MOLECULAR SPECIFIERS OF NEURAL CELL PLASTICITY IN THE HYPOTHALAMUS

By

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CHAPTER ONE

BACKGROUND AND SIGNIFICANCE

Chapter 1: Background and Significance:

General:

Mammalian puberty begins with an increased pulsatile secretion of luteinizing-hormone releasing hormone (LHRH), also known as gonadotropin-releasing hormone (GnRH), from specialized neurons in the basal hypothalamus into the portal vasculature, effecting release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from cells in the anterior pituitary [1]. The release of LH and FSH is essential for the production of mature gametes and sex steroid secretion from the gonads (**Figure 1**).

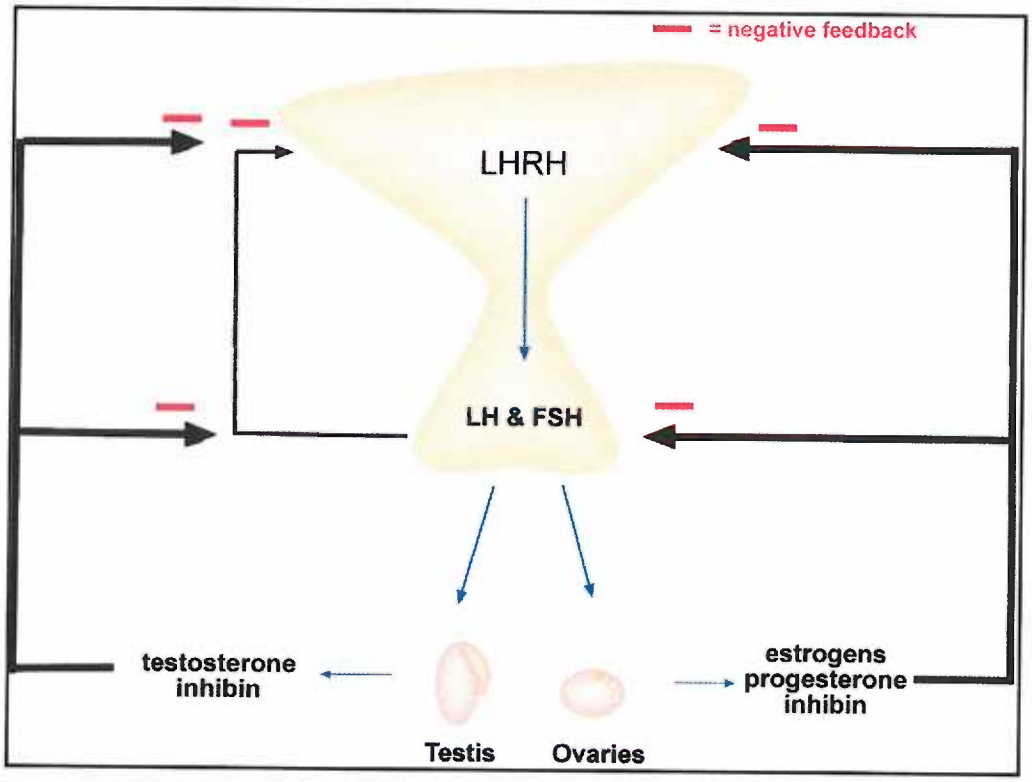

Figure 1. The hypothalamic- pituitary-gonadal (HPG) axis. LHRH (or GnRH) is secreted by LHRH neurons in the basal hypothalamus into the portal vasculature of the anterior pituitary. LHRH stimulated gonadatrope cells in the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) into the general circulation where it stimulates development and function of the testis and ovaries. Gonadal steroid hormones (such as estrogen and testosterone) can then provide negative feedback both at the pituitary and hypothalamus levels. As the arrows indicate, LH and FSH can also provide negative feedback to the LHRH neurons.

The initiation of puberty is a central event since it can occur in the absence of gonads and, thus, steroid sex hormones [2]. It appears that the initiation of puberty requires both the loss of a transsynaptic inhibitory tone and the activation of excitatory inputs to LHRH neurons [1,3] (**Figure 2**). Gamma-amino butyric acid (GABA) is the dominant inhibitory neurotransmitter in the hypothalamus [4]. Most of the known actions of GABA on LHRH neurons appear to involve activation of $GABA_A$ receptors and have been shown to either inhibit or stimulate LHRH neuronal

function. In nonhuman primates GABA, acting via the $GABA_A$ receptor, restrains LHRH release during female sexual development [5] and the removal of this restraining influence results in increased LHRH secretion [6]. Seemingly in contrast, other studies have shown excitation of both isolated embryonic [7] and adult [8] LHRH neurons by GABA via the $GABA_A$ receptor. It is now clear that while GABA's inhibitory influences upon LHRH release may require neuronal circuits functionally connected to the LHRH neuronal network, excitatory influences mediated by $GABA_A$ receptors can be directly exerted on LHRH neurons [8].

The main trans-synaptic excitatory input to LHRH neurons is provided by glutamatergic neurons. Glutamate is the major excitatory amino acid neurotransmitter in the hypothalamus [9]. LHRH neurons receive direct glutamatergic innervation [10,11] and isolated LHRH neurons respond to glutamate and NMDA by firing action potentials [12]. Activation of each of the three ionotropic glutamate receptor subtypes increases LHRH secretion in adult animals (for review see [1]). Glutamate, kainate and NMDA all stimulate LHRH release in sexually immature animals including monkeys [13], rats [14] and sheep [15]. An important role for glutamate in the initiation of mammalian puberty is provided by studies showing the induction of precocious puberty in rats [16] and monkeys [17] with stimulation of NMDA receptors. Puberty can also be delayed in rats with the NMDA receptor blockers AP-5 and MK-801[18,19].

Neuronal Plasticity:

Synaptic specificity: One family of adhesion proteins implicated in both GABAergic and glutamatergic synapse function consists of the neurexins. Neurexins are highly polymorphic cell-surface proteins, expressed in neurons, which serve as both cell-adhesion molecules and signaling receptors. Alpha-neurexins serve as postsynaptic receptors for neurexophilins, a family of neuropeptide-like proteins encoded by four different genes [20] and that are preferentially expressed in inhibitory interneurons [21]. Beta-neurexins serve as receptors for a family of transmembrane proteins named neuroligins [22,23], which are encoded by three different genes. Of the four known neuroligins, neuroligin 1 is expressed in the post-synaptic membrane of excitatory glutamatergic synapses [24,25] and has been shown to be required for their specification [26,27] and maturation [27]. On the other hand, neuroligin 2 is localized preferentially to GABAergic synapses [28,29] and induces inhibitory synapse formation [27]. Neuroligins have been shown to bind to a number of postsynaptic proteins, including the synaptic cell adhesion molecule SynCAM [26,30,31], and the scaffolding proteins Pick1 [24] and PSD-95 [32]. Recently, mutations in neuroligin genes have been associated with autism [33,34]. Thus, these proteins represent crucial components of the molecular code governing the formation and remodeling of excitatory and inhibitory synapses [35].

It then follows that by defining changes in the neurexin/ neuroligin/neurexophilin make-up of the developing primate hypothalamus, one should be able to take significant steps towards both resolving the issue of whether or not synaptic plasticity is a major component of the

neuroendocrine control of puberty, and identifying key molecular determinants responsible for synaptic reconfiguration at this critical phase of hypothalamic development.

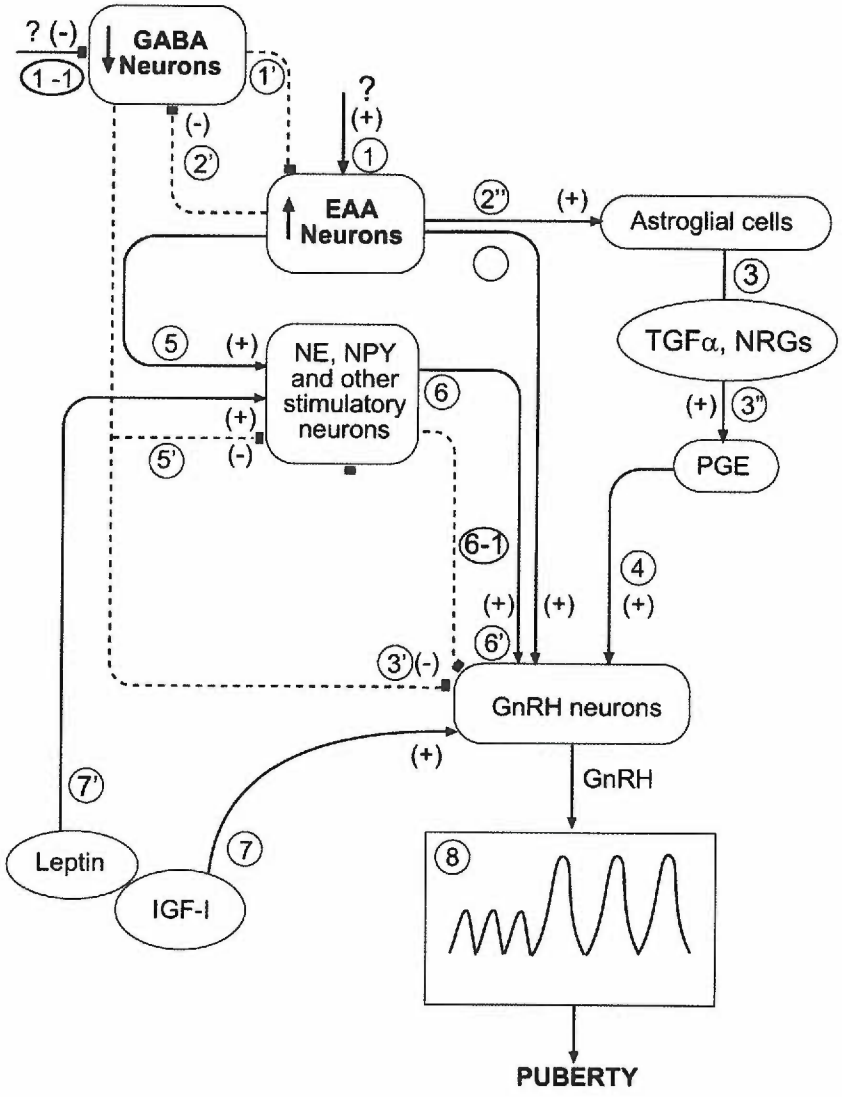

Figure 2. The central events underlying the initiation of female puberty. An increase in episodic GnRH secretion is activated by an integrative mechanism involving both neuron-to-neuron and glia-to-neuron communication processes. The neuron-to-neuron (transsynaptic) component involves the simultaneous activation of excitatory transsynaptic inputs and a reduction in inhibitory inputs to GnRH neurons. The main excitatory transsynaptic input affecting GnRH release is provided by neurons that use excitatory amino acids (EAA) as neurotransmitters. Noradrenergic (NE) and Neuropeptide Y (NPY)-containing neurons provide a complementary facilitatory input to the GnRH neuronal network. The chief inhibitory neuronal input restraining GnRH release is provided by GABAergic neurons (GABA). The glia-to-neuron component of the process involves the production of the epidermal growth factor (EGF)-related peptides, TGFα and neuregulins (NRGs), by astroglial cells, and the stimulatory effect of these peptides on the glial production of neuroactive substances, such as prostaglandin E_2, which act directly on GnRH neurons to elicit GnRH secretion. Current evidence suggests that an increase in EAA neurotransmission (1) may be a primary event responsible for the pubertal activation of pulsatile GnRH secretion in the rat, whereas a decrease in an inhibitory GABAergic tone (1-1) may be the initial transsynaptic event underlying the process in the rhesus monkey. Evidence collected in rodents indicates that excitatory amino acids can act directly on GnRH neurons (2) to enhance GnRH release, and on GABA neurons (2') to inhibit GABA neurotransmission. It is also possible, however, that a primary decrease in GABA release (1') triggers the pubertal increase in EAA neurotransmission. EAA acting on astroglial cells (2") appear to facilitate the glial production of TGFα/NRGs (3). While the decrease in GABA transmission begins to relieve GnRH neurons from GABAergic inhibitory control (3'), the increased production of EGF-related glial peptides stimulates the formation of

prostaglandin E_2 (3"), which acts directly on GnRH neurons to elicit GnRH release (4), amplifying the stimulatory effect of EAA (2) and magnifying the loss of GABAergic inhibitory control (3'). Upon the initiation of these changes, a further increase in EAA stimulation (5) and further decrease in GABAergic inhibitory tone (5') lead to activation of NE and NPY neuronal systems (6). Both NE and NPY would then contribute to the progression of the pubertal process by stimulating GnRH secretion (6'). There is also evidence that an inhibitory NPY influence (6-1) on GnRH release is lifted at the end of juvenile development, and thus, contributes to the initiation of the pubertal process. The metabolic signals, Leptin and IGF-I further the process along by stimulating GnRH secretion either directly (7), or *via* functionally connected neuronal networks (7'). The resulting increase in pulsatile GnRH secretion (8) then results in the initiation of puberty. = (-) = Inhibition; (+) = stimulation. (Modified with permission from Ojeda and Bilger, In Neuroendocrinology in Physiology and Medicine, PM Conn and ME Freeman, eds, Totowa, NJ; Humana Press Inc. 197–224).

Synaptic adhesiveness: The identification of a group of cadherin-related molecules localized at CNS synapses displaying a high degree of adhesive diversity, but also endowed with signaling capabilities, has lead to the expectation that these molecules may serve as components of the "lock-and-key" interactions used by neurons to recognize their synaptic partners [36]. The family of protocadherins-α (PCDHα, H for Human) are structural homologs of the murine family of Cadherin-Related Neuronal Receptors (CNRs) [37]. PCDHαs/CNRs are type 1 transmembrane proteins that share a common intracellular domain encoded by three small exons and a variable extracellular domain differentially encoded by one of at least 15 different exons (Figure 1A) [38]. The variable and constant exon are combined by *cis*-splicing of the mRNA, which is generated by promoters upstream of each PCDHα/CNR gene [39,40]. Thus, the complete extracellular domain of each PCDHα/CNR is encoded by a single exon and is composed of six ectodomains, the first of which may serve as an integrin-binding element. The intracellular domain, which is common to all members of the family, appears to link each family member to at least one postsynaptic intracellular pathway mediated by Fyn, a Src-related nonreceptor tyrosine kinase protein [37]. One interpretation for this unusual arrangement is that, while the extracellular domain of each PCDHα/CNR provides a different Ca^{2+}-dependent adhesive specificity, this diversity converges intracellularly, via the common intracellular domain, to activate similar signaling pathways. The similarity of the first ectodomain observed among some PCDHαs suggests that they may engage in heterophilic as well as homophilic interactions *in trans* [41]. Pcdhαs also display some heterophilic binding to β1 integrin [42], an extracellular matrix protein. Different neurons can express different CNRs [37], even within the same neuronal phenotype [43], a feature that provides a powerful tool for the profiling of synaptically connected neuronal networks.

Glial-Neuronal Plasticity:

The LHRH neurons remain plastic after migrating from the olfactory placode to the basal hypothalamus during development. The LHRH decapeptide must be transported to LHRH nerve terminals in the median eminence and released into the capillaries of the primary plexus of the hypophyseal portal vessels. These terminals are prevented from contact with the capillaries by the processes of specialized glial cells called tanycytes that line the ventral portion of the third ventricles and extend "end-feet" to the median eminence to envelop the LHRH terminals [44]. It has been shown in the rat that during proestrus, when estrogen levels are highest, the tanycytic

end feet allow physical contact between the LHRH nerve terminal and the pericapillary space of the portal vessels either by retraction of the end-feet [45] or an evagination of the basal lamina [46]. This plasticity is caused, at least partially, by the sequential activation of tanycytic erbB1 receptors by TGFα and the subsequent production of TGFβ [47]. From this evidence of LHRH neuronal plasticity, we hypothesize that there are important neuronal-glial molecules modulating this relationship that are yet to be examined.

The neurexin-related CASPR (contactin-associated protein) family of proteins has been found to be an important component of neuronal-glial adhesion complexes. CASPR (henceforth referred to as CASPR-1) was originally identified as a type I transmembrane protein associated *in trans* with the neuronal cell-surface protein contactin (also called F3), which in turn binds to the glial transmembrane protein Receptor Protein Tyrosine Phosphatase-β (RPTP-β) [48]. Subsequent studies led to the identification of CASPR-2, the second member of the family [49]. Both CASPR-1 and 2 have been implicated in the physiology of myelinated axons, as they are abundantly expressed in adjacent structural subdomains required for the saltatory conduction of myelinated nerve fibers [49,50]. Because the organization of these functional and structural subdomains of myelinated axons requires the presence of glial cells, it has been postulated that CASPRs are essential components of the signaling code utilized by neurons and glial cells to communicate with one another [51]. The function that CASPRs may be playing in the neuronal and glial systems of the brain remains to be established.

Glial Signaling and Neuronal Plasticity:

It has become increasingly clear of late that astrocytes play a much more active role in neural signaling than previously supposed. Astrocytes have the ability to specifically modulate neuronal activity and synaptic plasticity, particularly through glutamate signaling [52-54]. Astrocytes also contain all the cellular components necessary for cell signaling, such as the expression of a number of neurotransmitter receptors [55-57] and the capability to produce substances such as prostaglandins, cyclic AMP, calcium and growth factors known to influence neuronal synaptic activity [58]. Our laboratory has focused on the role that the astrocytes and tanycytes surrounding the LHRH neurons play in the facilitation of LHRH secretion and the timely onset of puberty and reproductive capacity. LHRH neurons receive a sparse synaptic input, comprising less than 2% of the cell membrane [59] as measured with electron microscopy. Newer techniques of dye-filling have recently shown that this may be an underestimation [60]. Glial cells appose between 20% and 80% of the LHRH neuronal cell membrane and this association is especially strong along the LHRH neuronal axons, which project to the median eminence [61]. Plasticity of this association is evident from studies showing changes in the physical relationship between LHRH neurons and surrounding glial cells during the estrous cycle of the rat [44].

Two glial pathways have been shown to regulate LHRH neurosecretion: those involving transforming growth-factor β (TGFβ) and transforming growth-factor α (TGFα) / Neuregulins (**Figure 3**). Upon release from astrocytes, $TGF\beta_1$ stimulates LHRH release by activating serine/threonine kinase receptors located on LHRH neurons [62,63]. The epidermal growth-factor related peptides TGFα and Neuregulins elicit LHRH secretion indirectly [64] via a juxtacrine / paracrine mode of communication consisting of the activation of astroglial erbB-1 [65] and erbB-4 [66] receptors, formation of heterodimeric complexes with erbB-2 [67], release of bioactive substances such as prostaglandin E_2, and stimulation of LHRH secretion by a direct action of those substances on LHRH neurons. Our laboratory and others have provided evidence that astroglial erbB-1, erbB-2 and erbB-4 receptors are required for normal sexual development [65,66,68].

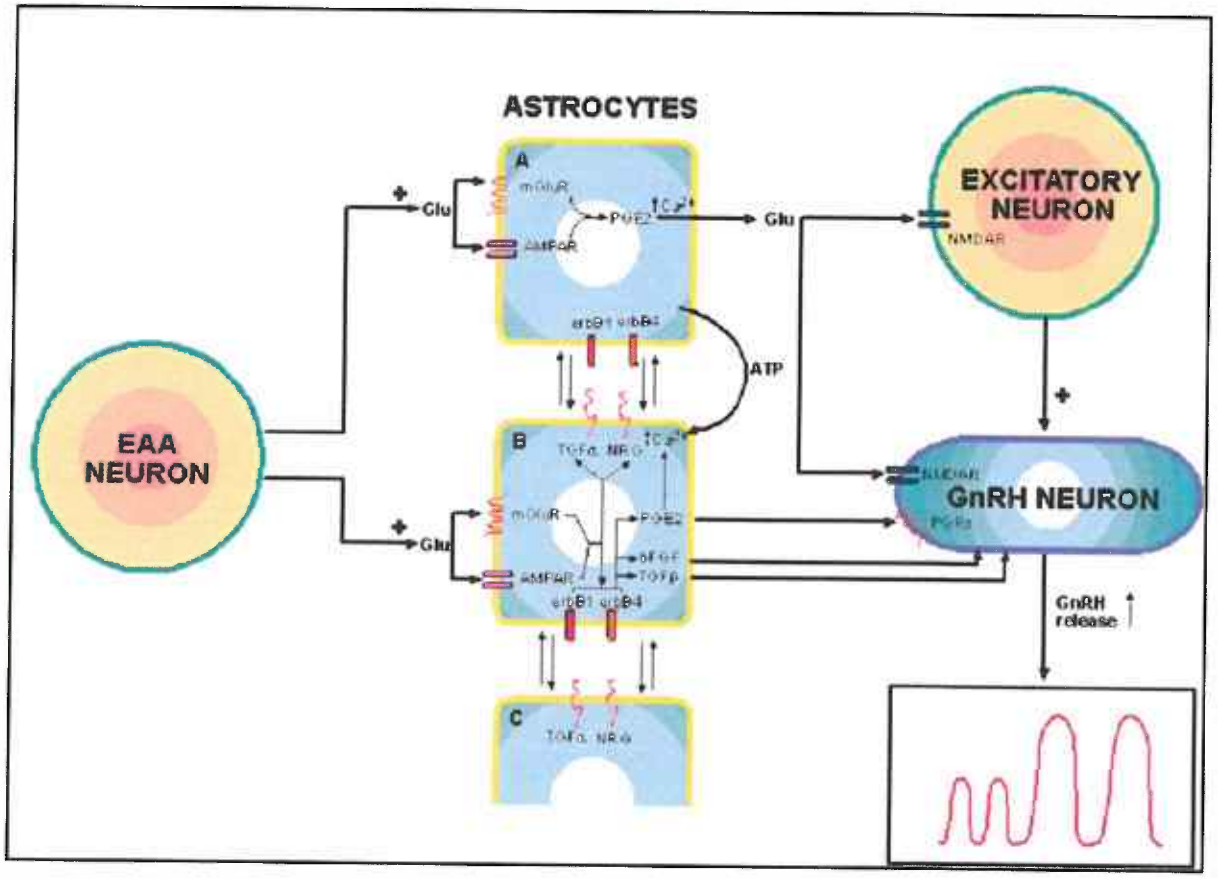

Figure 3. Postulated cell-cell signaling mechanisms involved in hypothalamic neuron-glia reciprocal communication. Astrocyte A mostly depicts information obtained by other authors working with glial cells derived from regions of the brain other than the hypothalamus. Astrocyte B contains information derived from studies using hypothalamic astrocytes. The partially drawn astrocyte C is shown for completeness of the concept that astrocytes contain both erbB receptors and their respective ligands. Glutamate acting *via* metabotropic and AMPA receptors causes PGE_2 release [69], calcium (Ca+2) waves [70,71] and ligand-dependent activation of erbB receptor signaling [56] in astrocytes. The calcium waves initiated by neuronal glutamate are PGE_2-dependent; however, they can also be initiated spontaneously in the absence of neuronal inputs [72]. Propagation of the calcium waves within astrocytic networks requires ATP and gap junction communication [70,71,73]. PGE_2 causes glutamate release in a calcium-dependent manner [69], in turn glial glutamate activates NMDA receptors on neighboring neurons [74], presumably also including those GnRH neurons that express these receptors. In addition, PGE_2 acts directly on GnRH neurons to stimulate GnRH release [64]. Like glutamate, activation of erbB1 and erbB4 receptor signaling by their respective ligands TGFα and neuregulins (NRGs) elicits PGE_2

release [66,75]; however, this effect occurs at a much later time than that of glutamate. Activation of erbB-signaling also causes increased synthesis of both TGFβ1 and bFGF [47,76], two growth factors involved in the regulation of GnRH neuronal function. Reproduced from [77].

There are four known members of the erbB receptor family. Three of them, erbB1, erbB3 and erbB4, bind and are activated by cognate ligands. In contrast, erbB2 acts as a co-receptor or auxiliary subunit recruited by ligand binding to the other receptors (for review see [78]. Hypothalamic astrocytes express erbB1, erbB2 and erbB4 receptors but not erbB3. In addition, cells in the hypothalamus express the erbB1 ligand TGFα, and several forms of the erbB4 ligand neuregulin (NRG or heregulin, [67]). There are about 12 ligands that bind the erbB receptors (**Figure 4**). Neuregulins 1 and 2 bind both erbB3 and erbB4. Neuregulins 3 and 4 are specific to erbB4 receptors [79,80]. Neuregulin 3 is the only specific erbB4 ligand present in the brain[81,82].

