadal steroids modulate Cx43 synthesis is not yet clear, although two principal pathways have been proposed. Yu et al. (1994) demonstrated that the Cx43 gene is responsive to estrogen, despite the lack of a palindromic sequence homologous to the estrogen response element within the Cx43 promoter. The 5' promoter region of the Cx43 gene does, however, exhibit a consensus activator protein-1 (AP-1) binding site. Within the uterus, estrogen is known to induce the expression of the AP-1 proteins, *c-fos* and *c-jun* \(^{112,280}\). Piersanti and Lye (1995) demonstrated an increase in myometrial Cx43 mRNA following
expression of mRNA encoding c-fos and c-jun. Spontaneous and premature labour was also associated with a coincident increased expression of Cx43 and c-fos within the myometrium of pregnant rats.  

In addition to the crucial role of gonadal hormones within the term uterus, steroids exert a variety of morphological, biochemical and electrophysiological effects in a number of different regions in mammalian brain. As previously discussed, estrogen is one of the most potent steroids, and exerts a range of effects, including the modulation of key enzymes for neurotransmission, neuro-glial connectivity, and synaptic remodelling. Such genomic effects are mediated by intracellular estrogen receptors present in both neurons and glia. Alternatively, gonadal steroids can exert rapid and reversible effects on their target cells, often via interactions with neural membrane activity. It is well documented that estrogen modulates neuronal activity within the CNS, specifically in estrogen-sensitive regions important in the regulation of different aspects of reproductive function of the animal. Estrogen priming, for example, has the ability to alter neuronal excitability and sensitivity. Within the CNS, the expression of c-fos by individual neurons is often used as a marker for cell activation. Following estradiol administration to OVX rats, Insel (1990) reported an increase in staining for c-fos-like protein in a variety of regions rich in estradiol-concentrating cells, including the anterior medial POA, the medial POA, the medial amygdala nucleus, and the ventromedial nucleus of the hypothalamus. Estrogen priming has also been shown to increase the expression of c-fos in the CA1 region.

Thus, the above evidence suggests that like the uterus, the brain may also possess the requisite mechanisms for a hormonal regulation of connexin expression, potentially mediated through the steroidal induction of proto-oncogenes, which act on connexin promoter AP-1 sites to regulate transcription. However, the current state of knowledge with respect to gonadal hormone regulation of gap junctions in the central nervous system is poor, and furthermore, possible mechanisms for such effects remain unexplored. Evidence consistent with the potential for hormonal regulation of connexin mRNA expression in the central nervous system was first described by Matsumoto et al. (1991),
who examined Cx32 mRNA expression in the androgen-sensitive motoneurons of the spinal nucleus of the bulbocavernosus. Castration of male rats was shown to dramatically reduce the expression of Cx32 mRNA in the SNB motoneurons, a change which was counteracted by treatment with testosterone. More recently, Micevych et al. (1996) demonstrated a dramatic up-regulation of Cx32 mRNA expression in the rat supraoptic nucleus (SON) of the hypothalamic magnocellular nucleus, during late pregnancy and lactation, both periods characterized by increased neuronal membrane apposition and dye-coupling. These results suggest that connexons may form between SON neurons in response to increased activation. The increased expression of Cx32 mRNA on the day before parturition, may mediate perinatal activation, resulting in the release of approximately 60% of the oxytocin and vasopressin from the neurohypophysis. On postpartum day 13, there are once again high levels of Cx32 mRNA, a time when the SON is in a continual state of activation induced by suckling stimuli and the homeostatic maintenance of maternal water levels. Estrogen has also been shown to modulate dye coupling within the female rat SON and may also influence Cx32 mRNA levels in virgins and in postpartum lactating dams. Although this proposed mechanism of connexin mRNA regulation may be consistent with observations of elevated Cx32 mRNA and dye coupling during the lactating period when there is low estrogen, the hypothesis breaks down in the prepartum condition, when there is high levels of both maternal estrogen and Cx32 mRNA.