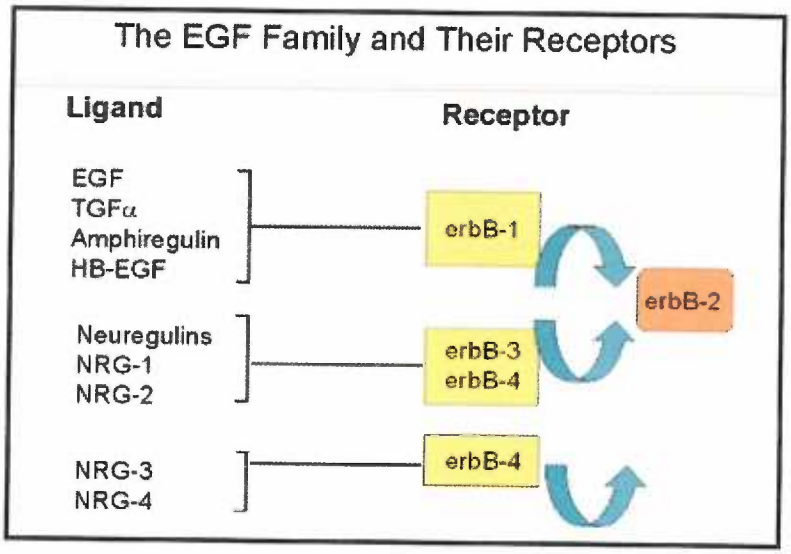

Figure 4. Each member of the family of erbB tyrosine kinase receptors binds a variety of ligands. ErbB1, erbB3 and erbB4 dimerize preferentially with erbB2 upon ligand binding. Neuregulins 3 and 4 are the only specific ligands for erbB4; only Neuregulin 3 is expressed in the brain.

ErbB4 has been implicated as playing a role at the neuronal synapse. The erbB4 receptor is the only erbB receptor containing a PDZ-binding domain in its intracellular portion [83]. This domain has been shown to interact with post-synaptic density proteins such as PSD-95 [84]. ErbB4 is able to signal intracellularly though the intracellular domain through various pathways, the use of which is determined by the erbB4 isoform present [85-87], (**Figure 5**). The intracellular domain of erbB4 has also been shown to be subject to cleavage and translocation to the nucleus [88], where it associates with the co-transcriptional activator YAP [89,90]. ErbB4 is, therefore, a growth factor receptor with a complex signaling function that may play a role in synaptic communication and glial-neuronal communication.

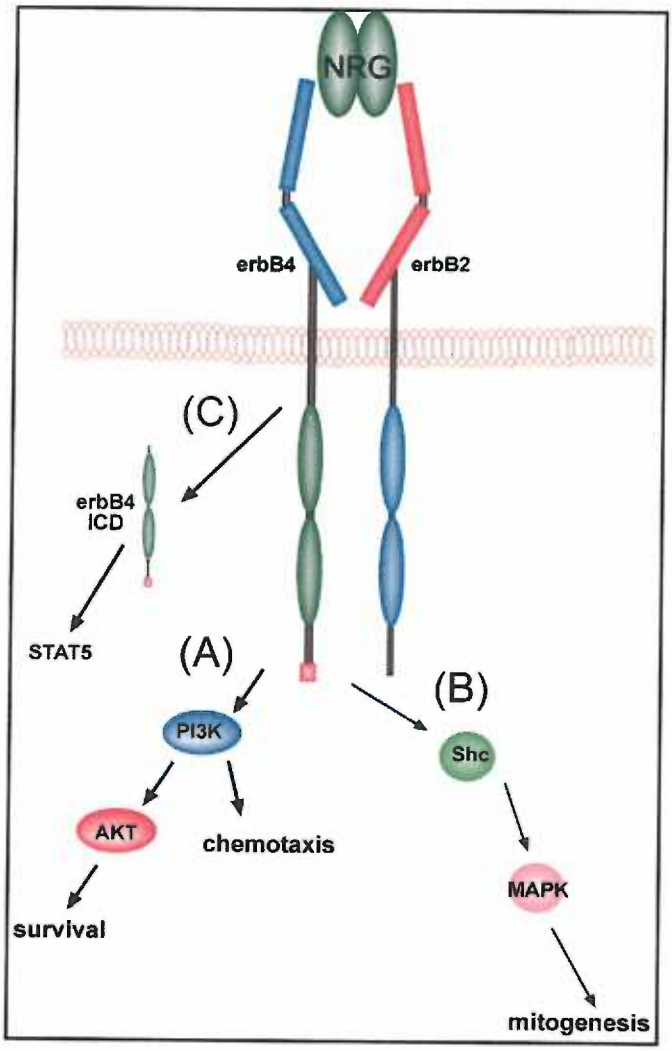

Figure 5. ErbB4 has multiple signaling capabilities. The ligand-induced dimerization of erbB4 with erbB2 can activate several different signaling pathways, depending on the erbB4 isoform involved. A. The CYT-1 isoform of the erbB4 receptor contains an intracellular phosphoinositide 3-kinase (PI3-K) binding site. The activation of PI3-K by erbB4 leads to numerous cellular responses including survival, membrane ruffling and chemotaxis, and activation of signaling molecules such as Akt [85]. The CYT-2 isoform does not bind PI3-K and is unable to mediate these responses [85]. B. Both CYT-1 and CYT-2 isoforms of erbB4 are able to activate the Shc/MAPK pathway which leads to proliferation [91]. C. The JM-a isoform of erbB4 contains an extracellular juxtamembrane sequence which may be cleaved by matrix metalloproteases to produce a membrane-anchored 80kD fragment [92]. This fragment may then be further cleaved at an intracellular site by γ-secretases and released as a soluble cytoplasmic protein [93]. Recent evidence has indicated that this fragment is translocated to the nucleus where it interacts with transcription regulators such as STAT5 [94,95].

It is not known what functions erbB4 may have in the communication between neurons and glia. By creating a mutant mouse with an astrocyte-specific dominant-negative mutation of erbB4, we show that the function of LHRH neurons in the hypothalamus is disrupted by a loss of astrocytic erbB4 signaling [68]. In Chapter 3 we describe further experiments performed to elucidate the mechanisms by which erbB4 signaling can control the function of these neurons. We hypothesize that the relationship between glial erbB4 signaling and LHRH neuronal function may be impacted by changes in adhesion/signaling molecules important to communication between these cells types.

Glial-Neuronal Glutamate - erbB Signaling: Involvement of Adhesion Molecules?

The importance of glutamate in the functional relationship between the astrocyte and the neuron has become increasingly appreciated in recent years. Astrocytes surrounding synaptic contacts are able to both uptake [96] and release [74] glutamate. Thus, the simple absence, or presence, of astrocytes and their glutamate transporters can change both glutamate receptor-mediated current in the postsynaptic cell [97] and also the activity of metabotropic glutamate receptors on the presynaptic cell [96].

Glutamate receptors on the astrocytes themselves play an important role in the modulation of the astrocytic-neuronal relationship. In the cerebellum, Bergmann glia are intimately associated with the glutamatergic synapses formed between parallel fibers and Purkinje neurons. Stimulation of these cerebellar synapses leads to glutamate-dependent Ca^{2+} influx into the Bergmann glia via Ca^{2+} permeable (lacking GluR2) AMPA receptors [97]. This stimulation leads to the extension of fine processes from the Bergmann glia which wrap themselves around the cerebellar synapse [97]. Thus, synaptic activity leads to a morphological change in the surrounding glial cells that are then able to rapidly modulate the synapse.

The Ojeda lab has described a different astrocyte-neuronal signaling mechanism in the astrocytes that leads to activation of the LHRH neuronal signaling network (Figure 3, for review see [77]). The work of Dziedzic et. al. found that these astrocytes express the AMPA GluR2 subunit, and are thus impermeable to Ca^{2+} [56]. The metabotropic glutamate receptor mGluR5 is also present. The stimulation of both these receptors with glutamate leads to transactivation of erbB1 and erbB4 signaling in the astrocytes. This activation involves the recruitment of the erbB receptors to the cell membrane, physical approximation of the receptors to their respective membrane-bound TGFα and Neuregulin on nearby astrocytes, and the phosphorylation of each erbB receptor via activation by the ligands following protease cleavage of their precursors. Earlier findings showed that the combined activation of AMPA and metabotropic receptors in astrocytes cases PGE_2 release, which in turn increases glial glutamate release [69]. The Ojeda lab has shown that erbB receptor stimulation results in secretion of PGE_2 that then effects LHRH release from LHRH neurons [98]. This is a mechanism that may then be modulated by astrocytic glutamate stimulation, both from neighboring astrocytes and from glutamatergic neurons functionally connected to the LHRH neuronal system.

Glutamate stimulation of astrocytes results in Ca^{2+} fluxes that result in astrocytic glutamate release and synaptic modification [74]. These increases in Ca^{2+} levels can be from direct Ca2+ through GluR2-minus AMPA receptors, as in Bergmann glia [97]. A more likely mechanism in hypothalamic astrocytes is increased intracellular Ca^{2+} levels resulting from glutamate-receptor mediated activation of erbB receptors leading to PGE_2 production (**Figure 3**). These increases in Ca^{2+} then lead to both an increases in PGE_2 release as well as potential astrocytic glutamate release. Astrocytes are also able to initiate rapidly-propagating calcium

waves in connected glial networks in the absence of neuronal stimulation, waves that then evoke glutamate release from the astrocytes [72].

In Bergmann glia, glutamate-induced calcium signaling leads to extensions of the fine processes important in synaptic modulation [97]. Receptor tyrosine kinases may be necessary for this phenomenon [99]. Similar plastic rearrangements are seen in the glial cells which interact with LHRH neurons [46,61,100]. Elsewhere in the hypothalamus, activation of oxytocinergic neurons in the PVN leads to a reduction in their astrocytic coverage, leaving the neurons directly apposed [101,102]. These changes are inhibited by the enzymatic removal of polysialic acid from the neural adhesion molecule NCAM [103]. It is not yet known how this modification is affected by neuronal activation in this system. Previous experiments in oligodendrocyte precursors show that glutamate signaling can upregulate PSA-NCAM expression [104], demonstrating a relationship between glutamate signaling and adhesion molecule regulation. No such relationship has yet been elucidated in astrocytes. We would like to propose that the plastic rearrangements between astrocytes and LHRH neurons described above are effected by changes in adhesion molecules, and subjected to modulation via astrocytic erbB signaling. Such erbB signaling may represent the downstream effects of glutamate stimulation in astrocytes [56].

Consequences of Astrocyte-LHRH morphological plasticity: Astrocyte connectivity and influences on LHRH signaling.

How can glial adhesion molecules regulate LHRH neuronal function? We know that the proximity of astrocytes and their processes to both inhibitory and excitatory synapses can profoundly affect those synapses' behaviors through a number of mechanisms, including glutamate uptake and release, potassium buffering (for review see [105-108]) and modulation of intersynaptic cross-talk [106]. Simple co-culture of neurons with astrocytes will increase the number of functional synapses formed by the neurons [109]. In a number of cases, glial coverage of neurons has been found to decrease as a result of neuronal activity (ie. oxytocinergic neurons in the PVN [101]) or hormonal status (ie. glial ensheathment of LHRH processes during the estrus cycle [61,100,110]). Therefore, in addition to providing growth factors and PGE_2 to the LHRH neurons to facilitate LHRH release, the presence of astrocytes could influence the function of synapses formed onto LHRH neurons by afferent neurons. In the oxytocinergic system of the PVN, astrocytic processes retract from the synapse at times of neuronal stimulation [111]. A similar process may be occurring in LHRH neurons, as glial contact with the neuronal processes diminishes during proestrus [61,100] and glial processes retract from LHRH neuronal terminals preceding the release of LHRH [110]. In another hypothalamic area, the ventromedial nucleus, synaptic density increases with estrogen levels at proestrus [112]. This could be resulting from a proestrus-related glial withdrawal like that in the median eminence. In additional to a modulatory role on synapse function, the very presence of astrocytes closely apposed to the neuronal cell

membrane could inhibit the formation of synapses. In addition to effects upon synapse formation, the proximity of astrocytes to LHRH neurons is important because of the soluble molecules such as growth factors, glutamate and PGE_2 with which astrocytes are known to modulate LHRH neuronal function. This plasticity must involve adhesion molecules that interact between the LHRH and astrocyte membranes, proteins that can be regulated by the conditions of physiological activation described above. The work described in this thesis is aimed at examining several groups of adhesion /signaling proteins that are likely, by their locations and functions, to be involved in this glial-neuronal plasticity.

In conclusion, we propose this work to investigate the roles of a variety of adhesion/ signaling molecules in the mammalian hypothalamus in the complex machinery underlying LHRH – glial signaling and the control of the onset of puberty. In Chapter 2 we describe the presence and distribution of members of two neuronal protein groups, the protocadherin αs and the neurexins, important in neuron to neuron communication. We then consider the glial-neuronal signaling complex comprised of neuronal Caspr and Contactin, and glial RPTPβ, and the presence of these proteins in the hypothalamus and well as in LHRH neurons and astrocytes specifically. Chapter 3 brings us to a more specific examination of the glial erbB4 receptor system, which plays an important role in the timely onset of puberty. We examine the functional consequences in the hypothalamus of a loss of astrocytic erbB4 signaling, and especially how that loss may affect adhesion/signaling molecules in the astrocytes and LHRH neurons.

CHAPTER TWO

Expression of Three Gene Families Encoding Cell-Cell Communication Molecules in the Prepubertal Nonhuman Primate Hypothalamus

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Chapter 2. Expression of three gene families encoding cell-cell communication molecules in the prepubertal nonhuman primate hypothalamus.

Abstract

Transsynaptic and glial-neuronal communication are important components of the mechanism underlying the pubertal activation of luteinizing hormone-releasing hormone (LHRH) secretion. The molecules required for the architectural organization of these cell-cell interactions have not been identified. We now show that the hypothalamus of the prepubertal female rhesus monkey expresses a multiplicity of genes encoding three families of adhesion/signaling proteins involved in the structural definition of both neuron-to-neuron and bi-directional neuron-glia communication. These include the neurexin/neuroligin (NRX/NRL) and protocadherin-alpha (PCDHα) families of synaptic specifiers/adhesion molecules, and key components of the contactin-dependent neuronal-glial adhesiveness complex, including contactin/F3 itself, the contactin-associated protein-1 (CASPR1), and the glial receptor protein tyrosine kinase β (RPTPβ). Prominently expressed among members of the NRX family is the neurexin isoform shown to specify glutamatergic vs GABAergic synapses [23,113,114]. While NRXs, PCDHαs and CASPR1 transcripts are mostly detected in neurons, the topography of expression appears different. NRX1 mRNA-containing neurons are scattered throughout the hypothalamus, PCDHα mRNA transcripts appear more abundant in neurons of the arcuate nucleus and periventricular region, and neurons positive for CASPR1 mRNA exhibit a particularly striking distribution pattern that delineates the hypothalamus. Examination of LHRH neurons, using the LHRH-secreting cell line GT1-7, showed that these cells contain transcripts encoding NRXs and one of their ligands (NRL1), at least one PCDHα (CNR-8/ PCDHα10), and the CASPR1/contactin complex. The results indicate that the prepubertal female monkey hypothalamus contains a plethora of adhesion/signaling molecules with different but complementary functions, and that an LHRH neuronal cell line expresses key components of this structural complex. The presence of such cell-cell communication machinery in the neuroendocrine brain suggests an integrated participation of their individual components in the central control of female sexual development.

Introduction

Significant progress has been made in the last few years towards elucidating the basic cellular and molecular mechanisms underlying the neuroendocrine control of mammalian puberty (for reviews see [115-117]). A major conclusion derived from these investigative efforts is that the pubertal activation of LHRH secretion can no longer be considered as an event exclusively driven by transsynaptic influences. Cell-cell signaling molecules, produced in astroglial cells and able to facilitate LHRH secretion, have been identified [77,118], and genetic approaches have been employed to define the physiological contribution of some of these molecules to the pubertal process [68,119]. The search for the neuronal networks critical for the enhancement of LHRH release during sexual development has been narrowed down to those systems that utilize excitatory and inhibitory amino acids as neurotransmitters [115,120,121]. Some of the appropriate functional connections between these two systems [122], and with glial cells [56], have been identified.

Inherently tied to the concept that attainment of sexual maturity depends on the faithful execution of a developmental program requiring bi-directional cell-cell communication is the long-suspected, but still unresolved, issue of the role that neuronal and glial remodeling may play in the pubertal process. Available morphological evidence, though scanty, is consistent with the concept that hypothalamic cell plasticity might contribute to the acquisition and attainment of sexual maturation in both rodents [123,124] and primates [125].

The identification of multigene families that participate in the organization, recognition and adhesiveness of synapses, as well as in neuron-glia communication, raises the intriguing possibility that the functional connectivity of neuronal and neuro-glia networks controlling the pubertal process might be determined by a host of molecular determinants with different, but complementary functions. Neurexins and protocadherins appear as the molecular "specifiers" most likely to bear relevance to the transsynaptic control of LHRH secretion.

In addition to a well-recognized dependence on transsynaptic inputs, LHRH neurons are subjected to glial control. Glial cells facilitate LHRH secretion by both inducing plastic rearrangements within the median eminence, and activating specific glia-to-glia and glia-to-neuron signaling pathways (for reviews see [1,64,126]). It is also clear that both the switch from immaturity to adulthood and from one physiological endocrine condition to another are characterized by reversible rearrangements in glial-neuronal architecture [127-130]. What are the molecular determinants of such functional plasticity? The critical importance of the contactin/CASPR1/RPTPβ interaction for neuron-glia adhesiveness [131] has raised the possibility that such interaction may be required not only for the functional adhesiveness of neurons and glial cells in different regions of the central nervous system, but also for their bi-directional communication. While the physiological implications of this ternary association have been best characterized in the peripheral nervous system [48,132,133], the complex might also

operate in the neuroendocrine brain, as suggested by the increased contactin expression observed in actively secreting hypothalamic magnocellular neurons [134].

As an initial step towards understanding the role that hypothalamic neural cell plasticity might play in the control of sexual development, we have employed prepubertal female rhesus macaques to determine if any of these multigene families of adhesion/signaling molecules is expressed in the neuroendocrine brain. The results indicate that members of each family are present in the monkey hypothalamus. Family members are also present in the LHRH-secreting cell line GT1-7. It is thus possible that female reproductive neuroendocrine development may be regulated by cell-cell interactions involving these novel communication molecules.

Materials and methods:

Animals:

Monkeys: The hypothalamic tissue used in this study derived from female rhesus monkeys (Macaca mulatta) that had been euthanized for a variety of reasons and obtained through the Oregon National Primate Research Center (ONPRC) Necropsy Program. The animals were classified as being in the prepubertal (juvenile) stage of development according to the criteria reported by Watanabe and Terasawa [135]. Serum LH levels measured in a group of monkeys of similar age (2.57±∀0.3 ng/ml, n=10) were distinctly lower than those (6.48±∀1.64 ng/ml, n=10) observed at 38-43 months of age, i.e. in pubertal animals. All procedures were approved by the ONPRC Animal Care and Use Committee in accordance with the NIH guidelines for the use of animals in research.

The PCR-cloning experiments were all performed with RNA derived from 3 juvenile females monkeys from the group described above.

Mice: The mice used were of the FvB/N strain purchased from Taconic (Germantown, NY). Astrocytes cultures were prepared from the hypothalamus of neonatal (PN0-PN2) pups, both male and female. Tissue for RNA extraction was obtained from adult female mice.

Tissue collection:

The preoptic area (POA), medial basal hypothalamus (MBH) and prefrontal cortex (CTX) of rhesus monkeys were dissected as described [136] and preserved overnight at 4°C in RNA-later solution (Ambion, Austin, TX). The tissues were then removed from RNA-later and stored at -85°C until RNA extraction. Mouse hypothalamic tissue for astrocyte cultures and adult brain tissue for RNA extraction was obtained after killing the animals by decapitation.

RNA isolation:

Tissues were homogenized (100mg/ml) in TriReagent solution (MRC, Cincinnati, OH), and the aqueous and organic phases were separated by the addition of 0.1vol bromo-chloropropane

(BCP, Sigma Chemicals, St. Louis, MO) followed by centrifugation at 4°C. RNA was precipitated from the aqueous phase with 1 volume of isopropanol by centrifugation at 13,000 rpm for 30 minutes at 4°C. RNA was then resuspended in DEP-treated H_2O and treated with Ambion's DNA-free DNAse I (2U per reaction) for 30 minutes at 37°C. RNA concentrations were determined spectrophotometrically. Afterwards, 500ng of RNA from each sample was run on denaturing agarose gels to confirm RNA integrity.

Cell Culture:

Hypothalamic astrocytes were purified by the method of McCarthy and de Vellis [137], as reported by Ma et. al. [75]. Briefly, hypothalamic cells were cultured to confluency in T75 flasks (8-10d). Contaminating cells were then removed by shaking the cultures at 37°C for 24 hours at 250 rpm followed by a change of media and a further shaking period of 24 hours. The purified astrocytes were then seeded on 100mm plates and grown in a mixture of DMEM and F12 medium (1/1,v/v) containing 10% donor calf serum plus penicillin (100U/ml) and streptomycin (100µg/ml). After the cells reached confluency, the medium was removed, dishes were washed with PBS, and rapidly frozen on dry ice. TriReagent was then added to each dish and RNA was extracted as described above.

The immortalized LHRH-producing GT1-7 cells [138] were grown in DMEM containing 10% fetal calf serum and the antibodies indicated above under an atmosphere of 5% CO_2/95% air at 37°C. Cells were seeded on 100mm plates; upon reaching 80% confluency the RNA was extracted as above.

RT-PCR:

Each total RNA (500 ng) was reverse-transcribed with Omniscript RT (Qiagen, Valencia, CA) in a 20 µl reaction volume for 1 hour at 37°C. Samples without RT were run as controls to confirm the absence of contaminating DNA. PCR was performed in 100µl reactions using HotStar Taq polymerase (Qiagen) and 50 pmol of the appropriate primers (Table 1). PCR conditions were as follows: Enzyme activation = 95°C for 15 minutes, 35 cycles of denaturation at 94°C (30 sec), annealing at 50-60°C (30 sec), extension at 72°C (1 min), followed by a final elongation at 72°C (10 min). The resulting PCR products were run on 2% agarose gels and gel images were captured with a Gel Doc 2000 system (BioRad, Hercules, CA).

DNA Cloning:

Each PCR product was cloned into the pGEM-T vector (Promega, Madison WI), transformed into XL-1 Blue competent cells (Stratagene, La Jolla, CA) and spread onto Luria-Bertani (LB) broth (Fisher Chemicals, Fairlawn, NJ) / agarose plates containing 50µg/ml ampicillin. Positive colonies were grown overnight in 6ml of LB media with 50µg/ml ampicillin and plasmid DNA was isolated

using the Qiagen Plasmid Miniprep kit. The cloned cDNAs were sequenced on an ABI 3100 Genetic Analyzer DNA sequencer using M13 forward and reverse primers, and the sequences were analyzed using DNAStar software (DNASTAR, Inc. Madison, WI).

Hybridization histochemistry:

Immunohistochemistry (IHC): To localize PCDHs and CASPRs in the monkey hypothalamus we utilized "pan-probes", i.e., monkey-specific cRNAs that recognize mRNA regions common to all members of each family (Table 2). In the case of neurexins, we used a probe (GenBank accession number AF424848) that recognizes the carboxy terminus-encoding region of β-neurexin mRNA, but lacks the segment derived from the alternative use of splice site 4 in the neurexin genes [139]. Only in the absence of this insert are neuroligins able to recognize neurexins as receptors and trigger presynaptic development [140,141]. The probe corresponds to a sequence located between nucleotide (nt) 897 and 1484 in human neurexin-1 mRNA (GenBank NM_138735). PCDH mRNA was identified with a cRNA (GenBank accession number AY598414) corresponding to nt 4570 - 4759 in the common 3' region of human PCDHα mRNA (NM_018900); the cRNA used to detect CASPR1 mRNA (AF480426) corresponds to a segment located between nt 1369 and 1745 in human CASPR1 mRNA (HSU87223). The monkey brain sections used were kindly provided by Dr. Cynthia Bethea (Oregon National Primate Research Center) and derived from animals utilized in an unrelated study [142]. The brains were fixed by intracardiac perfusion of 4% paraformaldehyde-borate buffer, pH 9.5 as reported [143]. Both solutions were made with DEPC treated water (0.1% diethyl pyrocarbonate) to minimize RNase contamination. After dissection of the brain, tissue blocks were postfixed in 4% paraformaldehyde for 3 hours, then transferred to 0.02 M potassium phosphate-buffered saline (KPBS) containing 20% glycerol and 2% dimethyl sulfoxide (DMSO) at 4°C for 3 days to cryoprotect the tissue. After infiltration, the blocks were frozen in isopentene cooled to –55°C, and stored at –80°C until sectioning. The sections (25μm) were cut on a sliding microtome, mounted on Superfrost Plus slides (Fisher Scientific, Santa Clara, CA), dehydrated under vacuum overnight and then frozen at –80°C until processing for hybridization. Every tenth section (each 250μm) was stained with hematoxylin for morphological reference and anatomical orientation [144]. The hybridization procedure was that recommended by Simmons et al. [145], as described earlier by us [143,146], using ^{35}S-UTP-labeled cRNA probes. Control sections were incubated with sense probes transcribed from the same plasmid, but linearized on the 3' end to transcribe the coding strand of cDNA.

Following an overnight hybridization at 55°C, the slides were washed and processed for cRNA detection [143,147]. After dehydration, the slides were dipped in NTB-2 emulsion, and were exposed to the emulsion for three weeks at 4°C. At this time the slides were developed, counterstained with 0.1% thionin, quickly dehydrated in ascending concentrations of alcohol, and coverslipped for microscopic examination. Cells were considered to be positive when they

showed at least 10 silver grains overlying the nucleus and/or perikaryon, and the density of silver grains overlaying a positive cell is at least three times that of adjacent areas devoid of cells [8].

Plasma LH:

Plasma LH levels in monkeys were measured by the ONPRC Hormone Assay Core using a mouse LH bioassay [148] and an LH reference preparation (cynomologous LH-RP-1) distributed by the National Hormone Distribution Program, NIH. All samples were assayed in a single assay. The intraassay coefficient of variation was 10%.

Results:

Table 1. Oligodeoxynucleotide primers used to amplify DNA fragments encoding various cell-cell communication molecules from reverse-transcribed RNA extracted from the rhesus monkey hypothalamus.

Protocadherin (Pcdh) alpha 1:FOR= gaccaggccgtatacagagt; REV=ttcttgaaggtggacaccagctt
Protocadherin (Pcdh) alpha 2: FOR= tcagaggctacgctggtggat; REV= atactgttggccactgctgat
Protocadherin (Pcdh) alpha 3: FOR = cactggtggatgtcaacgtgt; REV= atactgttggccactgctgat
Protocadherin (Pcdh) alpha 4: FOR=aatcccggcgtctgctgctcttac;REV=ggcgccctctagtggaaacc
Protocadherin (Pcdh) alpha 5: FOR= ggccgcggggaccttctgga; REV=ttctggggcgttatcattagcatc
Protocadherin (Pcdh) alpha 6: FOR= cacagatgggggcaaacc; REV= gaggcgtcagcgagcagt
Protocadherin (Pcdh) alpha 7: FOR=agttccacatggacccccttaagt; REV=ttcttgaaggtggacaccagctt
Protocadherin (Pcdh) alpha 8: FOR= tgggatcctggcgactactactct; REV=gtcttttacccggaacactgg
Protocadherin (Pcdh) alpha 9: FOR= tccaccccaatgcctcagat; REV= ttcttgaaggtggacaccagctt
Protocadherin (Pcdh) alpha 10: FOR=gcagtcggcggcggttggt; REV=ggtggaaggctggggctgaagg
Protocadherin (Pcdh) alpha 11: FOR= agtcttcctctaggtctgaat; REV= atactgttggccactgctgat
Protocadherin(Pcdh) alpha12: FOR=tcagagaaagggaacaaaaggta; REV=ttctcactcactggcaaaatca
Protocadherin (Pcdh) alpha 13: FOR= cttcctccttgtctgggttct; REV = atactgttggccactgctgat
Protocadherin (Pcdh) alpha C1: FOR = tgtggggtggcagttttatgtttg; REV = gggctccgggcgtcagg
Protocadherin (Pcdh) alpha C2: FOR= catgccctggctgctgctact; REV= cgccaccgattcgcttacct

Caspr1: FOR= tatcagctgggcaccagtccct; REV= atcaagctctggtggcacctc
Caspr2: FOR= tacaacctgggtggcacccgagagcc; REV= aggcgtttgtctgcctcaaggc
Caspr4: FOR= aagaagtcacggctggtcaataag; REV= catacatccgcgctaagttctcc

Neurexin (NRX) 1α and β: FOR= tgcagggcgtcagctcacaatc; REV= acagggcggcagcggctactatcc
Neurexin (NRX) 2α and β: FOR= cgctccccggcccaacctca; REV= cagccggggccttctctttcacca

Neurexin (NRX) 3: FOR= aggactggagggcttgaggaatgt; REV= gtgtagcccgtcgtggtaagaatg
Neurexin (NRX) 1α: FOR= cccgcggcctcgtgctctact; REV= gctgttgggcggctcatcgtc
Neurexin (NRX) 2α FOR= accgccgccgctgctgttga; REV= gcggaagggcggctcgta
Neurexin (NRX) β1 spanning splice sites 4: FOR= tctggccctgctctggatagtc; REV= tggctggccctgctctttc

Neuroligin (NL) 1: FOR= ttgtggggataaaggaaactg; REV= ttaccaaaaggaacaaaataca
Neuroligin (NL) 2: FOR= ccaagttgtcggtgaaccac; REV= tgggccccgtcgtgcagttcttg
Neuroligin (NL) 3: FOR= cgctacggctcgcctacctacttc; REV= gctcctggcgccgtttgtcct
Neuroligin (NL) 4: FOR= tggagaccccgcgtgaagatgaaa; REV= gacgcagaccggggtgaacaacaa

Neurexophilin (NXPH) 1: FOR= ggtgccccccaacaaaaatc; REV= acaagagcagcaaggtagacacat
Neurexophilin (NXPH) 3: FOR= gtggccctgaacctgctc; REV= gccgatagtccgtgctgtagaa
Neurexophilin (NXPH) 4: FOR= caattgccacgtggagtatgagaa; REV= ccacaggagcggcagaacac

Contactin I: FOR=gagcccagtataccatctaaca; REV= agcccaataccgaatctga
RPTPβ: FOR=agtggggcctgttgtcg; REV= ataggaggcattgatgtagtctgt

FOR = forward; REV = reverse

Table 2. GenBank accession numbers for rhesus macaque neural cell adhesion molecules.

GenBank Acc. No.	Gene	cDNA length (bp)
AF480426	Caspr 1	433
AF424844	Contactin 1	470
AF424848	Neurexin 1 β without insert	590
AF424849	Neurexin 1 β with insert	685
AF424835	Neurexin 1 α	448
AF424846	Neurexin 2 α	498
AF424847	Neurexin 3	513
AF424833	Neurexophilin 1	436
AF424823	Neurexophilin 3	362
AF424820	Neurexophilin 4	333
AF424827	Neuroligin 1	410
AF462607	Neuroligin 2	196
AF424850	Neuroligin 3	614
AF462606	Neuroligin 4	658
AF424839	Protocadherin alpha 1	474
AF424838	Protocadherin alpha 2	444
AF424837	Protocadherin alpha 3	453
AF424836	Protocadherin alpha 4	445
AF424840	Protocadherin alpha 5	506
AF424818	Protocadherin alpha 6	229
AF424821	Protocadherin alpha 7	334
AF424824	protocadherin alpha 8	388
AF424831	Protocadherin alpha 9	424
AF424842	protocadherin alpha 11	583
AF424841	Protocadherin alpha 12	474
AF424817	Protocadherin alpha 13	201
AF424828	Protocadherin alpha C1	417
AF424845	RPTPβ	473

All members of the PCDHα family of synaptic adhesion molecules are expressed in the prepubertal female monkey hypothalamus

RT-PCR experiments using primers directed against the human sequences (**Table 1**) revealed that mRNA for each of the 15 described PCDH-αs is expressed in the juvenile rhesus macaque hypothalamus at different levels **(Figure 6B)**. In situ hybridization using a pan-PCDH-α cRNA probe (i.e., complementary to the common intracellular domain of the PCDH-α family) showed that PCDH-α mRNAs are more abundantly expressed in the hypothalamus than in adjacent regions of the primate brain (**Figure 7A**, dotted line). Within the hypothalamus, the transcripts appear to be more prevalent in the region of the arcuate nucleus **(Figures 7A and B,** arrows). For the most part, cells exhibiting hybridization signals had large, pale nuclei (**Figure 7C**, arrows), suggesting a neuronal localization of the PCDH-α transcripts.

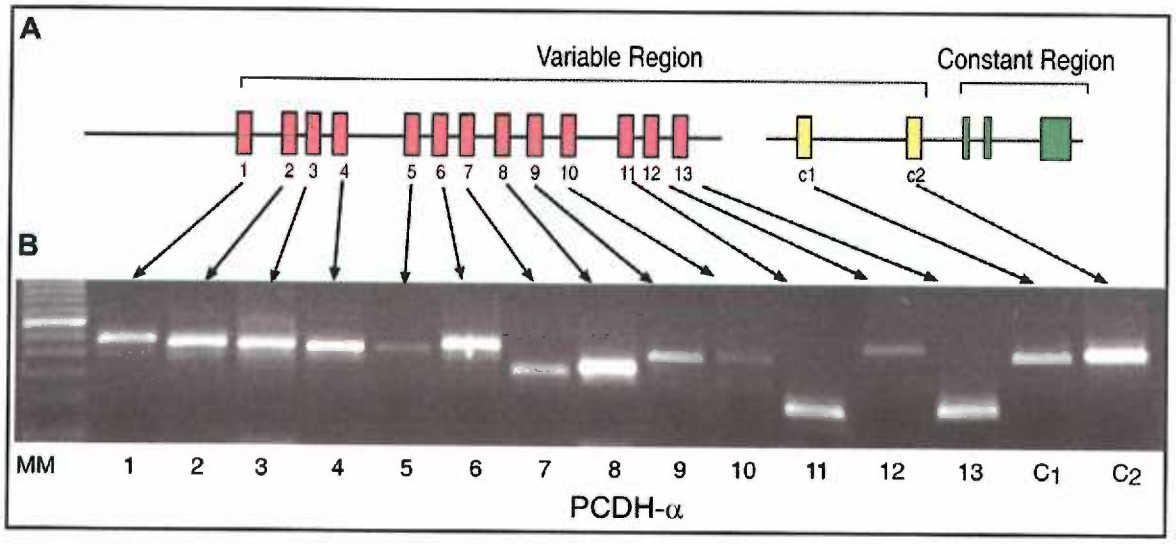

Figure 6. **A**. The genomic organization of the human PCDH-α gene family [38]. The family is composed of 15 different variable regions encoded by large single exons of about 2400 bp in length organized in tandem, and a constant downstream region encoded by three small exons. The PCDH-α gene cluster spans about 230kb of genomic DNA. Each of the large 15 exons encodes a different extracellular domain, which is individually joined to the common downstream region encoding a C-terminal cytoplasmic domain that is identical in all members of the family. Arrows point to the corresponding PCR products of each variable domain expressed in the rhesus monkey hypothalamus. **B**. RT-PCR of hypothalamic RNA extracted from juvenile female monkeys (1-2 years of age) demonstrating the presence of all 15 members of the PCDH-α family. MM = 100 bp molecular marker.

The synaptic specifiers, NRXs and their ligands, are expressed in the juvenile monkey hypothalamus

To simultaneously amplify both the α and β isoforms of each of the neurexin genes, we designed primers recognizing a conserved region corresponding to the sixth LNS domain adjacent to the cell membrane in each neurexin mRNA coding sequence (**Figure 8A**). RT-PCR amplification of total hypothalamic RNA demonstrated that the three neurexin genes are expressed in the prepubertal monkey hypothalamus (**Figure 8B**). Notwithstanding the non-quantitative nature of these RT-PCR reactions, it is interesting to note that the level of expression of neurexin 1 and 2 appears to be

much higher than that of neurexin- 3, suggesting that the three genes are differentially expressed in this region of the brain. Moreover, specific amplification of α-neurexins 1 and 2 with primers directed against the first (N-terminus) LNS domain (**Figure 8A**) revealed that these neurexin forms are much less abundant than the total neurexin mRNA content (i.e., α plus β forms), implying that much of the neurexin mRNAs expressed in the prepubertal monkey hypothalamus encode β-neurexins. Studies have not yet been conducted to determine if the expression of β-neurexin mRNAs predominates in the hypothalamus of monkeys of other ages.

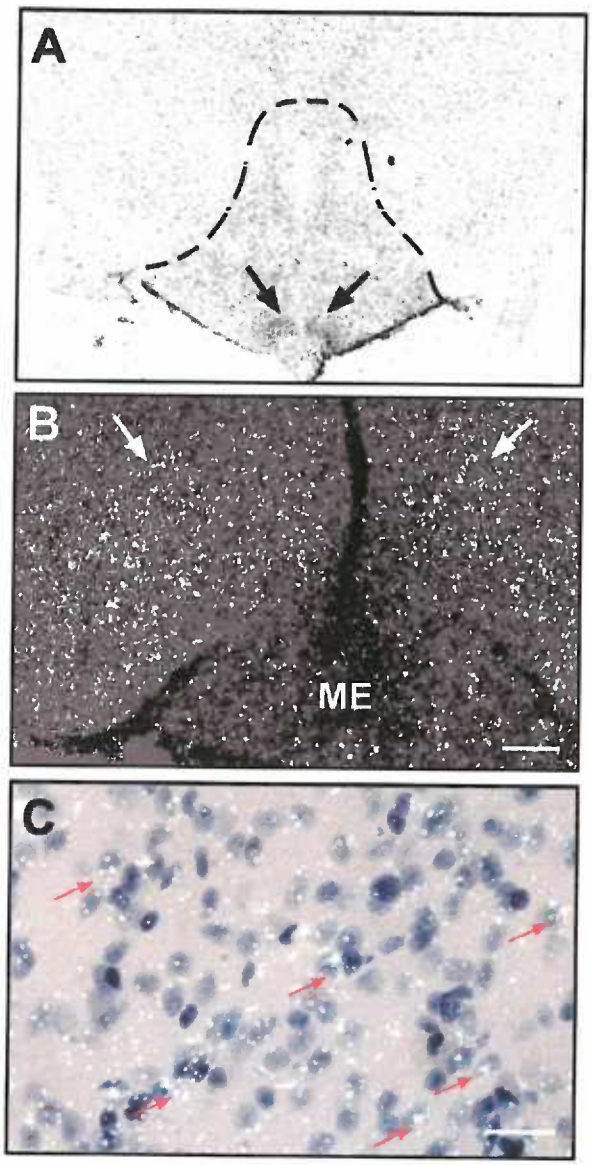

Figure 7. **A.** Detection of PCDHα mRNA in the female rhesus monkey hypothalamus by hybridization histochemistry using a ^{35}S-UTP-labeled rhesus monkey-specific cRNA transcribed from a panPCDH-α cDNA template obtained from the monkey hypothalamus by RT-PCR cloning. Notice the abundance of PCDHα mRNA transcripts in the hypothalamus as a whole (outlined by dashed line), and in the arcuate nucleus-median eminence region in particular (arrows). **B.** Image showing the presence of PCDHα mRNA transcripts in cells of the arcuate nucleus. Bar = 200μm. **C.** Higher magnification image showing PCDHα mRNA-containing cells in the arcuate nucleus. The section was counterstained with thionin. In most cases the positive cells have pale nuclei and, thus, appear to be neurons. Bar = 20μm.

Using monkey primers that detect the two NRX1β isoforms resulting from the alternative use of splice site 4 in the primary NRX1β mRNA transcript, both neurexin 1β isoforms are detected in the prepubertal monkey hypothalamus (**Figure 9A**). The amino acid sequence of the alternatively spliced insert is well conserved, as it is identical to the rat sequence (**Figure 9B**). As expected, based on the neurexin localization to glutamatergic synapses [149], neurexin- 1β mRNA is expressed throughout the hypothalamus in cells presumed to be neurons based on the pale thionin staining of their nuclei (not shown).

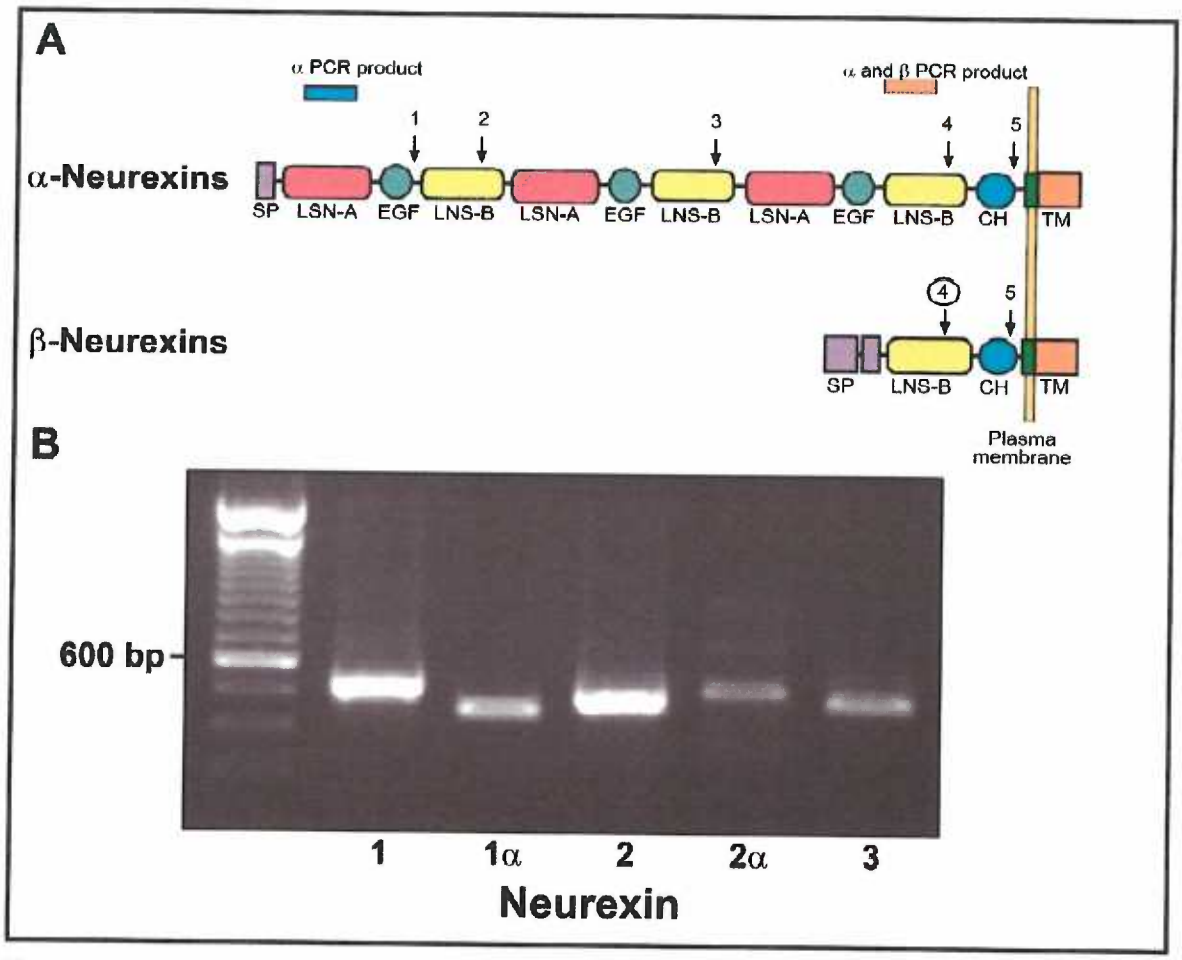

Figure 8. A. The structural characteristics of the NRX family. SP = signal peptide; LNS-A and LNS-B = two types of repeat domain found in laminin (L), neurexins (N), and sex-hormone binding protein-like domain (SHBG); EGF = epidermal growth factor-like repeat; CH = O-linked glycosylation region; ICD = intracellular domain. Numbers 1-5 point to the five alternative splice sites that are common to all members of the NRX family. Site 4 is circled to indicate its critical importance for neuroligin binding, as neuroligins only bind to β-NRXs lacking an insert in this site[141]. The locations of the PCR products shown in B are given above the α-NRX diagram. B. The three known NRX genes are expressed in the prepubertal rhesus monkey hypothalamus. RNA extracted from the hypothalamus of a 351 day-old female monkey was subjected to RT-PCR using primers complementary to a conserved region corresponding to the sixth LNS domain located near the cell membrane in the extracellular encoding portion of human NRX mRNA 1, 2 and 3. Specific identification of NRX1α and 2α mRNAs was achieved by using primers corresponding to the first LNS domain near the amino terminus of each α-NRX (see A). MM = 100 bp molecular marker.

In addition to NRXs, the prepubertal rhesus monkey hypothalamus expresses all four neuroligin genes, and three of the four known neurexophilins (**Figure 10, A and B**). Among the latter, and as previously observed in humans [150], neurexophilin 4 mRNA appears to be the predominant species expressed in the monkey hypothalamus at this stage of development (**Figure 10B**).

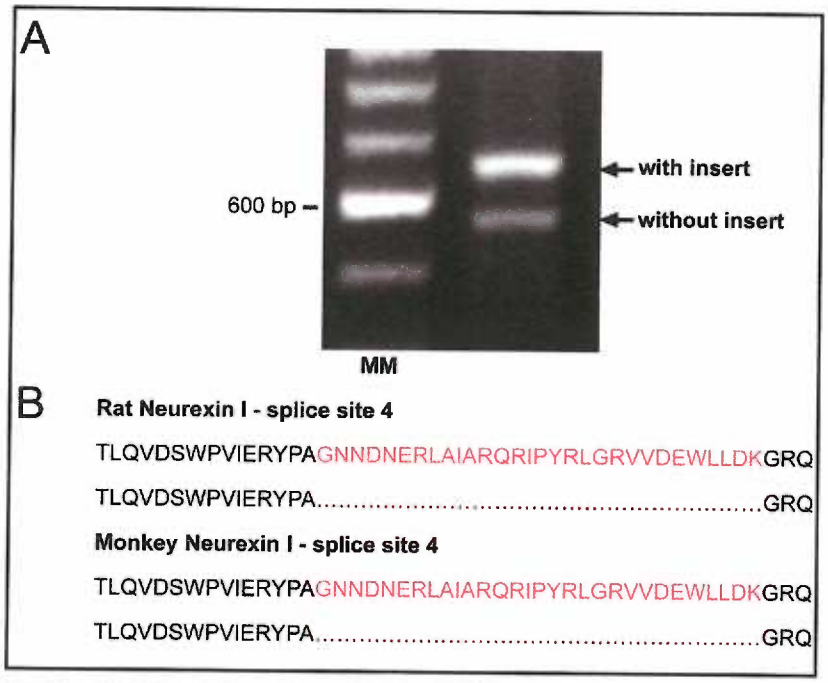

Figure 9 . Alternative splicing of exon 20 of the NRX1β gene (splice site 4) in the monkey hypothalamus, as assessed by RT- PCR amplification of the mRNAs using a 5' primer corresponding to the sequence encoding the unique signal peptide domain of NRX1β and a 3' primer complementary to a sequence downstream from splice site 4. The primer sequences used were those of the published human NRX1 mRNA (GenBank Accession NM_004801). A. Two distinct PCR products corresponding to the splice site 4 with and without an insert are observed (arrows). B. The amino acid sequence of both splice variants is identical in rats and monkeys. MM = 100 bp molecular marker.

The prepubertal monkey hypothalamus expresses the molecular complex required for contactin-dependent neuron-glial adhesiveness.

RT-PCR amplification of CASPR mRNAs from the hypothalamus of prepubertal 1-2 year-old female monkeys demonstrated the presence of mRNAs encoding three of the four known CASPRs (**Figure 11B**). Among them CASPR1 appears to be the most abundant and CASPR4 the least. As would be expected if hypothalamic neurons and astrocytes were to communicate via contactin/CASPR/ RPTPβ-mediated adhesiveness complexes, the juvenile monkey hypothalamus also expresses the contactin gene and abundant levels of RPTPβ mRNA (**Figure 11B**). In situ hybridization employing a CASPR1 cRNA probe demonstrated that CASPR mRNA is abundant in the monkey hypothalamus (**Figure 12A**). Surprisingly, the pattern of CASPR1 expression appears to delineate the hypothalamic region, as the abundance of CASPR1 transcripts sharply decreases at the boundaries of the lateral hypothalamus (**Figure 12, A and B**, arrows). Most CASPR1 positive cells appear to be neurons, as expected (**Figure 12C**, arrows pointing to hybridizing cells with pale nuclei).