Further evidence consistent with a possible hormonal regulation of gap junctions in the brain, was demonstrated by Pérez et al. (1990), who reported an estradiol-induction of gap junctions in the arcuate nucleus. This observation suggests that estrogen-sensitive neurons of the arcuate nucleus may form gap junctions in response to estradiol in order to prepare for synchronous activity, which occurs in this nucleus under the influence of estrogen. Cobbett et al. (1987) demonstrated that castration profoundly lowers the incidence of dye coupling among magnocellular paraventricular nucleus neurons in male rats, and that testosterone replacement reverses these effects.

In this study, we demonstrate that the presence or absence of gonadal steroids in the systemic circulation of rats is not associated with large-scale changes in connexin
mRNA expression, a result contrary to our initial hypothesis. The expression of connexin transcripts was examined in four groups of rats: cycling female rats; control and estradiol-treated ovariectomized adult female rats; gonadectomized and intact adult male rats, and; male and female rat pups, 18 hours post-partum. All Northern analyses and in situ hybridization assays focused on the regulation of connexin mRNA expression in areas of the brain reported in the literature to exhibit high levels of high estradiol binding. Northern analyses of several estrogen-sensitive regions of the cycling female rat brain demonstrated a small, but significant reduction of Cx43 mRNA levels in the amygdala on the days of estrus, diestrus and proestrus, with respect to metestrus. Northern analyses of brain tissue following hormone exposure (in the form of either testicular secretions in the male, or estradiol treatment in the female) from adult female and male rats exhibited no change in Cx43 or Cx32 transcript levels.

Further examination of several experimental groups (i.e. adult female and male rats; female and male rat pups) were performed using in situ hybridization with a [S\textsuperscript{35}]-labelled Cx43 riboprobe. Measurements of connexin mRNA labelling were performed by taking the average densitometric value over various estradiol-sensitive regions. Within the female ovariectomized rat, treatment with estradiol induced a small up-regulation of Cx43 mRNA expression in only a few, restricted regions, including the bed nucleus of the stria terminalis, the dorso-lateral septum, and the periventricular hypothalamus. No other gonadal steroid effects were observed in the adult male rats, female pups or male rat pups. However, visual inspection of riboprobe labelled sections after autoradiographic emulsion dipping demonstrate hormone-induced changes of connexin mRNA expression on a small-scale, very regionalized manner. Such small, region-specific alterations in connexin mRNA expression were indistinguishable when densitometrically analysed over relatively large areas. In order to investigate these highly regionalized regulatory effects of hormones, emulsion dipped brain sections of a greater number of animals should be quantified for the number reduced silver grains per unit area. Where connexin expression is regulated by hormones in the brain, the changes are highly localized and therefore undetectable by analysis on a large-scale.
This remarkably small hormonal regulation of connexin expression in all treatment groups, differs from the regulation observed in uterine tissues, in which hormone-induced changes in electrical activity involve dramatic changes in gap junction mRNA and protein synthesis. There are several possible interpretations. One explanation for the discrepancy between steroidal effects in non-neural and neural tissue is that, in fact, gonadal hormones may not regulate connexin transcript levels within the central nervous system. This explanation is at best only partially correct, as previously demonstrated in studies by Matsumoto et al. (1991), Micevych et al. (1996), Cobbett et al. (1987), and Pérez et al. (1990). These studies have provided the precedent for steroidal regulation of gap junctions, dye coupling and connexin mRNA expression in regions of the CNS; but it remains possible that these may be highly region-specific responses, that are not observed in other gonadal sensitive areas of the brain.