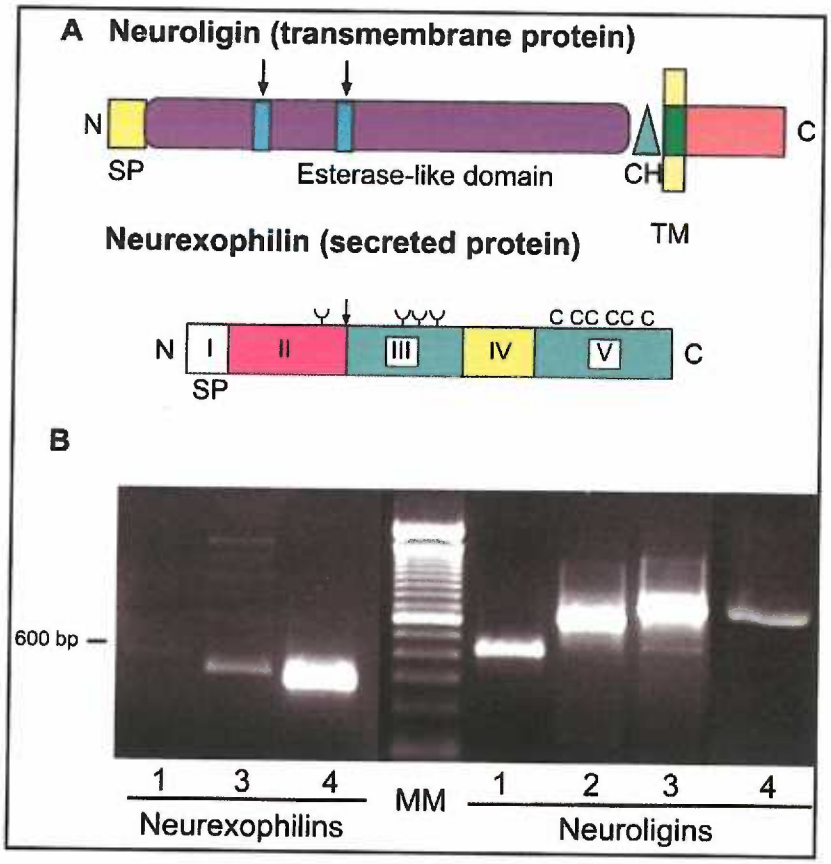

Figure 10. A. The structural characteristics of neuroligins [23] and neurexophilins [20]. N = amino terminus; C = carboxy terminus; SP = signal peptide; CH = O-glycosylation region; TM = transmembrane region. The arrows above the neuroligins diagram point to the two alternatively spliced sites common to all three neuroligins; the single arrow above the neurexophilin diagram identifies the putative site for the proteolytic cleavage of neurexophilins. B. The genes encoding neuroligins and neurexophilins are expressed in the prepubertal rhesus monkey hypothalamus. RNA extracted from the hypothalamus of a 351 day-old female monkey was subjected to RT-PCR using sets of primers complementary to domains of the human neuroligin and human/mouse neurexophilin genes that distinguish the different members of each family among themselves. MM = 100 bp molecular marker.

LHRH neurons express mRNAs encoding synaptic specifiers (NRXs) and their ligands (NRL1) in addition to molecules involved in trans-synaptic (PCDHαs) and neuron-glia (CASPR1) adhesiveness.

PCR analysis of GT1-7 cells using primers that recognize each of the eight CNR mRNAs originally described by Kohmura et al. [37] demonstrated that only the CNR5 and CNR8 genes (the orthologs of PCDHα-13 and 10, respectively) [151] are present at low levels in GT1-7 cells. In contrast, cultured mouse astrocytes contain CNR1, 3, 5 and 8 mRNA transcripts (**Figure 13A**). A similar analysis of the NRL1 and NRX1 genes showed that GT1-7 cells express NRL1, and, surprisingly, both NRX1α and NRX1β (**Figure 13B**). The relative abundance of each transcript appeared to be similar to that detected in the mouse preoptic area (POA) tissue, used as a positive control (**Figure 13B**). Finally, GT1-7 cells express the mRNAs encoding both CASPR1 and its partner contactin, but do not contain RPTPβ mRNA (**Figure 13C**), the glial receptor for the

CASPR1-contactin complex. As expected, hypothalamic astrocytes contain abundant levels of RPTPβ mRNA, but express neither CASPR nor contactin mRNA (**Figure 12C**).

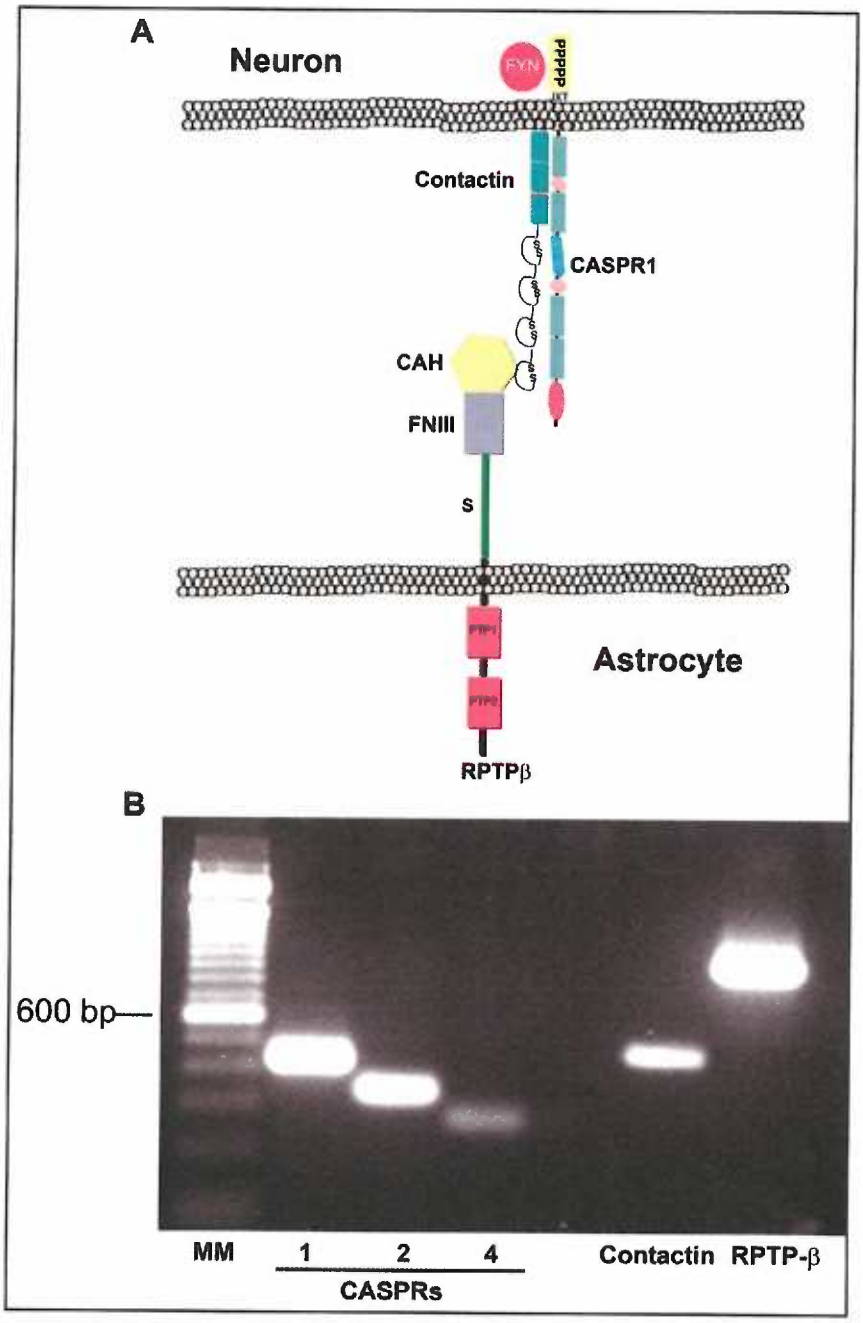

Figure 11. A. The carbonic anhydrase (CAH) region of astrocytic receptor tyrosine phosphatase beta (RPTPβ) binds *in trans* to the neuronal Contactin/CASPR1 heterodimer through the Ig domain of Contactin [48]. Contactin does not have an intracellular portion, but the proline-rich intracellular domain of CASPR1 interacts with the soluble tyrosine kinase Fyn for signaling [48]. PTP1 and 2 = protein tyrosine phosphatase domains, PPPP = proline-rich domain, CAH = carbonic anhydrase domain, FNIII = fibronectin III domain, S = spacer domain. B. The prepubertal female rhesus monkey hypothalamus expresses CASPR mRNAs at different abundance, in addition to contactin, the neuronal cell-surface protein partner of CASPR1. The monkey hypothalamus also contains RPTPβ mRNA, which encodes the glial receptor for the neuronal contactin/CASPR1 complex. MM = 100 bp molecular marker.

Discussion

The results of this study demonstrate that the prepubertal hypothalamus of female monkeys expresses a host of genes encoding molecules shown to have roles in establishing and maintaining neuron-to-neuron and neuron-glia adhesive communication. Earlier morphological examination of the rodent and primate hypothalamus led to the conclusion that both synaptic organization and glia-neuronal adhesiveness are highly plastic events, regulated by both developmental cues and gonadal steroids. For instance, the onset of puberty in the female rat is accompanied by extensive synaptic remodeling of neuronal circuitries in the arcuate nucleus [124,152], changes that reoccur during the adult estrous cycle [153,154] as a consequence of estrogen action. In the monkey hypothalamus the apposition of glial cells to LHRH neurons changes during the course of sexual development [127,155] and, in adult animals, as a consequence of estrogen action [156].

One of the most striking plastic event involving glial cells and LHRH neurons is observed at the median eminence of the hypothalamus. LHRH nerve terminals, which converge to the median eminence to release their secretory products into the portal system, are in close contact with astrocytes and modified ependymoglial cells known as tanycytes [157]. These cells line the ventral surface of the third ventricle and send their processes to the external region of the median eminence, where they establish contact with the endothelial wall of the portal vessels via "end-feet" specializations. The relationship between glial cells of the median eminence and LHRH nerve terminals changes during both the estrous cycle [45] and in response to changes in the sex steroid milieu [61]. During periods of low LHRH secretion, tanycytic end-feet prevent the direct contact of LHRH nerve endings with the endothelial wall of the portal vessels; at times of increased LHRH output (for instance, during the preovulatory surge of gonadotropins), the end-feet retract allowing the nerve ending to directly contact the portal system [45,61]. Recently, tanycytic signaling pathways set in motion by ligand-induced activation of erbB-1 receptors and involving the sequential activation of PGE_2 and TGFβ1 formation have been shown to account for at least part of this plasticity [47].

While these findings reiterate the importance of regional and developmental specificity of both synaptic remodeling [124,152-154] and growth factors actions in the neuroendocrine brain [118,158], they also argue for the existence of an adhesion/recognition code able to provide specificity, coordination, and flexibility to hypothalamic cell-cell communication. Perhaps the first hint that changes in adhesiveness and/or cell recognition events could play a role in the control of primate puberty was provided by the work of Perera and Plant, [125], who demonstrated a greater abundance of the polysialated form of NCAM (polysialation is an extracellular modification of NCAM whose presence indicates a less adhesive, thus more plastic form of the molecule [159-162]) in the hypothalamus than in the cerebral cortex of prepubertal rhesus monkeys.

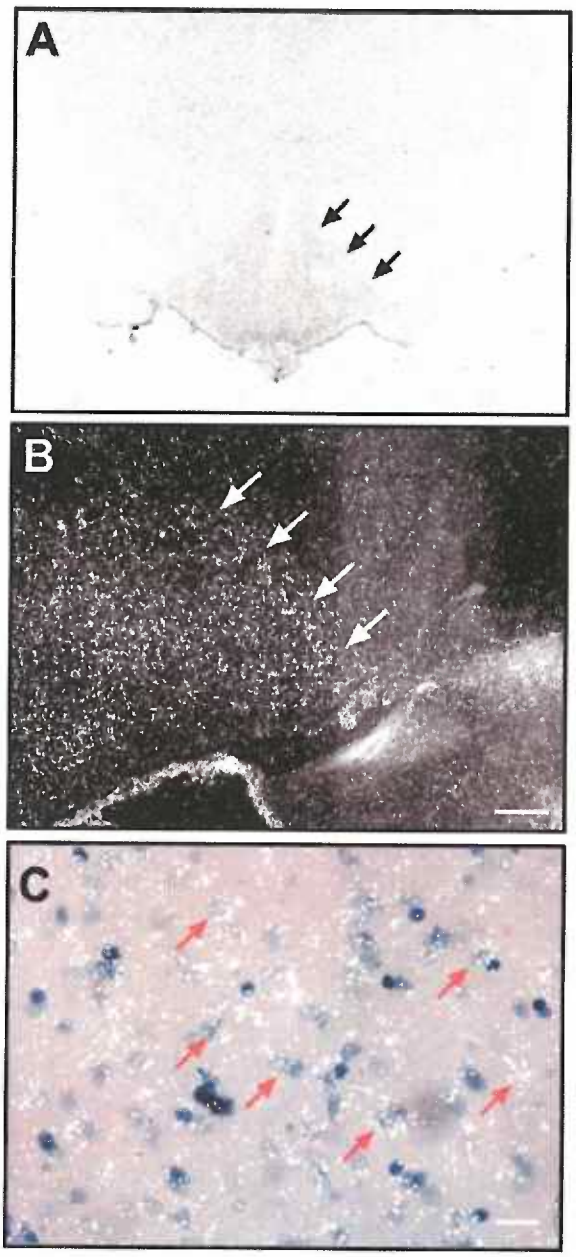

Figure 12 . A. Detection of CASPR1 mRNA in the rhesus monkey hypothalamus by hybridization histochemistry using a ^{35}S-UTP-labeled rhesus monkey-specific cRNA transcribed from a CASPR1 cDNA template obtained from the monkey hypothalamus by RT-PCR cloning. Arrows highlight the peculiar distribution pattern of CASPR1 mRNA, which appears to delineate the boundaries of the hypothalamus. B. Higher magnification image illustrating the sharp delineation of the hypothalamus by CASPR1 mRNA containing cells (denoted by arrows). Bar = 400 µm. C. Expression of CASPR1 mRNA in neurons of the hypothalamus as suggested by the presence of hybridization in cells with pale nuclei. The section was counterstained with thionin. Bar = 20µm.

As the initial step of an ongoing effort to acquire a more global understanding of such a molecular code, we now describe the existence in the prepubertal female monkey hypothalamus of three large families of adhesion / signaling molecules that may play a significant role in specifying, establishing and maintaining cell-cell interactions within the neuroendocrine brain. Our findings demonstrate that all 15 members of the PCDHα subfamily [163], the human counterparts of the murine family of Cadherin-Related Neuronal Receptors (CNRs) [37], are expressed in the

monkey hypothalamus before the initiation of puberty. PCR analysis of GT1-7 LHRH-secreting cells showed that only CNR5 (PCDHα13) and CNR8 (PCDHα10) are expressed in these cells. The low level of expression may be attributed to an immaturity of GT1-7 cells, which lack the synaptic input received by LHRH neurons in situ. PCDHαs are mostly localized to synaptic contacts [37,42,43,164,165], and, thus, their level of expression would be expected to increase as synaptic connectivity becomes established during development. Nevertheless, CNRs are highly expressed in discrete neuronal subsets of the olfactory bulb of the mouse brain; therefore it is entirely possible that at least CNR8/PCDHα10 is expressed in LHRH neurons in situ. Further investigation is needed to clarify this issue.

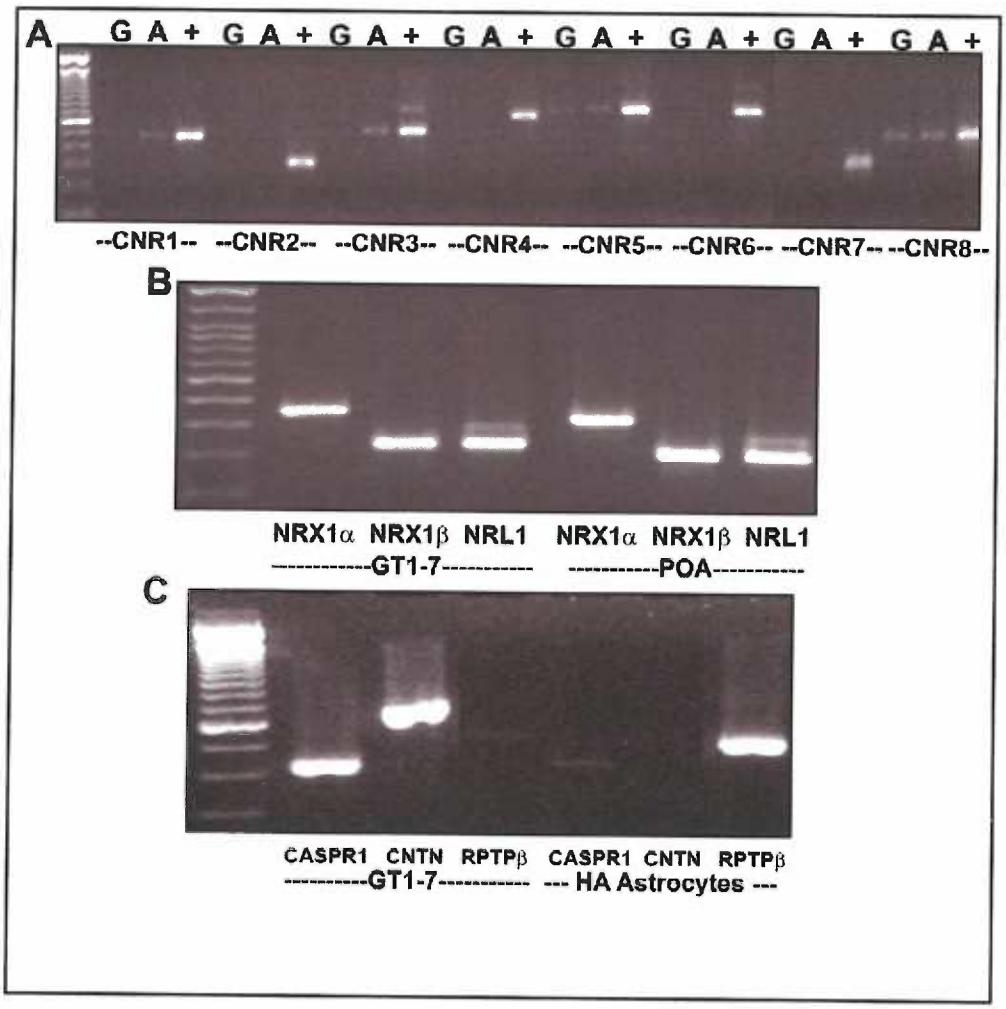

Figure 13. A. Detection of CNRs (the mouse orthologs of PCDHα) in GT1-7 cells (G). The levels of expression are compared to those detected in hypothalamic astrocytes (A), and mouse preoptic area (POA) used as a positive control (+). B. Detection of NRX and NRL1 mRNAs in GT1-7 cells and mouse POA. C. Selective expression of CASPR1 and contactin (CNTN) mRNA in GT1-7 cells and RPTPβ mRNA in mouse hypothalamic (HA) astrocytes. MM = 100 bp molecular marker.

The presence of more than one PCDHα in GT1-7 cells raises the possibility that LHRH neurons in situ might also express several members of this family and that, as envisioned for synaptic adhesiveness in general [36,165], LHRH neurons may maintain a diversity of synaptic contacts via the extracellular domain of each PCDH/CNR. Each PCDH would then converge intracellularly, via the common intracellular domain, to activate a similar signaling pathway [36,165]. The presence of all 15 members of the family in the hypothalamus as a whole further suggests the existence of an extensive network of transsynaptic connectivity mediated by both homophilic [166] and heterophilic PCDHα-dependent interactions [41]. Because different neurons can express different PCDHs [37,43,167], future studies will have to be performed to profile synaptically connected hypothalamic neuronal networks, define the PCDH-based adhesive code underlying the GABAergic and glutamatergic control of LHRH neuronal function, and identify potential changes in synaptic adhesiveness that may occur at the time of primate puberty.

Central to the issue of the dual excitatory/inhibitory transsynaptic control of LHRH secretion is an understanding of the molecular mechanisms underlying the establishment of this control. Well-suited to carry out this function is the neurexin family of adhesion / signaling molecules and their ligands because they are neuronal cell surface proteins differentially expressed in excitatory and inhibitory synapses [168-170], [21,149]. Although α-NRXs are not required for synapse formation, they are crucial for Ca^{2+}-dependent presynaptic vesicle exocytosis [168] and postsynaptic NMDA receptor function [170]. In contrast, the postsynaptic clustering of neuroligins and their interaction with β-NRXs results in the organization of excitatory synapses . Critical for this association to take place is the absence in NRX1β of an insert generated by the use of alternative spliced site 4 in the neurexin mRNA primary transcript [171,172]. It then follows that NRL-expressing neurons can form excitatory synapses only when their presynaptic partners contain the appropriate "insert-minus" splice form required for NRL recognition and binding [169]. Our results show that this form is present in the prepubertal monkey hypothalamus, and thus is able to encode proteins engaged in the organization of glutamatergic synapses.

The presence of NRL-1 in GT1-7 cells is consistent with the concept that LHRH neurons receive direct glutamatergic innervation [10,12], because NRL-1 is a postsynaptic protein of excitatory synapses [25,173] able to trigger the organization of presynaptic terminals [169,174]. More difficult to explain is the detection of NRX1β in GT1-7 cells. Because NRX1β is selectively expressed in presynaptic terminals [168,169], its expression in GT1-7 cells would imply that it forms part of the molecular complex used by LHRH neurons to synaptically target either other neurons of the LHRH network [175,176] or glutamatergic neurons [10]. Alpha-neurexins, on the other hand, have been found to play a role both pre and postsynaptically [168,170].

While protocadherins and neurexins are required for the structural and functional adhesiveness of neurons CASPR1, a member of the CASPR family of cell-recognition molecules, and its binding partners neuronal contactin and glial RPTPβ have been implicated in glial-neuronal adhesiveness [177,178]. Our results show that the three components of this intercellular signaling

complex are expressed in the juvenile monkey hypothalamus, and that LHRH neurons themselves contain contactin and CASPR1, the neuronal partners of glial RPTPβ required for the adhesive recognition of glial cells [48]. The striking distribution of CASPR1-expressing cells that essentially delineates the hypothalamus from surrounding brain regions reinforces the notion that the hypothalamus is a preferred site of glial-neuronal interactions [179,180]. Recognition events mediated by CASPR1/contactin association have been shown to be important for the functional integrity of paranodal junctions of myelinated axons [133]. The hypothalamus, however, is not a highly myelinated brain region, and yet it expresses the three components of this glia-neuron communication complex. It is then likely that contactin/CASPR1-RPTPβ mediated interactions sub-serve functions different than those required for myelinated nerve conduction. The homeostatic regulation of contactin expression in magnocellular vasopressin secretory neurons in relation to shifts in neuronal-glial association [134] suggest that a function of contactin/CASPR1-RPTPβ complexes in the neuroendocrine brain is to facilitate neurosecretion via changes in glial-neuronal adhesive signaling.

In summary, this study describes the presence in the prepubertal female monkey hypothalamus of a multiplicity of adhesion molecules with signaling capabilities. Their molecular and functional diversity, and the far-reaching consequences of their actions suggest that these multigene families might play a significant role in specifying changes in neuronal and glial plasticity in the neuroendocrine brain during peripubertal development.

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CHAPTER THREE

The synaptic adhesion molecule SynCAM is associated with erbB4 dysregulation in the hypothalamus of mice with a delayed onset of puberty.

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Chapter 3. The synaptic adhesion molecule SynCAM is associated with erbB4 dysregulation in the hypothalamus of mice with a delayed onset of puberty.

Abstract:

The erbB family of EGF-like growth factor receptors plays an important role in the timely onset of mammalian puberty. TGF-α and the Neuregulins elicit luteinizing hormone-releasing hormone (LHRH) release from the hypothalamus via a juxtacrine/paracrine mode of communication that involves activation of glial erbB1 and erbB4 receptors, recruitment of erbB2 co-receptors into an heterodimeric complex, and release of bioactive substances such as prostaglandin E_2 that then acts directly upon LHRH neurons to enhance LHRH secretion. Transgenic mice in which erbB4 signaling has been impaired in astrocytes via astrocyte-specific expression of a dominant-negative erbB4 receptor lacking the intracellular domain show delayed sexual development and a delayed initiation of reproductive capacity. To identify hypothalamic proteins whose expression may be affected in erbB4-deficient mice, hypothalamic proteins from wild-type and mutant mice were extracted and subjected to a quantitative proteomics procedure based on the use of isotope-coded affinity-tag (ICAT) labeling followed by micro-liquid capillary tandem mass-spectrometry. The content of a synaptic adhesion molecule known as SynCAM was prominently reduced in the mutant hypothalamus in comparison with wild type mice. Further analyses revealed that SynCAM is not only expressed in neurons, but also in astrocytes, and showed that both SynCAM mRNA and SynCAM protein content are reduced in hypothalamic astrocytes of mutant mice. Hypothalamic astrocytes mostly express one of the five reported alternatively spliced SynCAM mRNA transcripts, and the expression of this transcript (isoform 2) is decreased in the erbB4-deficient mice. Promoter assays demonstrated the SynCAM promoter can be regulated by methylation, but this does not appear to be the main mechanism for lowered SynCAM mRNA levels in the DNerbB4 hypothalamus. The SynCAM promoter is activated by ligand-dependent stimulation of the wildtype erbB4 receptor. In agreement, erbB4 activation by Neuregulin β1 in astrocytes increases the expression of SynCAM mRNA. Yeast-two hybrid and immunoprecipitation experiments showed that SynCAM is physically associated with the erbB4 receptor in hypothalamic astrocytes. Both astrocytes and the LHRH neuronal cell line GT1-7 express the same SynCAM isoform, suggesting that SynCAM may be a required component of cell-cell communication between LHRH neurons and their astrocytic entourage. In vitro adhesion assays demonstrate that wildtype hypothalamic astrocytes adhere preferentially to SynCAM fusion proteins, and this binding is greatly diminished in the erbB4-disrupted astrocytes. These results suggest that SynCAM plays an important role in the erbB4-mediated LHRH neuronal-glial interactions underlying pubertal timing. Support Contributed By: NIH Grants MH-65438, HD-25123, and HD-18185.

Introduction:

The erbB4 receptor tyrosine kinase and its ligand, Neuregulin, have been shown to be important for synaptic function (for review see [181]). Neuregulin is involved in the specification and function of synapses [182,183], as evidenced by its ability to downregulate postsynaptic $GABA_A$ receptors at the hippocampal inhibitory synapse [183], and appears to exert this effect by the activation of erbB4 receptors. The erbB4 receptor is the only erbB receptor containing a PDZ-binding domain in its intracellular portion [184]. This domain has been shown to interact with post-synaptic density proteins such as PSD-95 [84]. ErbB4 is able to signal intracellularly though the intracellular domain through various pathways, the use of which is determined by the erbB4 isoform present [85,185], [87]. The intracellular domain of erbB4 has also been shown to be subject to cleavage and translocation to the nucleus [88], where it associates with the co-transcriptional activator YAP [89,90]. Particular interest has been paid of late to the role of neuregulins and erbB4 in the pathogenesis of schizophrenia (for review see [186,187]). Work in our lab has also shown erbB4 to interact with the glial adhesion molecule RPTPβ [188], the binding partner of the neuronal Caspr/Contactin complex [177]. ErbB4 is, therefore, a growth factor receptor with a complex signaling function that may play a role both in synaptic and neuronal-glial communication.

Astrocytes in the hypothalamus produce both Neuregulin and erbB4, and interact with each other in a juxtacrine manner through these molecules **(Figure 3)**. Previous work in the Ojeda lab has shown that impaired erbB4 function specifically in astrocytes leads to a delay in the onset of puberty and diminished reproductive capacity in early adulthood. This is true in mice [68,189], and preliminary data also suggests that a polymorphism in the erbB4 gene, occurring normally in a subset of the human population, is correlated with a later, though not pathologically so, onset of puberty in girls (unpublished results). The mechanisms by which defects in this receptor disrupt the function of LHRH neurons in the pubertal hypothalamus are a subject of intense scrutiny. We have shown that the release of prostaglandin E_2 from hypothalamic astrocytes, important in effecting LHRH release, is reduced in these animals [68]. This could be a result of reduced communication between LHRH neurons and the closely apposed astrocytes that surround them.

Astrocytes have recently been shown to have a profound effect on synaptic formation and plasticity [52,54]. This could be due in part to their complement of neuregulins and erbB4 receptors. Neuregulins act as bi-directional signaling molecules in neurons that, upon binding erbB4 on nearby cell membranes, influence neuronal excitability [190]. We have also shown astrocytic erbB4 receptors to be intimately associated with glutamate receptor function [56]. Defects in glial erbB4 receptor function could therefore directly affect the activity of nearby neurons through a lack of erbB4 binding to neuregulin on those neurons, or from a loss of excitatory influences from the astrocyte itself.

Additionally, loss of erbB4 could influence astrocytic adhesion molecules important in physical association with the LHRH neurons. The three-dimensional relationship of astrocytes to neurons can play a major role in neuronal function, as the relative proximity of glial cells affects glutamate uptake, K+ balance, and the availability of juxtacrine signaling molecules like the erbBs [159,191-193]. These findings have thus lead us to search for molecules, particularly those which have been shown to function as adhesion molecules, which may be affected by the disruption of astrocytic erbB4 function.

What are the cellular proteins affected by the disruption of erbB4 signaling in the developing hypothalamus? Although other studies in our laboratory are aimed at identifying mRNAs whose expression is affected by the loss of erbB4 function, it is extremely important to study the actions of the relevant genes at the protein level. Differences in mRNA levels do not necessarily reflect differential protein expression [194] and give little indication of how protein activity may be modulated by post-translation modifications such as phosphorylation or proteolytic cleavage. Recently, the field of comparative proteomics has undergone technological advances that allow relatively simple, high-throughput approaches to examining differences in protein expression and regulation in tissues [195]. In particular, the isotope-coded affinity-tag (ICAT) reagent allows investigators to detect differences in protein expression large, heterogeneous protein samples [196]. We established a valuable collaboration with the Institute for Systems Biology (ISB) in Seattle, WA that allowed our laboratory (which had previously specialized in molecular approaches to this problem) to explore changes in protein expression and regulation in the hypothalamus relating to the onset of mammalian puberty.

With this technique we were able to screen the hypothalamic proteome for proteins that were affected in animals carrying an astrocyte-specific disruption of the erbB4 receptor (DNerbB4) relative to controls. **Figure 14** describes the various steps of this experiment. Of particular interest was one synaptic adhesion molecule, SynCAM, which was greatly reduced in the mutant animals.

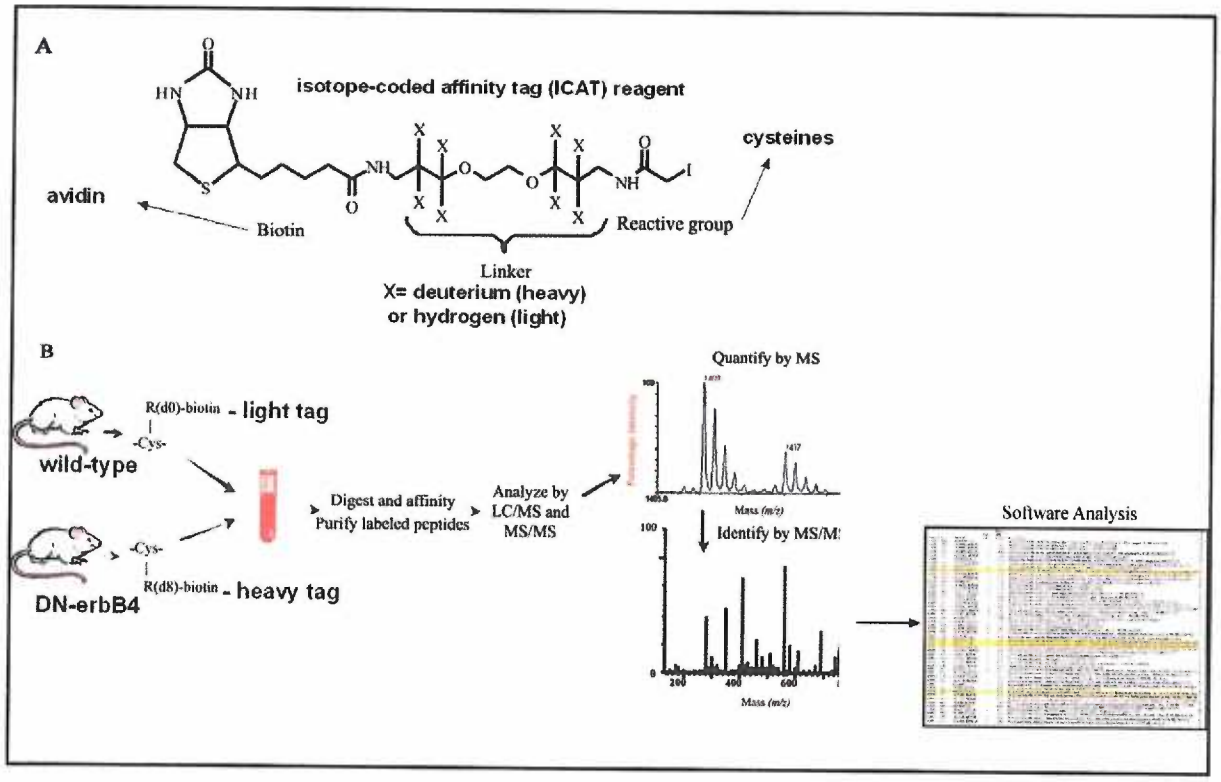


Figure 14. Scheme for isotope-coded affinity tag (ICAT) experiment. A. The ICAT reagent attaches to cysteines in proteins via its reactive thiol group. The linker region contains either 8 hydrogens (the "light" tag) or 8 deuteriums ("heavy" tag), giving the two tags a mass difference of 8 Daltons. A biotin moiety allows the labeled peptides to be isolated on an avidin affinity column. B. Proteins were extracted from the hypothalami of wild type and DNerbB4 female mice, labeled with the ICAT light or heavy tags, and pooled. The pooled proteins were digested into tryptic fragments and labeled peptides were isolated with an avidin affinity column. Labeled peptides were further purified on a strong ion-exchange column and fractions from the column were analyzed by tandem micro-capillary mass spectrometry (μLC-MS/MS). Peptide abundance was measured on the first mass spectrometer run, and peptide peaks were then sequences through collision-induced dissociation (CID) in the tandem mass spectrometer. The INTERACT software package then matches identified peptides with their peptide peaks and provides a list of sequenced peptides and the relative abundance of the light and heavy labeled portions.

Materials and Methods:

Animals:

The dominant-negative erbB4 (DNerbB4) mice were generated on an FvB background by introduction of a dominant-negative erbB4 receptor protein which lacks an intracellular domain [68,197]. The transgenic construct carried a GFAP promoter to drive expression exclusively in astrocytes. These mutant animals have been shown to have a delayed onset of puberty and a diminished reproductive capacity [198].

Astrocytes:

Astrocytes were isolated from the hypothalamus of neonatal wild type and transgenic mice, and cultured as previously described [66,199]. After a growth period of 8-10 days in 75 cm culture flasks containing DMEM/F12 medium supplemented with 10% donor calf serum, the astrocytes were isolated from contaminant cells by 48 hours of shaking at 250 rpm, and were replated in either 10cm dishes for protein extraction, or six-well plates for RNA extraction. Upon

reaching 80-90% confluence, the medium was replaced with a serum-free, astrocyte-defined medium consisting of DMEM devoid of phenol red, supplemented with 2 mM L-glutamine, 15 mM HEPES, 5 μg/ml insulin, and 100 μM putrescine [66]. The cells were kept in astrocyte-defined media, with daily changes, for at least two days before treatment. Some cultures were stimulated with 100ng/mL Neuregulin (Heregulin) β1 (Lab Vision Corp., Freemont, CA), an effective ligand for erbB4, in astrocyte-defined medium.

Cell Lines:

GT1-7[138], HEK293 [200], HiB5 [201] and BAS8.1 [202] were cultured at 37°C in 5% CO_2 in DMEM/10% fetal calf serum (FCS) medium (GT1-7, HEK293) or at 33°C in 5% CO_2 in DMEM/10% FCS medium (HiB5, BAS8.1) with 30ng/ml puromycin (BAS8.1). BAS8.1 cells were shown via RT-PCR to express erbB1 and erbB2, but neither erbB3 nor erbB4 (data not shown).

Tissue and Protein:

For the mass spectrometric studies, the preoptic area (POA) was dissected from four DNerbB4 and four wildtype FvB pubertal (day 30) female mice and rapidly frozen on dry ice. The tissue was then transported to the Institute for Systems Biology (ISB) in Seattle, WA, where the protein from each group was extracted with the MPER mammalian protein extraction reagent following the manufacturer's protocol (Pierce, Rockford, IL). The protein samples were spun to pellet insoluble protein and then dried down to 100μl. 400μl of labeling buffer was added to each sample (0.05% SDS, 200mM Tris pH 8.3, 5mM EDTA, 6M Urea). Proteins were quantified with the Pierce BCA reagent. They were reduced with 5mM tris(2-carboxyethyl) phosphine (TCEP) and 535 nanomoles of the ICAT reagent were added to each sample [195] and incubated for 90 minutes at room temperature. An aliquot of each sample was run on a SDS-PAGE gel to check for labeling, indicated by a small shift in the size of the Coomassie-stained protein bands. The ICAT reaction was then quenched with an excess of DTT and the proteins were digested overnight at 37°C with trypsin (1:50, w/w). Complete digestion was verified via SDS-PAGE and Coomassie staining. The resultant tryptic peptides were subjected to separation on a strong-ion exchange column and ICAT-bound peptides were isolated with an avidin-affinity column. These purified peptides were lyophilized and processes as described below.

The rest of the protein used in these studies was obtained using a protocol designed to maximize retention of membrane proteins [203] and described previously by our laboratory [68]. Briefly, cultured cells were grown to 80% confluency in 60mm plates, rinsed with PBS, and snap-frozen on dry ice. Proteins were then extracted with lysis buffer consisting of 25 mM Tris pH 7.4, 50 mM β-glycerophosphate, 1% Triton X100, 1.5 mM EGTA, 0.5 mM EDTA, 1mM sodium pyrophosphate, 1mM sodium vanadate, 10 μg/ml Leupeptin and Pepstatin, 10 μg/ml aprotinin and 100 μg/ml phenylmethanesulfonyl fluoride (PMSF). Lysates were homogenized by passage through a 27g syringe 4-6 times and insoluble proteins were pelleted by centrifugation; protein

concentration was measured using a Bradford assay (BioRad). Tissue was homogenized in the same lysis buffer using a PowerGen 700 homogenizer (Fisher), then centrifuged and measured as above.

Mass Spectrometry:

Mass spectrometry was performed in the proteomics core facility at the ISB and the data gathered was transmitted to the ONPRC. The lyophilized peptides were solubilized in 50µl of an aqueous solution of 0.4% acetic acid and 0.005% HFBA. Approximately 2µl of this solution was pressure-bomb loaded onto a 75µm i.d. reverse phase capillary column packed with 10cm of Magic C18 resin (Michrom Bioresources, Auburn, CA). The peptides were subsequently eluted off of the column using a linear solvent gradient of increasing acetonitrile concentration, and ionized using electrospray ionization using a LC set-up previously described [204]. The ionized LC stream was analyzed by tandem mass spectrometry on a LCQ-Classic (Thermofinnigan, San Jose, CA). Amino acid sequence assignments were generated from the tandem mass-spectrometry analyses by searching the mass spectrometer data files against the mouse genomic sequence using the SEQUEST algorithm [205]. The SEQUEST generated lists were processed for easier user analysis using the INTERACT interface [206].

Antibodies:

A rabbit polyclonal antibody against SynCAM was created directed against the sequence CNNSEEKKEYFI corresponding to the C-terminal amino acids of SynCAM plus an N-terminal cysteine for coupling (Sigma-Genosys). The other antibodies used in these studies were; mouse monoclonal anti-GAPDH (Chemicon), rabbit polyclonal anti-erbB4 sc283 (Santa Cruz), rabbit polyclonal anti-erbB2 Ab-1 (Neomarkers), mouse monoclonal IgM anti-PSA 5A5 (a kind gift from Dr. Urs Rutishauser [207]), goat polyclonal anti-human IgG, Fc portion (Sigma-Aldrich), guinea pig polyclonal anti- human GFAP (Chemicon), and mouse monoclonal anti-LHRH 4H3 (provided by Dr. Henryk Urbanski [208]) .

Immunoprecipitation and Western Blotting:

Protein extracts were size-fractionated by SDS-PAGE, as described [66,209]. The separated proteins were then transferred to PVDF membranes and immunoblotted with different antibodies (see above) to identify SynCAM, GAPDH and the different erbB receptors. Immunoprecipitations were performed using the polyclonal anti-SynCAM antibody describe above. Cell lysates (750-1,000 µg protein) were incubated overnight at 4°C with 4 µµg of the SynCAM antibody. Thereafter, the receptor-antibody complexes were incubated with a slurry of protein A-sepharose (60 µl sepharose beads/750 µl immunoreaction for 2 hours at 4°C). The sepharose beads were collected by centrifugation, washed twice with lysis buffer, resuspended in 2x sample buffer (SDS, β-mercaptoethanol, bromophenol blue, glycerol), and boiled for 5 minutes before

loading onto pre-cast 10% or 8-16% polyacrylamide-SDS gels (Invitrogen). After electrophoresis, the size-fractionated proteins were transferred to PVDF membranes and subjected to immunoblotting using the polyclonal anti-erbB4 antibody sc283 or the polyclonal anti-erbB2 Ab-1 antibody. The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch), incubated with an enhanced chemiluminescence reagent (Renaissance, NEN), and exposed to film to develop the immunoreaction. After stripping (62.5 mM Tris HCl, pH 6.7, 2% SDS, 100 mM β-mercaptoethanol, 30 min at 60°C), the membranes were reprobed with the SynCAM antibody used for immunoprecipitation. The housekeeping protein GAPDH, used as a loading control for the blots, was detected with the monoclonal anti-GAPDH antibody.

RNA extraction:

Tissues were homogenized (100mg/ml) in TriReagent solution (MRC, Cincinnati, OH), and the aqueous and organic phases were separated by the addition of 0.1vol bromo-chloropropane (BCP, Sigma Chemicals, St. Louis, MO) followed by centrifugation at 4°C. RNA was precipitated from the aqueous phase with 1 volume of isopropanol by centrifugation at 13,000 rpm for 30 minutes at 4°C. RNA was resuspended in DEP-treated H_2O and treated with Ambion's DNA-free DNAse I (2U per reaction) for 30 minutes at 37°C. RNA concentrations were determined spectrophotometrically. Afterwards, 500 ng of RNA from each sample were run on denaturing agarose gels to confirm RNA integrity. Astrocytes and BAS8.1 cells in culture were rinsed with PBS and snap-frozen on dry ice before RNA extraction.

Cloning and sequencing of SynCAM cDNA fragments:

Five hundred nanograms of RNA extracted from mouse hypothalamus, FvB and DNerbB4 astrocytes, or GT1-7 cells was subjected to reverse transcription with random hexamer primers (Invitrogen) using Qiagen's Omniscript reverse transcriptase. PCR was performed with mouse primers flanking the alternatively spliced sequence, nts 781-1204, in the published mouse SynCAM sequence, AF539424 [210]. The resultant bands were cloned the pGEM-T vector (Promega, Madison WI), transformed into XL-1 Blue competent cells (Stratagene, La Jolla, CA) and spread onto Luria-Bertani (LB) broth (Fisher Chemicals, Fairlawn, NJ) / agarose plates containing 50μg/ml ampicillin. Positive colonies were grown overnight in 6ml of LB media with 50μg/ml ampicillin and plasmid DNA was isolated using the Qiagen Plasmid Miniprep kit. The cloned cDNAs were sequenced on an ABI 3100 Genetic Analyzer DNA sequencer using M13 forward and reverse primers, and the sequences were analyzed using DNAStar software (DNASTAR, Inc. Madison, WI).

Real-time PCR:

Real-Time PCR primers and a fluorescent TaqMan MGB probe were designed using Primer Express software (ABI) to recognize the portion of mouse SynCAM encoding SynCAM isoform 2. The probe was designed with ABI's MGB quencher system, which allows the primers and probe to span a much shorter region of DNA, making isoform discrimination more reliable. Real-time PCR assays were performed in 96 or 384 well plates on a 7900HT Fast Real-Time PCR System (ABI) using Roche's Taqman Universal PCR Master Mix. Primers for 18S ribosomal RNA were used as internal controls.

Constructs:

The human SynCAM promoter was cloned into pGL3 as described below. Full-length erbB4 (JM-a) in pcDNA3 was provided by Dr. Gabriel Corfas (Children's Hospital, MA). The SynCAM Fc fusion proteins were a kind gift from Dr. Thomas Biederer (Yale), as well as the full-length SynCAM isoform 2 in pCMV5. The dominant-negative erbB4 mutant in pcDNA3 has been previously described by our laboratory [68].

Promoter Assays:

Primers were designed to amplify a 1.9kb sequence upstream of the human SynCAM mRNA; this fragment was isolated from human genomic DNA and cloned into the luciferase expression vector pGL3 (Promega). Luciferase assays in HEK293 cells demonstrated that this DNA segment was transcriptionally active.

To examine the role of erbB4 stimulation upon SynCAM promoter activity, HiB5 cells were co-transfected with the SynCAM promoter-pGL3 luciferase construct and either the full-length erbB4 receptor or the dominant-negative DNerbB4 receptor in pcDNA3 [68]. 24 hours after transfection and incubation in 5% FCS, lysis buffer was added to cells and they were frozen at -85°C. Luciferase assays were performed according to the manufacturer's protocol.

To examine the effects of erbB4 stimulation with Neuregulin upon SynCAM promoter activity, HiB5 cells were co-transfected with pGL3-SynCAM and the wildtype erbB4 and stimulated with 100ng/mL Neuregulin β1 in serum-free media for 0, 4, 8 and 12 hours. Cells were then lysed and processed as above.

To examine the effect of methylation on the activity of the SynCAM promoter, the pGL3 construct was incubated with SssI methylase for 18 hours at 37°C. Digestion with the methylation-sensitive restriction enzyme HhaI was used to confirm complete methylation of the promoter construct (**Figure 15**). This methylated construct was then used in promoter assays with comparison to the unmethylated SynCAM-pGL3 construct.

5-aza-cytidine treatment.

Hypothalamic astrocytes from wildtype and DNerbB4 mice were cultured in the presence of 2μM 5-aza-cytidine (Aldrich), a demethylating reagent [211]. Control cells were cultured in the absence of 5-aza-cytidine. mRNA from each set of astrocytes was extracted using the TriReagent protocol (MRC) and 500ng was reverse-transcribed with OmniScript RT from Qiagen. SynCAM transcripts in both samples were measured by Real-Time PCR using the primers and probe designed for SynCAM isoform 2 described above.

Bisulfite sequencing and methylation-specific PCR:

To examine the methylation state of the SynCAM promoter in wildtype and DNerbB4 cultured hypothalamic astrocytes, genomic DNA was extracted and subjected to methylation-specific PCR. DNA from cultured astrocytes was extracted with the DNeasy DNA extraction kit from Qiagen. Bisulfite conversion of DNA was performed as follows [212]: DNA (up to 2μg) was diluted into 50μl distilled H2O and denatured by incubation with 5.5μl of freshly prepared 2M NaOH at 37°C for 10 minutes. Thereafter, 30μl of 10mM hydroquinone (Sigma Co, St. Louis, MO), freshly prepared by adding 55mg of hydroquinone to 50ml of H2O, and 520μl of 3M Sodium Bisulfite (Sigma S-8890), freshly prepared by adding 1.88g of sodium bisulfite per 5ml of H2O, pH adjusted to 5 with NaOH, were added. The mixture was incubated at 55°C for 12 hours, with 5 minutes at 94°C every 3 hours to keep the DNA denatured. The converted DNA was then purified using Promega's DNA Wizard cleanup columns and precipitated overnight with 0.5vol 7.5M NH_4Ac, 3 volumes of ethanol and 1μl glycogen (Boehringer) as a carrier. Bisulfite-treated DNA was resuspended in TE buffer and 2 μl was used for both methylation-specific PCR and bisulfite-sequencing PCR.

Primers for both methylation-specific PCR (MSP) and for bisulfite sequencing were designed using the MethPrimer online program (http://www.ucsf.edu/urogene/ methprimer/ index1.html). The MSP primers were against overlapping segments of the SynCAM promoter, encompassing a number of CpG sites. Each primer set recognized either the methylated (protected from sodium bisulfite, thus remaining as a cytosine) or non-methylated (converted with sodium bisulfite from a cytosine to a thymine) cytosine in a CpG dinucleotide. The presence or absence of bands on an agarose gel after MSP indicate whether or not those sequences were methylated in the genomic DNA. The primers for bisulfite sequencing spanned a 468bp fragment of the predicted CpG island in the predicted mouse SynCAM promoter.

Bisulfite sequencing is a more precise method of determining the methylation status of the SynCAM promoter in astrocyte genomic DNA. Genomic DNA is treated with sodium bisulfite as described above to convert nonmethylated Cs to Ts. Primers flanking the predicted CpG island amplify the region via PCR. The amplicon was cloned into pGEMT and sequenced in the ONPRC's molecular biology core. Methylated CpGs were detectable in the sequence via their protection from the bisulfite treatment; the resulting sequence contains a "CG" instead of a "TG".

Fusion protein isolation:

Fusion protein constructs provided by Dr. Thomas Biederer [210] expressing either the extracellular domain of SynCAM isoform 2 bound to the human Fc protein or a mutant SynCAM isoform 2 lacking the extracellular Ig domains but with the spliced region intact [213] were produced in CHO cells and affinity-purified with Protein G columns. Proteins were then dialyzed overnight against PBS, concentrated by centrifugation in Millipore Amicon columns, and the protein concentration was measured using the NanoOrange fluorometric assay (Pierce). Protein production was confirmed by Western Blotting using an anti-human Fc antibody **(Figure 15)**.