A second interpretation is that a large-scale change in connexin mRNA expression may occur at a different time point than was investigated in this study. Comparison of data among cycling females demonstrated only negligible changes in Cx43 and Cx32 transcript levels, when the animals were sacrificed and brain tissue collected in the morning hours (9am to 11am) of the respective days of the estrous cycle. We chose this time of day very specifically in an attempt to minimize the potential confounding effects of alterations in neuronal activity, which could indirectly driving changes in connexin expression via altered removal or astrocytic c-fos synthesis. We were interested initially in this study in the specific effects of the steroids on Cx43 mRNA levels, as a potential causative factor, rather than as the result of changes in the electrical activity of the brain induced by steroid action. In female cycling rats, a sharp rise in estradiol and progesterone that occurs in proestrus stimulates the surge in luteinizing hormone (LH) in the late afternoon hours of proestrus. This LH surge is a result of a sudden synchronization of electrical activity within LHRH neurons dispersed in the preoptic area and hypothalamus. During the afternoon of proestrus, when luteinizing hormone releasing hormone (LHRH) output is significantly increased, c-fos expression rises dramatically in LHRH neurons around the time of the LH surge. Thus, it is possible that, while we saw very little evidence of altered connexin mRNA levels in the present
study, Cx43 and/or Cx32 expression might change later in the day, closer to the expected
time of alterations in neuronal electrical activity associated with the estrogen-induced LH
surge. Future studies should investigate the time course of connexin expression in
ovariectomized rats with estradiol replacement to test this hypothesis.

The third, related interpretation for the lack of steroidal regulation of connexin
expression in the brain, may lie in the status of electrical activation of the CNS.
Regulation of connexin expression may only be apparent when the exposure to gonadal
hormones is concomitant to a change in CNS electrical activity. The results reported
here are consistent with the assumption that in the absence of altered electrical activity,
the administration of gonadal hormones is insufficient to induce a change in connexin
mRNA expression. This hypothesis is also consistent with results previously reported in
the literature. Micevych et al. (1996) reported an up-regulation of Cx32 mRNA levels in
the SON prior to parturition and during lactation, as well as a down-regulation of
transcript on post-partum day 1 of the rat. On the day prior to the onset of labour when
there is increased Cx32 mRNA in the SON, high levels of circulating estrogen coincide
with synchronized high-frequency bursts of activity within oxytocin neurons. Postpartum
day 1 is characterized by a sudden reduction in Cx32, consistent with low levels of both
estrogen and SON neuronal activity. On postpartum day 13, there are once again high
levels of Cx32 mRNA, a time when the SON is in a continual state of activation induced
by suckling stimuli.

This interpretation of electrical activation as a necessary coincident stimulus for
connexin regulation by hormones could also be consistent with studies in uterine tissue.
The onset of coordinated uterine contractions during the process of labour is correlated
with both an increased ratio of estrogen:progesterone, as well as increased levels of Cx43
expression. Full induction of Cx43 expression and the assembly of functional Cx43 gap
junctions may require both estrogen action and increased myocyte electrical activity.
Piersanti and Lye (1995) found that premature, delayed, and spontaneous term labour
were associated with a coincident increase in Cx43 and c-fos mRNA. The increase in
estradiol prior to parturition induces a dramatic increase in electrical excitability and
contractility of the uterus. Estradiol is also known to increase the electrical excitability of
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non-pregnant rats, which may account for the increase in $c-jun$ prior to the elevation in Cx43 mRNA expression \textsuperscript{212}. The question of whether a change in Cx43 mRNA expression would be observed in the uterus if electrical activity was blocked following estrogen exposure remains to be answered experimentally. If a quiescent uterus exhibited a much smaller rise in gap junction expression following estrogen exposure than one in which alterations in membrane conductivity were allowed to occur, this might indicate convergence of the mechanisms regulating connexin expression. The same hypothesis could also be consistent with the known interactions between the effects of estrogen and stretch \textsuperscript{279}.