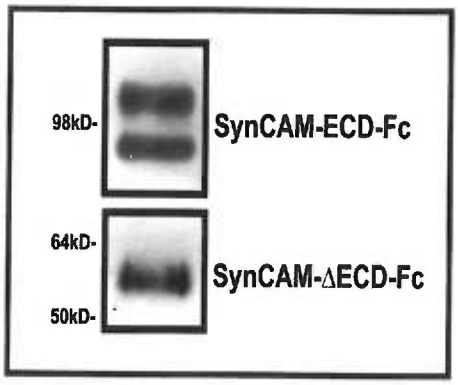


Figure 15. Fc fusion proteins were purified by protein-G affinity and verified by Western blotting with an anti-human Fc antibody. The complete extracellular domain of SynCAM fused to human Fc presents two bands, one glycosylated and one not, around 98kD. The mutated extracellular domain of SynCAM, lacking the Ig domains, is detected as a single band around 58kD.

Adhesion assays:

Adhesion assays were performed similarly to those described by Peles et. al. [177]. Circular glass coverslips were cleaned overnight in concentrated nitric acid and washed in distilled water. Identical small circles were drawn on each coverslip, coverslips were sterilized, and 3 µl of fusion protein (80ng/µl) was added to circles. Coverslips were placed in a 24-well culture plate and allowed to dry for 4 hours at room temperature. They were rinse twice in PBS, and the adherence of the fusion proteins to the coverslips was confirmed by Western blotting. To confirm the adherence of the fusion proteins, after drying for 4 hours to the cleaned coverslips, the circles containing the fusion proteins were rinsed twice in 40µl of PBS, and then 50µl of boiling 1X sample buffer was added and the coverslips were scraped. Ten microliters of 5X sample buffer were added to the two 40µL PBS rinse samples. All three samples were boiled and subjected to SDS-PAGE followed by western blotting with the anti-human Fc antibody. The presence of both wildtype and mutant SynCAM fusion proteins in the scraped sample, but not the two rinses, confirmed the adherence of the fusion proteins to the glass coverslip (data not shown).

Fifty thousand wildtype or DNerbB4 hypothalamic astrocytes were added to each well and allowed to settle overnight in serum-free, astrocyte-defined medium. Cells were then stained with

the fluorescent membrane dye DiO (Molecular Probes, Eugene, OR) for 20 minutes at 37°C, fixed with 4% paraformaldehyde for 20 minutes at room temperature, and stained with the fluorescent nuclear dye Hoescht (Molecular Probes) for 1 minute at room temperature. Coverslips were rinsed in PBS and mounted onto Superfrost microscope slides in aqueous media. Five frames per circle were captured at 20X on a Zeiss Axioplan fluorescent microscope. Cells were counted using the ImageJ software package with the cell-counter plugin (NIH) and averaged as number of cells / field. To control for cell loading when plating DNerbB4 and wildtype astrocytes in the same assay, fields were also captured outside of the ring of fusion protein on each coverslip; these cells were plated on the plain glass. Cells in these fields were also counted and averaged as number of cells/field, and a correction factor was derived the equation : (mean wildtype counts outside ring) / (mean DNerbB4 counts outside ring). This correction factor was then applied to the DNerbB4 inside-ring averages. Statistics were performed with a two-tailed Student's t-test assuming equal variance.

Immunohistochemistry:

Wild-type mice were perfused intracardially with 4% paraformaldehyde in PBS. Sections were cut at 30μm and processed for immunohistochemistry as described ([98]). Image stacks were acquired with a Leica TCS SP confocal system, using a 40x NA 1.25 PI APO objective, and exposed to adaptive blind deconvolution using AutoDeblur (Autoquant, Troy NY). Stacks were then imported in Volocity (Improvision, Lexington, MA), where only the SynCAM signal co-localized with either LHRH (A) or GFAP (B) was used for 3-D rendering.

Yeast-two-hybrid assay:

Two-hybrid assays were performed in the laboratory of Gabriel Corfas at Children's Hospital, Boston using the yeast strain EGY48 harboring LacZ and LEU2 reporters, as described [214]. The erbB4 intracellular domain (amino acid residues 676-1308, [215]) was generated by PCR, subcloned into NotI /EagI sites in pEG202, and used as a bait. The intracellular domain of SynCAM [30] and PSD-95 PDZ domains 1 and 2 (a gift from Dan Pak and Morgan Sheng, MIT) were subcloned into the EcoRI/ XhoI site in pJG4-5. Oligonucleotide-directed PCR mutagenesis was used to create the mutant (K751M) kinase-dead erbB4 receptor. All constructs were verified by DNA sequencing.

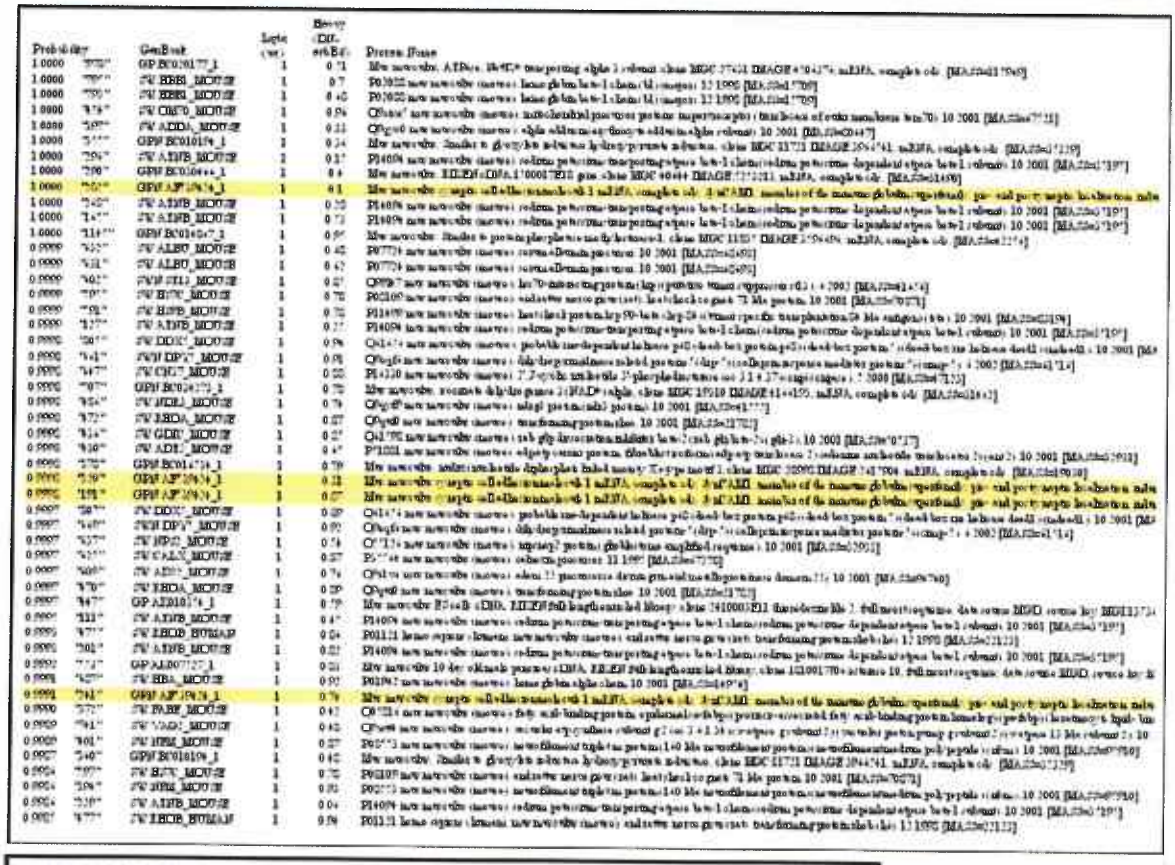
Results:

Differential mass spectrometry:

The results of this study revealed that protein levels of SynCAM, an immunoglobulin-like adhesion molecule recently described to play a critical role in homophilic adhesion and synapse formation and function [26,30,210], were strikingly decreased in the hypothalamus of DNerbB4 mice (**Figure 16 A and B**). **Figures 17 A and B** show the sequencing and the database matches for the

SynCAM peptides. The SynCAM results were the most consistent of the proteins identified, with 6 hits all showing a reduction in the mutant hypothalamus.

A.



B.

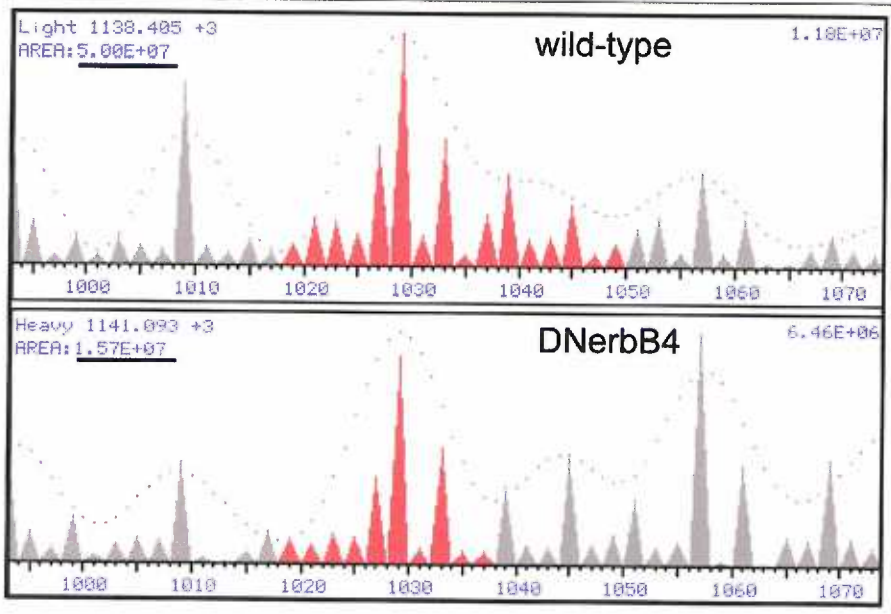


Figure 16. A. Database matches for ICAT-labeled peptides detected by μLC-MS/MS. The probability score refers to the likelihood that the peptide has been correctly identified. Only peptides scoring in the 95%-100% range were considered. The "heavy" and "light" scores refer to the relative abundance of the heavy (DNerbB4) and light (wildtype)-labeled peptides. B. Representative comparison of ICAT-labeled SynCAM peptides. As measured by the area under the curve on the chromatogram by the INTERACT software package (ISB, Seattle), the abundance of the SynCAM peptide in the DN-erbB4 sample ("heavy" label) was found to be 40 % - 60% reduced compared to wild-type ("light" label).

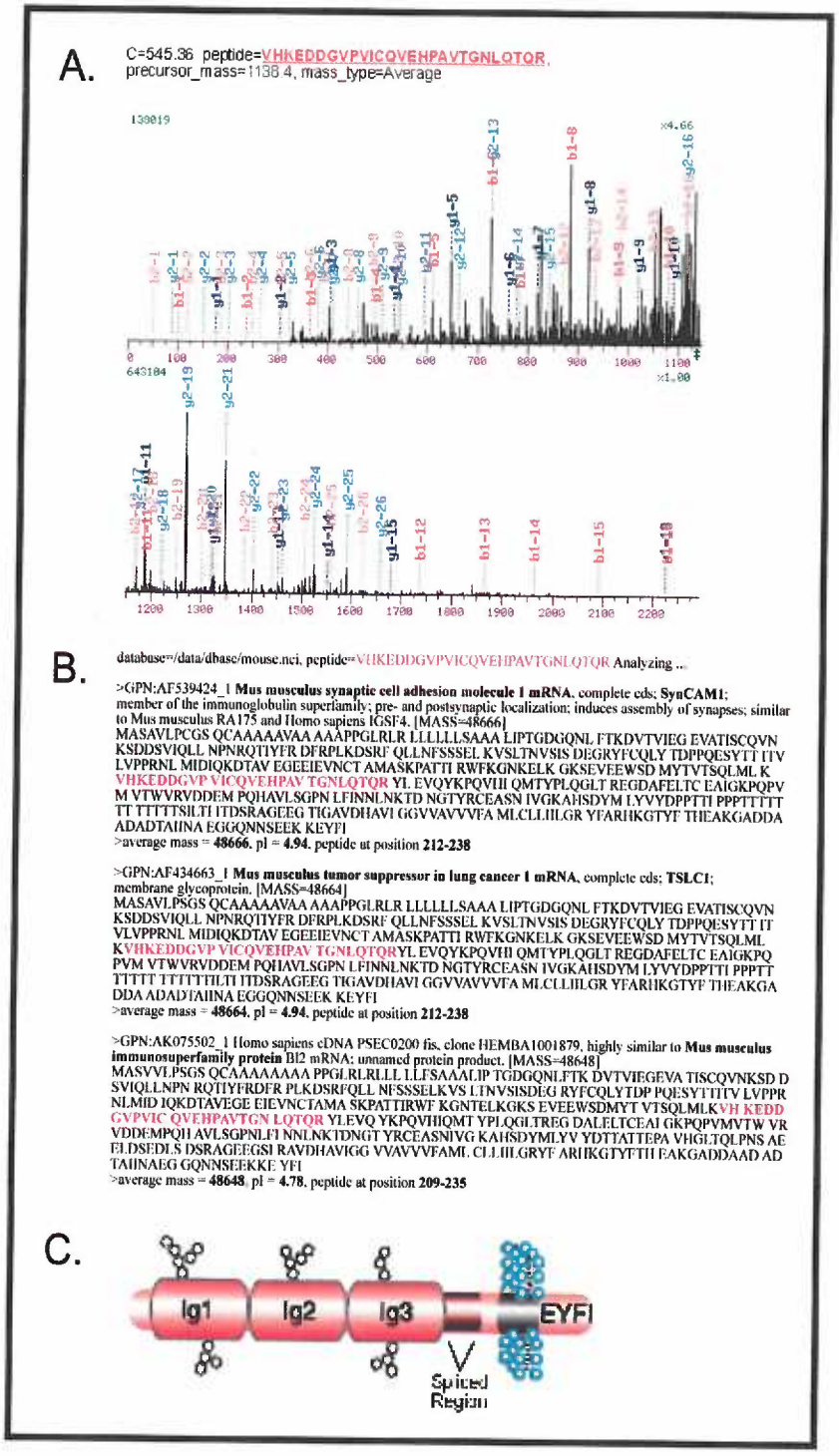


Figure 17. A. C- and N-terminus ions resulting from collision-induced dissociation (CID) of one of the SynCAM peptides selected from the first mass spectrometric analysis shown in Figure 16B. B. Database matches for the SynCAM peptides isolated by μLC-MS/MS (Fig. 16B) and identified by CID (Fig. 17A). C. Model of the SynCAM protein with three extracellular immunoglobulin (Ig) domains containing N-linked glycosylation, a juxtamembrane alternatively spliced region, and an intracellular PDZ-binding domain (EYFI).

Confirmation of MS results:

To verify these high-throughput results by an independent method, we used the SynCAM antibody we had generated to compare, in Western blots, the abundance of SynCAM in the preoptic area of wild-type and DNerbB4 mice. The protein pattern was the same as that observed in Biederer et. al., with a core protein band, higher molecular weight glycosylated bands and a band indicating dimerization [30]. As shown in **Figure 18**, all forms of the SynCAM protein were decreased in the hypothalamus of the mutant animals as compared with wild-type controls.

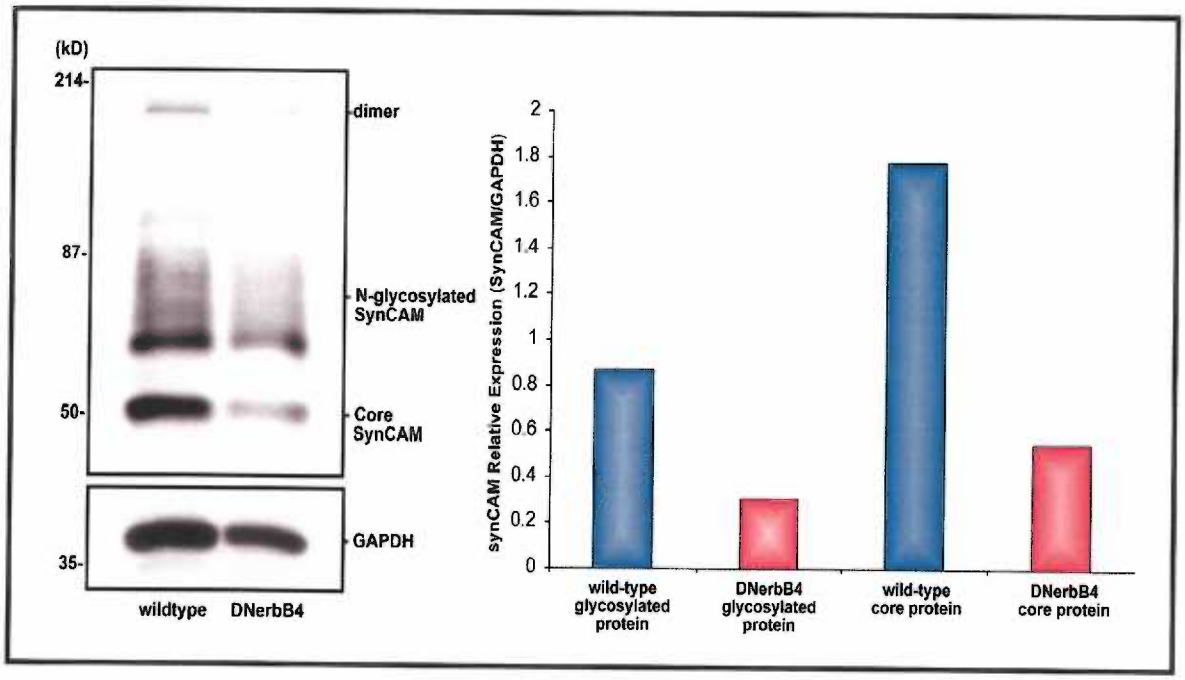


Figure 18. A. Decreased SynCAM protein levels in the preoptic area region of DN-erbB-4 mice as determined by Western blot analysis using antibodies against the C-terminus of SynCAM. Each lane was loaded with 50µg protein. GAPDH was the housekeeping protein used to control for protein loading. B. The amounts of each SynCAM form relative to GAPDH were quantified by densitometry.

SynCAM gene expression in wildtype and DNerbB4 hypothalamus:

Primers were generated against the published mouse SynCAM sequence [210] spanning the alternatively spliced sequence (**Figure 19A**). Sequencing of the resultant PCR fragments demonstrated the presence of isoforms 2, 4 and 6 in the mouse preoptic area (**Figure 19B**). Only 2 and 4 appear to be present in astrocytes and the LHRH neuronal cell line GT1-7 (**Figure 20C**). Isoform 2 is the form which has been described to play a role in synaptic formation and tumoregenicity [210,216,217]. Interestingly, it appears that the decrease in SynCAM mRNA displayed in DNerbB4 hypothalamic astrocytes is limited to isoform 2 (**Figure 19C**). In the preoptic area, all 3 SynCAM isoforms change in relative abundance during the first weeks of life (**Figure 20A**). Concomitantly, the glycosylation pattern of the protein changes over time (**Figure 20B**), perhaps reflecting differential glycosylation of the various isoforms.

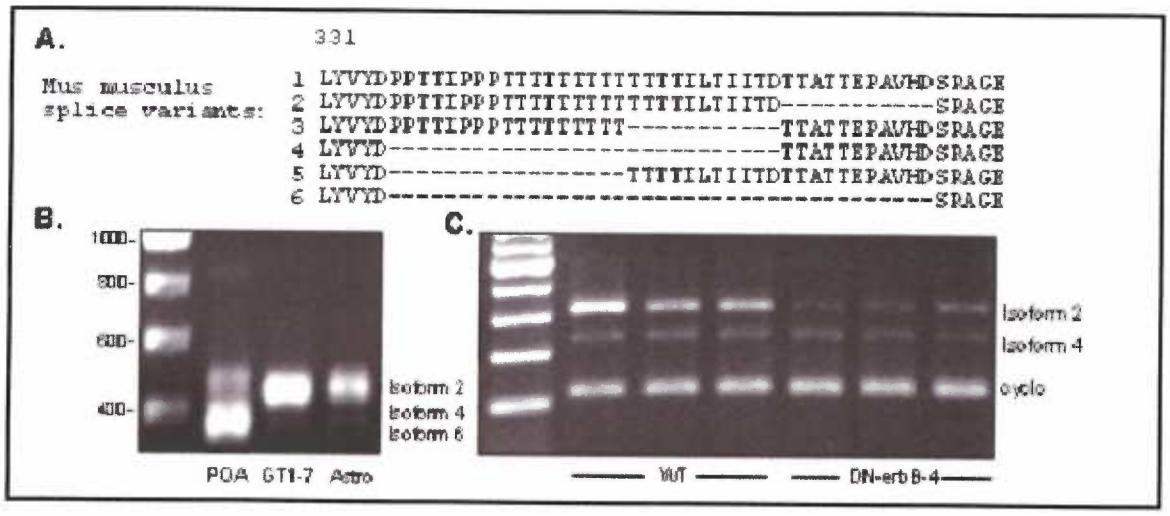


Figure 19. A. The six mouse SynCAM splice variants. B. While in the mouse preoptic area (POA) the main SynCAM mRNA variant expressed is isoform 6, LHRH neurons (GT1-7) and hypothalamic astrocytes (Astro) mostly express isoform 2. C. SynCAM Isoform 2 mRNA abundance is decreased in astrocytes from DN-erbB-4 mice as compared with wild-type (WT) astrocytes Isoform 4 in astrocytes shows no change between genotypes. Cyclophilin was used as an internal control. Cyclo = cyclophilin mRNA.

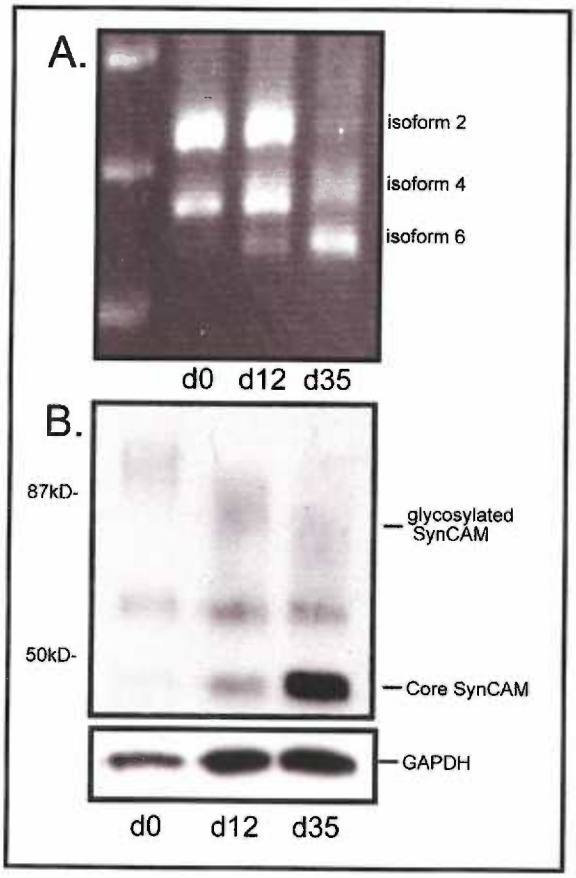


Figure 20. The relative abundance of SynCAM isoforms and the glycosylation pattern of the SynCAM protein change during development in the preoptic area. A. PCR with primers spanning the alternative spliced region show the changing proportions of isoforms 2, 4 and 6 from day 0 to day 35 of age in the mouse POA. B. During the same period of time in the mouse POA, the glycosylation of the SynCAM protein, as indicated by the heavier MW bands on the gel, shifts from more glycosylated at day 0 to less glycosylated at day 35 of age.

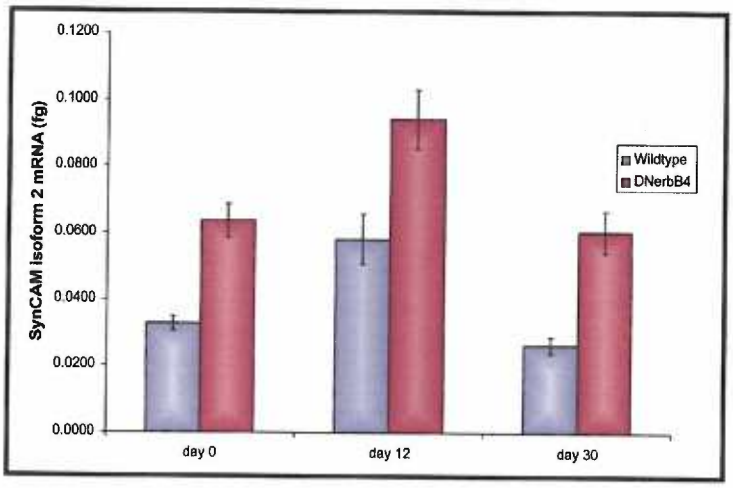


Figure 21. mRNA levels of SynCAM isoform 2 are increased in mRNA from the whole preoptic area (POA) in the mutant animals. mRNA levels reach a maximum at postnatal day 12 in both wildtype and DNerbB4 animals. However, levels of SynCAM isoform 2 in the DNerbB4 POA are significantly increased over wildtype levels at all three ages. n=5 for each age and genotype.

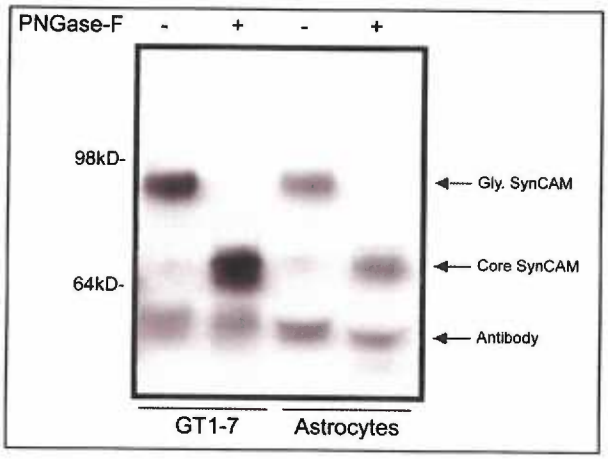


Figure 22. Proteins from LHRH neurons and hypothalamic astrocytes express the same electrophoretic pattern of SynCAM. Proteins were extracted from cultured GT1-7 cells and hypothalamic astrocytes and subjected to immunoprecipitation with an anti-SynCAM antibody. Half the immunoprecitates were treated with PNGase-F to remove N-linked glycosylation.

SynCAM in astrocytes and LHRH neurons:

SynCAM protein is decreased in the hypothalamus of DNerbB4 mice as compared to wild-types (**Figure 18**). Figure **19C** demonstrates the decrease of SynCAM isoform 2 in the astrocytes of DNerbB4 mice. Western blotting with the SynCAM antibody shows the SynCAM protein to be present in hypothalamic astrocytes and GT1-7 cells, in a less glycosylated form than is seen in the whole hypothalamus (**Figure 22**). In accordance with the PCR findings, the SynCAM protein is markedly reduced in hypothalamic astrocytes cultured from DNerbB4 mice (**Figure 23**). To verify

the presence of SynCAM in astrocytes and LHRH neurons in vivo, we performed double immunohistochemistry followed by adaptive blind deconvolution using AutoDeblur to colocalize SynCAM and GFAP on hypothalamic astrocytes, as well as SynCAM and LHRH in hypothalamic LHRH neurons (**Figure 24**). In both cell types, SynCAM is distributed in a punctate pattern reminiscent of synaptic proteins.

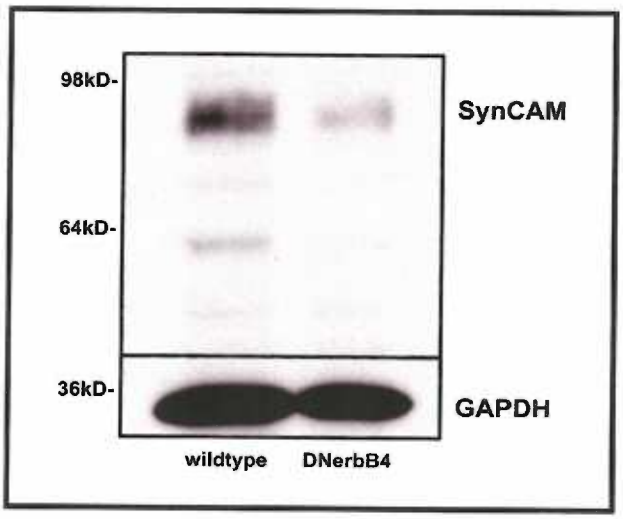


Figure 23. SynCAM protein expression is greatly reduced in hypothalamic astrocytes from DNerbB4 mice. Proteins were extracted from primary cultures of hypothalamic astrocytes from either wild-type or DNerbB4 mice and run on SDS-PAGE. Proteins were blotted onto a PVDF membrane and probed with anti-SynCAM antibodies. Membranes were then probed with an anti-GAPDH antibody as a loading control.

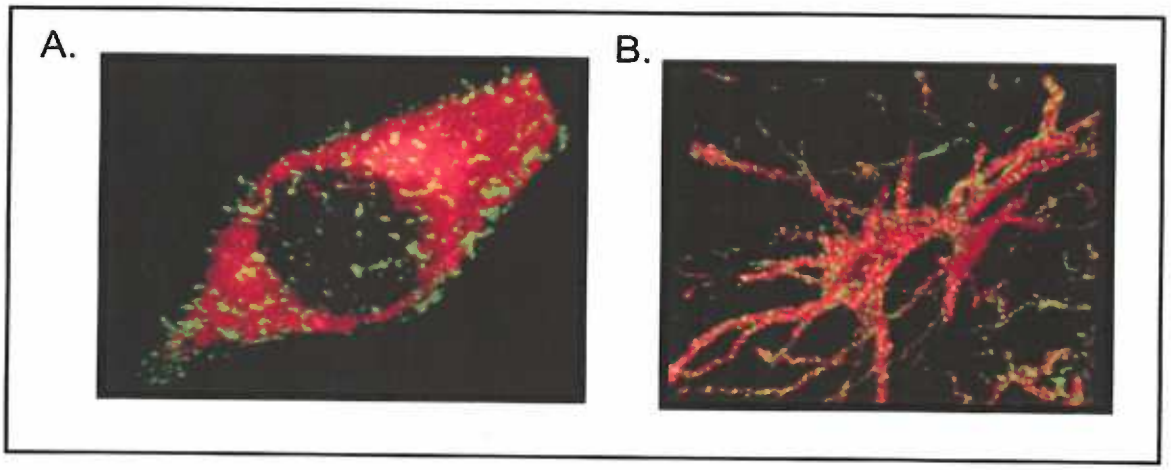


Figure 24. The SynCAM protein is present in LHRH neurons and hypothalamic astrocytes in vivo. A. SynCAM (green) and LHRH (red) immunoreactivity are present in a LHRH neuron. B. SynCAM (green) and GFAP (red) are present in a hypothalamic astrocyte.

The form of SynCAM described in the literature as critical for synapse formation is isoform 2 [30]. We have shown that this isoform is selectively reduced in cultured astrocytes from the DNerbB4 mouse hypothalamus. What is the significance of a change specifically in isoform 2? Glycosylation has been shown to be a major factor affecting the adhesive properties of immunoglobulin adhesion molecules [218]. The heavily N-glycosylated portion of SynCAM is outside of the spliced region of the molecule (**Figure 17C**). However, we show that as the SynCAM isoforms change with development, the glycosylation pattern also changes (**Figure 20**). By digesting with glycosidases, we can see that SynCAM is likely to also contain O-linked glycosylation (**Figure 25).**

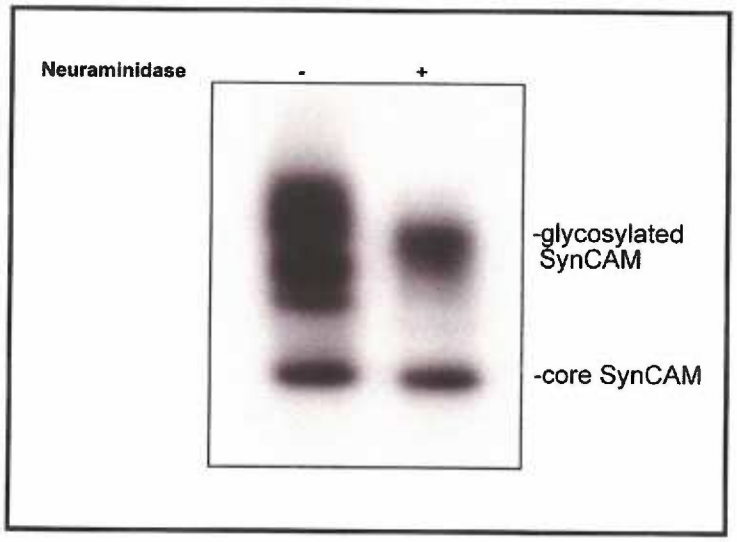


Figure 25. SynCAM contains some O-linked glycosylation groups. Neuraminidase removes sialic acid residues from O-linked glycans. The reduction in the amount of glycosylated SynCAM in protein from the mouse POA indicates that these groups are present on the SynCAM protein.

Using the Net-O-Glyc glycosylation prediction server (http://www.cbs.dtu.dk/services/-NetOGlyc), we can see that the alternatively spliced site in SynCAM is likely to contain this O-linked glycosylation (**Figure 26**). Moreover, the glycosylation likely to be carried by isoform 2 is significantly greater than the other astrocyte isoform, isoform 4. This could also explain why, as the abundance of isoform 2 decreases, the molecular weight of the glycosylated bands on the western blot also decreases (**Figure 20**). Digestion of mouse hypothalamic protein with neuraminidase, an O-glycosidase, demonstrates that SynCAM does contain O-linked glycosylation (**Figure 25**). O-linked glycosylation is very complex, and associated groups such as polysialic acid have been shown to be extremely important in developmental plasticity [159,219,220]. SynCAM appears to have some amount of polysialation (**Figure 27**), but digestion with endo-N, a rare endonuclease specific for polysialic acid [221], is necessary to confirm this finding.

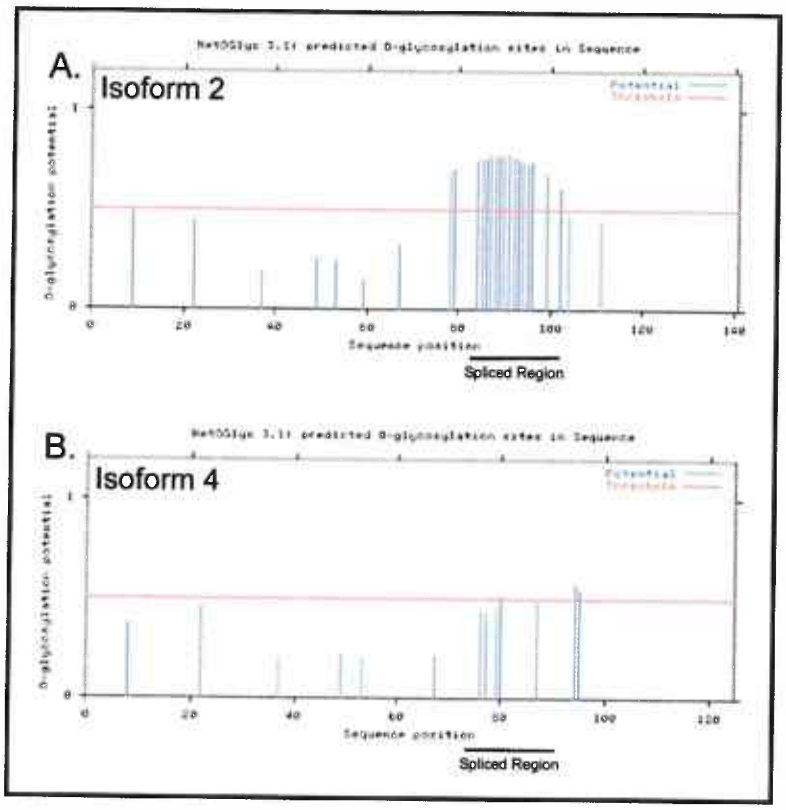


Figure 26. Of the two SynCAM isoforms expressed in hypothalamic astrocytes, only isoform 2 is predicted to contain O-linked glycosylation. The regions of isoform 2 and isoform 4 containing the alternative spliced sequence were amplified by PCR, cloned into pGEMT, and sequenced. The resulting sequences were entered into the NetOGlyc O-linked glycosylation prediction server (http://www.cbs.dtu.dk/services/NetOGlyc/). The analyses shown that the alternatively spliced sequence of isoform 2, but not 4, is predicted to contain O-linked glycosylation.

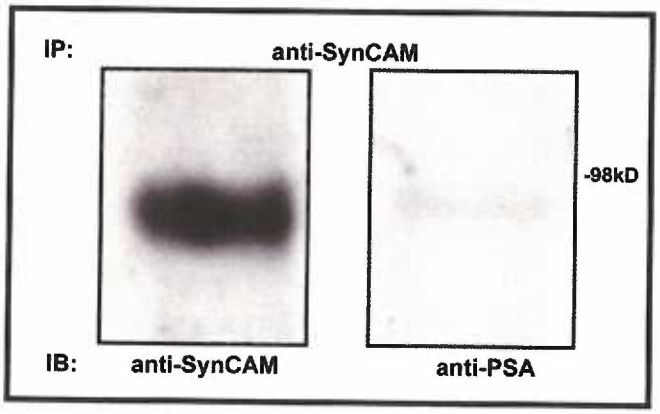


Figure 27. SynCAM contains polysialic acid. Proteins from wild-type cultured hypothalamic astrocytes were subjected to immunoprecipitation with the anti-SynCAM antibody. Proteins were then transferred to a PVDF membrane and blotted with the anti-PSA mouse monoclonal antibody 5A5 [222]. The membrane was then stripped and probed with the anti-SynCAM antibody.

Effects of erbB4 receptor function upon SynCAM gene expression and promoter function:

Figures **19C** and **23** show the abundance of both SynCAM mRNA and protein to be reduced in astrocytes of mice carrying an astrocyte-specific dominant-negative mutation of the erbB4 receptor. When astrocytic erbB4 receptors are stimulated in culture with 100ng/ml neuregulin β1 (NRGβ1) for 8 hours, a significant increase in SynCAM isoform 2 mRNA is detectable by real-time PCR (**Fig 28**).

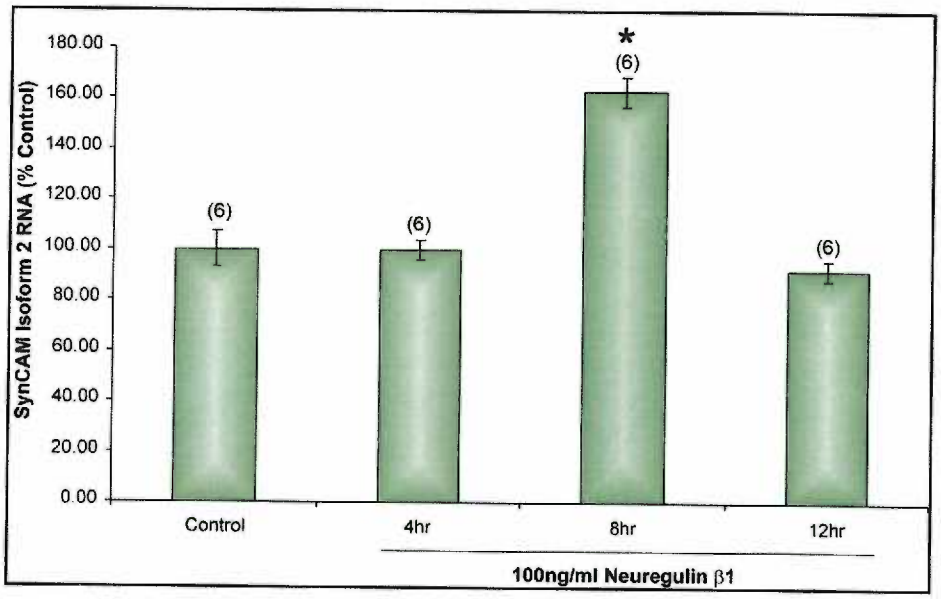


Figure 28. Stimulation of astrocytic erbB4 receptors results in an increase of SynCAM mRNA. Real-Time PCR for SynCAM isoform 2 demonstrates an increase in SynCAM mRNA in cultured wildtype astrocytes following 8 hours of NRGβ1 stimulation (100ng/ml). SynCAM isoform 2 mRNA amounts are expressed as a percentage of the control (unstimulated). * = p< .05.

The human SynCAM promoter was cloned into the pGL3 luciferase vector, and showed a dose-dependent increase in luciferase expression in culture (**Figure 29**.) Co-transfection of HiB5 cells with this promoter construct and a pcDNA3-erbB4 vector and incubation in regular media (containing serum) leads to an increase in SynCAM promoter activity (**Figure 30**). This effect is abolished when the dominant-negative erbB4 receptor (pcDNA3-DNerbB4) is co-transfected in place of the wild-type (**Figure 30**). The increase in SynCAM promoter activity can be duplicated with stimulation of the transfected cells in serum-free medium with neuregulin β1 to specifically activate the erbB4 receptor (**Figure 31**). Stimulation with TGFα has no significant effect (**Figure 32**), indicating that the SynCAM promoter is not activated by erbB1 signaling.

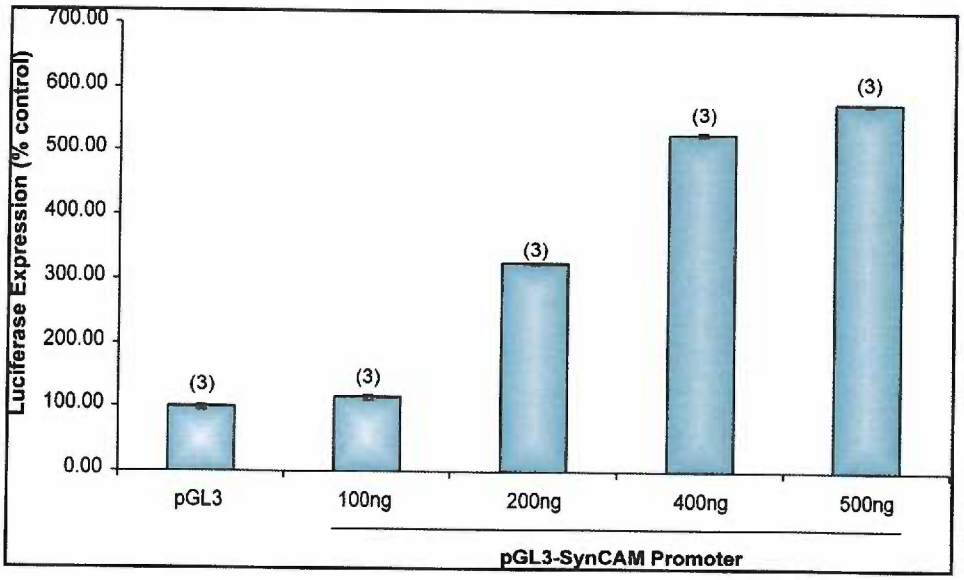


Figure 29. Dose-related increase in SynCAM promoter activity 48h after transfection of different amounts of pGL-3-SynCAM promoter into HEK-293 cells. Cells were transfected with 100-500ng of the pGL3-SynCAM promoter construct and incubated for 48 hours in regular (10% FCS) medium before snap-freezing and processing for luciferase expression by luminometry. The amount of luciferase expression increases each increasing amount of the pGL3-SynCAM vector, demonstrating that the cloned SynCAM sequence has promoter activity.

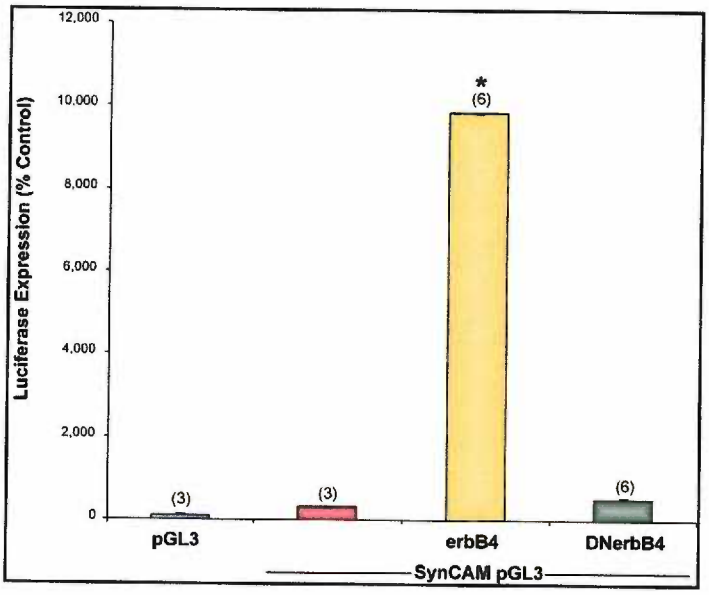


Figure 30. The dominant negative erbB4 mutant (DNerbB4) does not stimulate the SynCAM promoter. The effect of cotransfection with erbB4 on SynCAM promoter activity is not evident when the promoter was co-transfected with the mutant construct lacking the intracellular domain in medium containing 10% serum. * = $p < .01$.

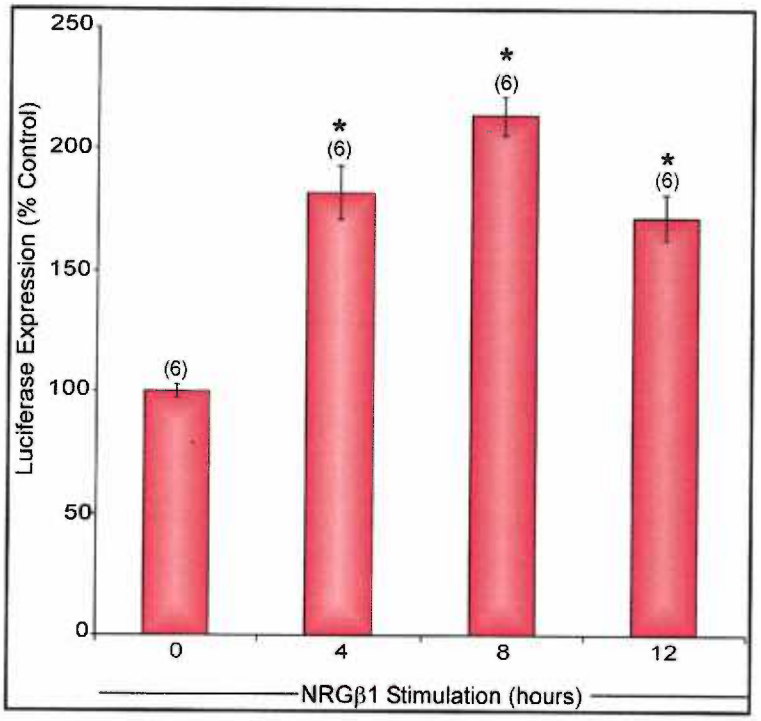


Figure 31. Stimulation of erbB4 receptors increases SynCAM promoter activity. In HiB5 cells co-transfected with the SynCAM promoter construct and the erbB4 receptor, stimulation with NRGβ1 in serum-free media leads to an increase in luciferase expression, with a peak effect at 8 hours of stimulation. * = p< 01 compared to 0 hours.

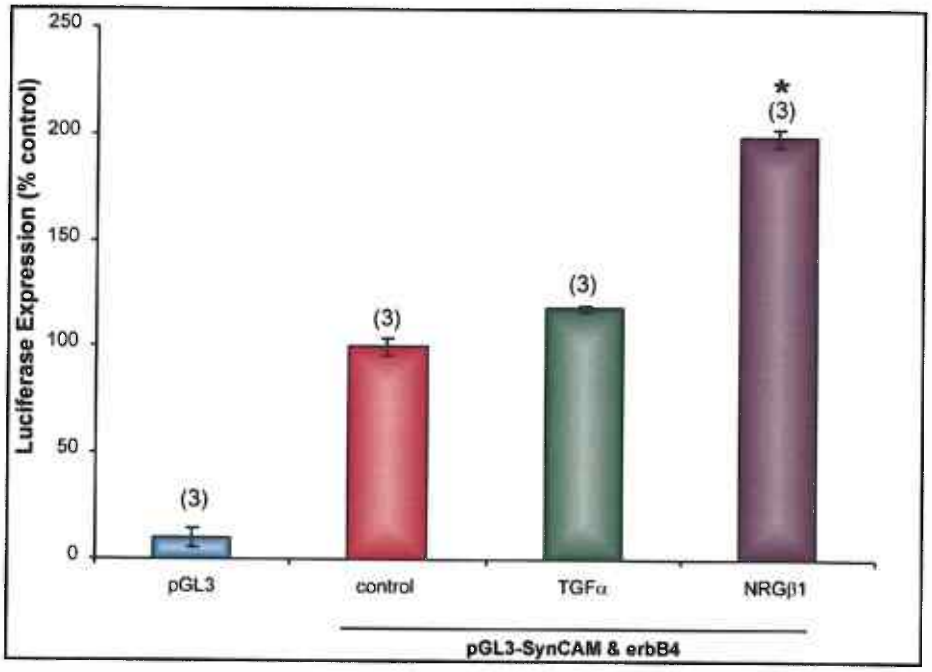


Figure 32. Stimulation of endogenous erbB1 receptors in erbB4-transfected BAS8.1 cells does not increase SynCAM promoter activity. BAS8.1 cells contain erbB1 receptors, but stimulation of these receptors for 12 hours with TGFα does not significantly increase luciferase expression. Stimulation of transfected erbB4 receptors with NRGβ1, however, does activate the promoter, as previously shown. * = p < .01 compared to control.