The most compelling support for the hypothesis that electrical activity coupled with estrogen action is required for altered connexin expression in the brain comes from recent unpublished data from our laboratory (H. Edwards). Based on the hypothesis that there might be synergistic effects of estrogen and electrical activation on gap junction expression, perhaps contributing to epileptic seizure activity, experiments were initiated using the kindled rat model of epilepsy. Kindling involves delivery of a small electrical stimulus to regions of the limbic system on a daily basis, until eventually the stimulus results in a spreading after-discharge that recruits a full-scale motor seizure that resembles epilepsy in humans. Kindling was combined with varying steroid treatment regimens. Preliminary results suggest that there is a dramatic up-regulation of Cx43 mRNA in estrogen target regions of the female rat brain, when estradiol treatment is provided to OVX epileptically kindled animals, but not when the estrogen is given to non-kindled animals, confirming the data I have obtained. Furthermore, progesterone treatment seems to yield an opposing, down-regulatory effect on Cx43 transcripts as demonstrated by \textit{in situ} hybridization. Similar effects were found whether the rat had been given the kindling stimulus from the dorsal hippocampus, or from the dorso-lateral amygdala. Thus, large-scale electrical activation of the brain may be necessary for hormonal induction of connexin expression.

This hypothesis, if correct, has major clinical implications in the field of epileptiform activity in the brain. Recent evidence suggests a possible role for electrical gap junctional coupling in the etiology of epileptic seizures \textsuperscript{84,192}. Patients with
cataleptical epilepsy experience a fluctuation in seizure frequency which varies within the menstrual cycle, showing a positive correlation with the ratio of estradiol:progesterone. Furthermore, estrogen priming is known to stimulate the expression of c-fos in the CA1, which is a marker correlated with seizure-like activity. This combination of evidence suggests that the epileptiform activity which is known to be induced by estrogen, may also be correlated with changes in connexin expression.

Although the data reported here indicates that there are no large scale changes in connexin transcript levels as a function of hormonal exposure, there are many other steps that may be hormonally influenced during the synthetic processes of connexins and gap junctions. Hormones may alternatively influence the production of connexin proteins, connexin assembly, phosphorylation, trafficking, or docking. Thus further studies should examine not only the gonadal steroid regulation of connexin transcript levels, but also protein levels, phosphorylation, and the presence of functional gap junctions. Further studies may also reveal a gonadal steroid regulation of CNS connexins other than those studied within this thesis.

In summary, these studies have documented the expression patterns of Cx43 and/or Cx32 mRNA in cycling female rats, castrate or hormone treated female and male rats and in neonatal rat pups. Changes in the brain induced by androgen or estrogen exposure are not associated with large-scale changes in connexin expression. In situ hybridization techniques demonstrate that estradiol up-regulates expression only in a few, restricted regions of the brain, including the bed nucleus of the stria terminalis, the dorsolateral septum, and the periventricular hypothalamus. There were no other significant changes in Cx43 or Cx32 mRNA in the other experimental groups investigated. It remains possible that for regulation of connexin expression in the brain by gonadal steroids to occur, there must first be electrical excitation in order to “prime” or accentuate the hormonal effects.
FUTURE DIRECTIONS

The work presented here clearly represents only a first step in attempting to elucidate the effects of gonadal steroids on connexin expression in the CNS. A considerable amount of additional work remains to be done. In particular, there are two specific additional studies that should be performed:

- **Time course experiment:** Examine the time course of connexin expression in the OVX rat with estradiol replacement, and during the 4-day estrous cycle of the female rat; this may reveal a potential association between connexin expression and neuronal electrical activity associated with fluctuating levels of gonadal hormones.

- **Mechanism of hormonal modulation of connexins:** Investigate a possible cascade mechanism of estrogen regulation of connexin expression, via estrogenic induction of the proto-oncogenes, *c-fos* and *c-jun*, which may subsequently alter connexin gene transcription. This may be achieved through cell culture experiments using tissues removed from specific regions of the brain. Connexin expression levels could be manipulated through treatment with hormones, and could be compared with patterns of expression following administration of antisense oligonucleotides that targets AP-1 expression on the connexin gene.


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