What are the possible mechanisms by which erbB4 might influence SynCAM promoter activity? The signaling capabilities of the erbB4 receptor are complex and diverse (for review see [223]). The SynCAM promoter has been shown to be hypermethylated, thus inactivated, in several tumor types [216,224,225]. We show that methylation of the SynCAM promoter in the pGL3 construct effectively silences it (**Figure 33**). Treatment of DNerbB4 hypothalamic astrocytes with the demethylating reagent 5-aza-cytidine [211] increased SynCAM gene expression in a dose-dependent manner (**Figure 34**). There was no effect of 5-aza treatment upon wildtype astrocytes (data not shown).

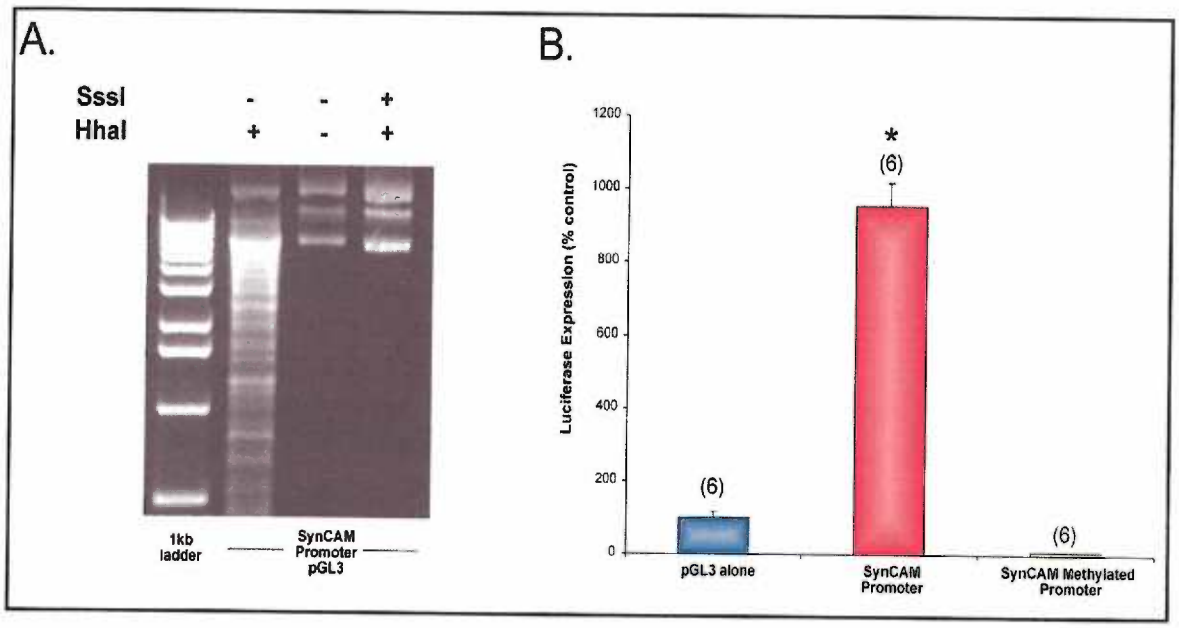


Figure 33. Methylation of the SynCAM promoter abolishes its activity. A. The human SynCAM promoter - pGL-3 construct was subjected to methylation by the SssI methylase for 16 hours at 37°C. The methylated and unmethylated promoters were then transfected into HEK293 cells and luciferase activity was measured as an indicator of promoter activity. B. The methylation of the SynCAM promoter greatly reduced its luciferase activity.

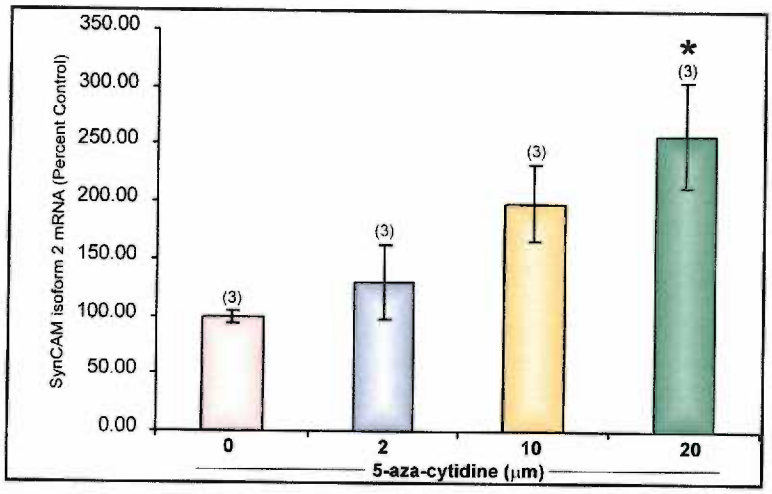


Figure 34. Treatment of DNerbB4 astrocytes with 5-aza-cytidine increases SynCAM mRNA expression as measured by real-time PCR. All treatments were for 7 days in regular medium, with daily changes of medium and drug. * = $p < .05$ compared to control.

These experiments do not tell us whether this increase in SynCAM mRNA is due to demethylation of the SynCAM promoter itself, nor if erbB4 signaling is able to decrease the methylation of the promoter. To study this, we have used methylation-specific PCR and bisulfite sequencing to examine the methylation status of the SynCAM promoter under different influences. Methylation-specific PCR showed no differences in the methylation status of the wild-type and the DNerbB4 SynCAM promoter in astrocytes (data not shown). Bisulfite sequencing revealed a small number of methylated CpGs in the SynCAM promoter, but there was no difference in the amount of methylation between the two genotypes **(Figure 35).** Therefore, the presence of a functional erbB4 receptor does not appear to have an effect on the methylation status of the SynCAM promoter in hypothalamic astrocytes.

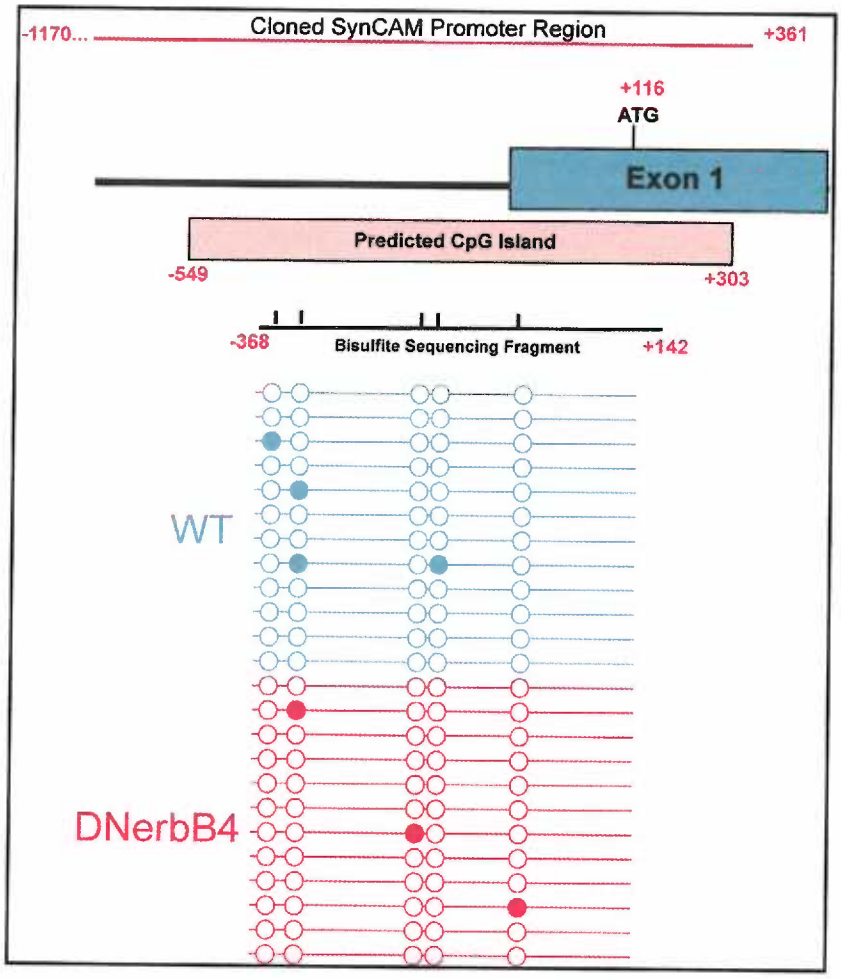


Figure 35. Bisulfite sequencing of the SynCAM promoter in DNA from wildtype or DNerbB4 astrocytes. The SynCAM promoter contains methylated CpG sites in both genotypes (filled-in circles), but these sites are few compared with potential CpG methylation sites and the number of sites do not differ between the genotypes.

SynCAM and erbB4 protein interactions:

SynCAM and erbB4 protein co-immunoprecipitate (**Figure 36**) in astrocytes. In co-transfected BAS8.1 cells, stimulation of erbB4 with NRGβ1 increases the strength of this association (**Figure 37**). This finding was then confirmed in hypothalamic astrocytes by the stimulation of erbB4 by β-cellulin (**Figure 38**).

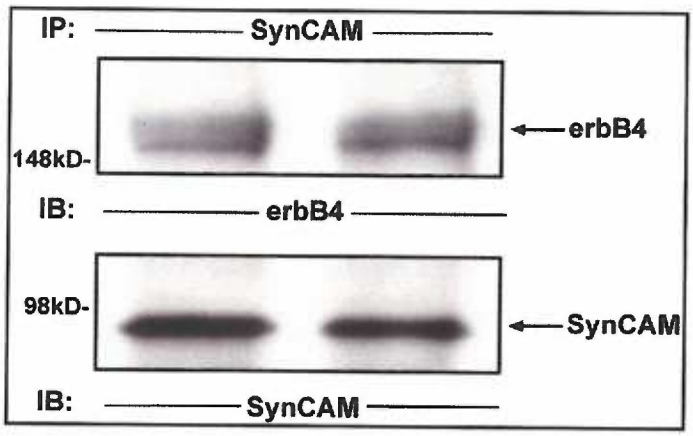


Figure 36. erbB4 and SynCAM are physically associated in hypothalamic astrocytes. Proteins extracted from cultured astrocytes from wildtype hypothalami were subjected to immunoprecipitation with an anti-SynCAM antibody and immunoblotted with anti-erbB4. The membrane was then stripped and probed with anti-SynCAM to verify the immunoprecipitation. Samples were run in duplicate.

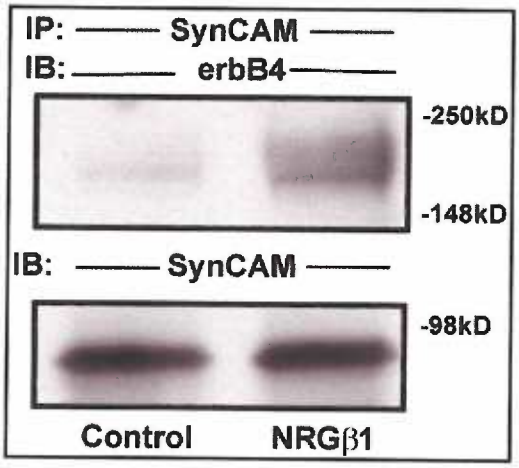


Figure 37. Neuregulin 1β stimulation increases the physical association of SynCAM and erbB4 proteins in co-transfected BAS8.1 cells. BAS8.1 cells transfected with SynCAM and erbB4 were stimulated with 100ng/ml NRGβ1 for 30 minutes. Proteins were extracted and subjected to immunoprecipitation with the anti-SynCAM antibody followed by immunoblotting with the anti-erbB4 antibody. The membrane was stripped and probed for SynCAM to verify equal loading of protein.

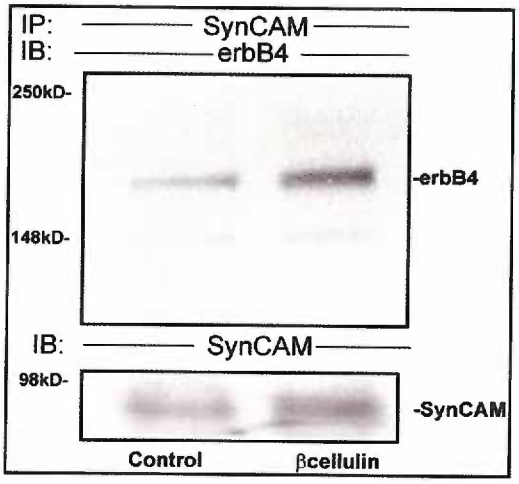


Figure 38. Stimulation of erbB4 receptors with betacellulin increases their association with SynCAM in hypothalamic astrocytes. Cultured astrocytes from wildtype hypothalamus were stimulated for 30 minutes with 100ng/ml betacellulin in serum-free media. Extracted protein was subjected to immunoprecipitation with an anti-SynCAM antibody and immunoblotted for erbB4. The blot was then stripped and probed with the anti-SynCAM antibody as a loading control.

This experiment does not tell us if the interaction is direct; since both proteins have PDZ intracellular domains, there are a number of intermediate proteins that they could both bind. To determine the nature of the interaction, we conducted a targeted yeast two-hybrid assay with constructs expressing the intracellular domains of erbB4 and SynCAM. We found that these proteins do interact directly (**Figure 39A**). Moreover, there is a functional component of this interaction, as SynCAM does not bind an erbB4 mutant with a disrupted kinase domain (**Figure 39B**). Not surprisingly, the association of SynCAM with erbB2, the preferred binding partner of erbB4, is also reduced in DNerbB4 astrocytes (**Figure 40**).

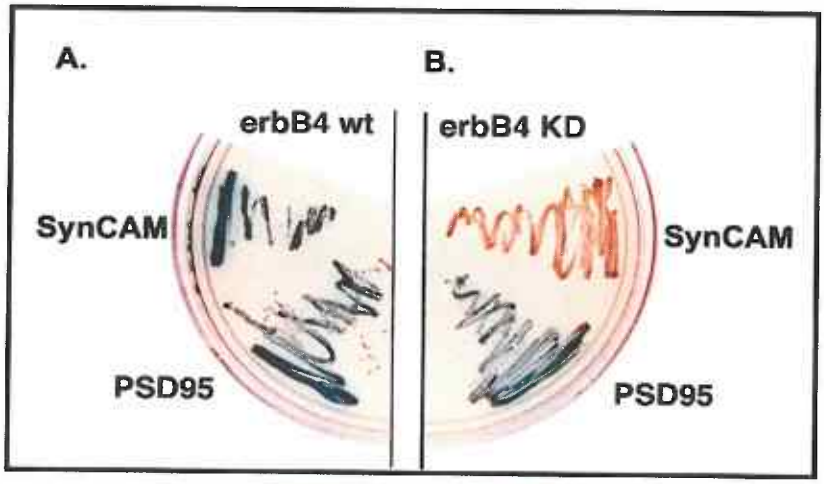


Figure 39. SynCAM interacts directly with the erbB4 receptor. A targeted yeast two-hybrid assay using the SynCAM intracellular domain as the bait and the erbB4 intracellular domain as the prey showed that these two proteins interact directly. The wild type-erbB4, but not the kinase dead-erbB4, interacts with SynCAM in yeast (reflected by activation of LacZ, blue color). PSD95, which is known to interact with erbB4 through its C-terminal PDZ binding motif, was used as a positive control for both baits. This interaction is thus dependent upon a functional erbB receptor.

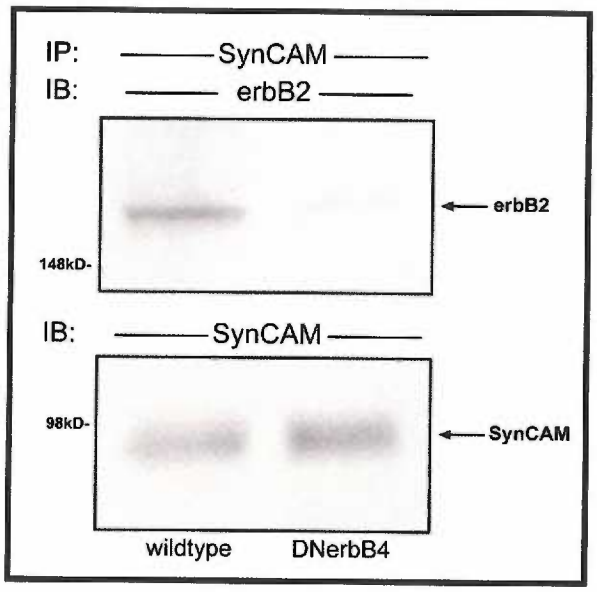


Figure 40. The physical association between SynCAM and erbB2 is reduced in hypothalamic astrocytes from DNerbB4 animals. Proteins extracted from cultured astrocytes from wildtype and DNerbB4 hypothalami were immunoprecipitated with the anti-SynCAM antibody and immunoblotted for erbB2. The results show that less erbB2 protein is associated with the SynCAM protein pulled down from the mutant astrocytes compared to wildtypes.

Physiological activity of SynCAM in hypothalamic cells:

To examine the possible adhesive role that SynCAM plays in the interactions of astrocytes and LHRH neurons, we have incorporated an adhesion assay [177] utilizing SynCAM Fc fusion proteins [210]. The SynCAM and control Fc fusion proteins were purified as described in the Methods. The wildtype fusion protein was digested with PNGase-F to confirm its N-linked glycosylation (**Figure 41**). When wildtype hypothalamic astrocytes were plated onto either the SynCAM fusion protein with an intact extracellular domain, or a mutant protein, they adhered significantly more to the intact SynCAM (**Figure 42**). The DNerbB4 astrocytes displayed a much-reduced adhesion to the SynCAM extracellular domain in these assays (**Figure 43**). Interestingly, qualitative observation of astrocytes plated at a low density upon the intact SynCAM fusion protein revealed a difference in morphology between the wildtype and DNerbB4 astrocytes (**Figure 44**), perhaps indicating an ability of SynCAM to promote plastic rearrangements of the actin cytoskeletal via interaction with the intact erbB4 receptor.

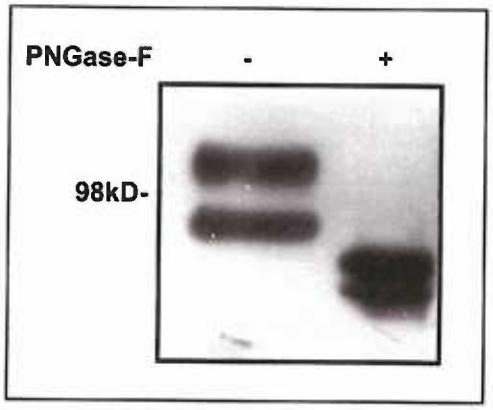


Figure 41. The wild-type SynCAM ECD Fc fusion protein is glycosylated normally. PNGase-F was used to remove N-linked glycans from purified wild-type SynCAM ECD Fc fusion proteins.

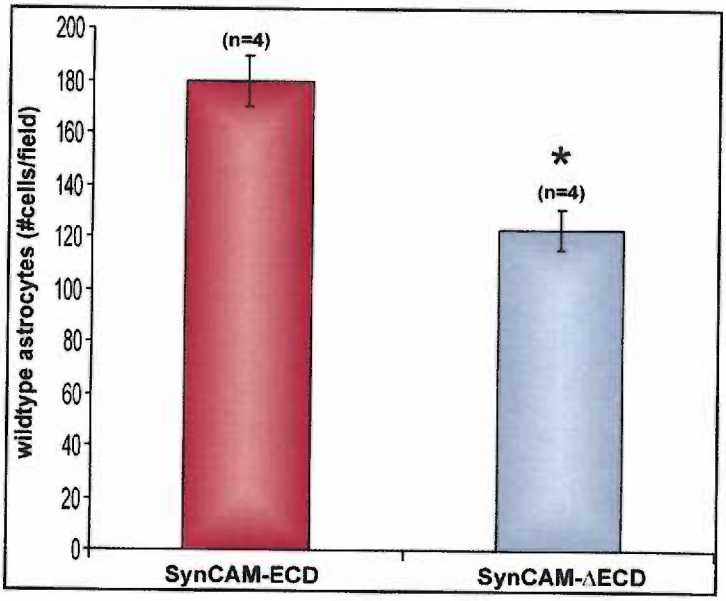


Figure 42. Wildtype hypothalamic astrocytes bind preferentially to the intact extracellular domain of SynCAM. Adhesion assays were performed by plating astrocytes on coverslips coated with either the wild-type extracellular domain (ECD) of SynCAM or a mutated control (ΔECD) in which the immunoglobulin domains were deleted. After 18 hours in culture, more astrocytes were adherent to the coverslips coated with the wildtype SynCAM fusion protein. * = $p < .05$.

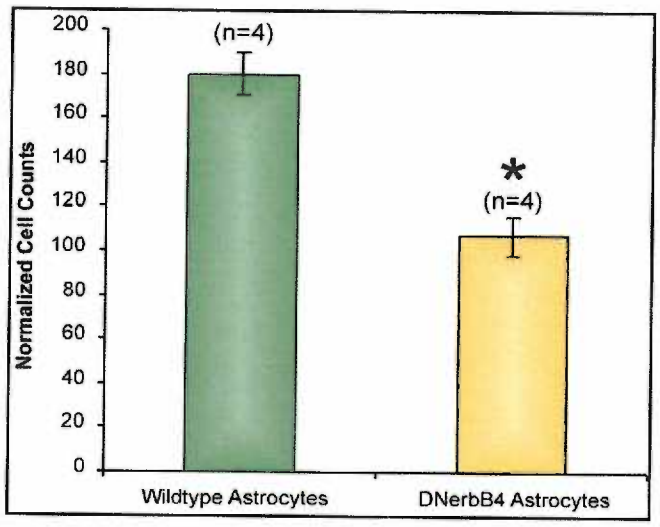


Figure 43. The adherence of astrocytes to SynCAM is impaired in the DNerbB4 mutants. When plated onto coverslips coated with the wildtype extracellular domain of SynCAM, significantly more wildtype than DNerbB4 astrocytes adhered after 18 hours in culture. Cell counts were normalized to the number of cells per field outside of the ring of protein for both genotypes to control for cell loading. * = $p < .05$.

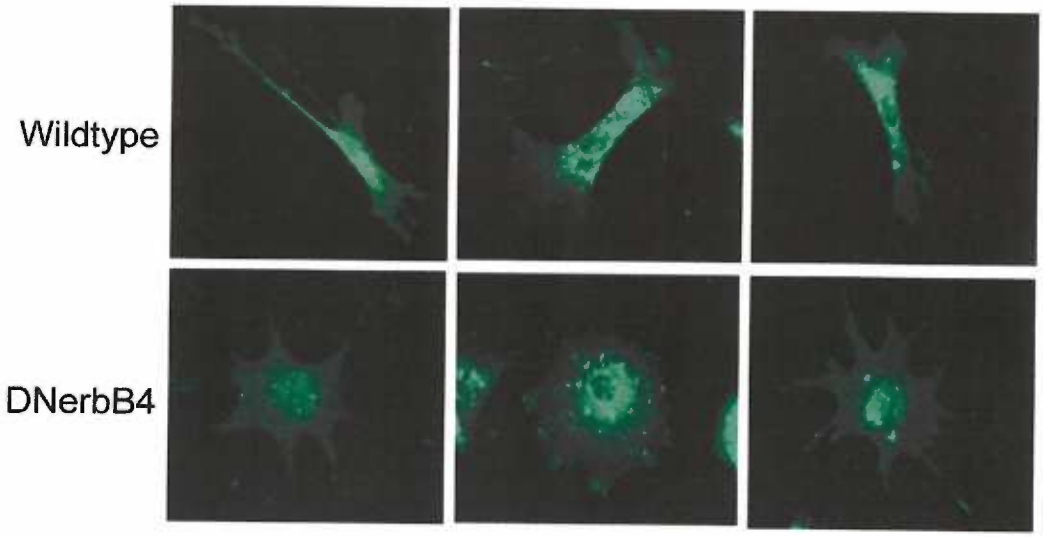


Figure 44. Wildtype astrocytes display a more differentiated phenotype than the DNerbB4 astrocytes when in contact with SynCAM fusion proteins. A greater proportion of wildtype astrocytes displayed a high degree of cytoskeletal organization when plated upon the SynCAM fusion proteins than the DNerbB4 astrocytes. Astrocytes from the mutant animals tended to be more unpolarized and spread-out.

Discussion:

The dynamic interaction between the adhesion molecule SynCAM (TSLC1, Igsf4, RA175, NECL2) and the growth factor receptor erbB4 has numerous implications in areas of research from synaptic plasticity to cancer. Work in other model systems has previously suggested interactions between adhesion molecules and growth factor receptors [226-228]. However, little of this research has taken place in the brain; these studies mostly are concerned with signaling in cancer cells of various types. This is the first study demonstrating a functional interaction between erbB4 and a cell adhesion protein. In addition, this is the first study to examine the role of SynCAM, a tumor-suppressor gene and synaptic protein, in astroglial cells. In the pubertal animal, we have shown that this association is disrupted in mutant mice carrying an astrocyte-specific defect in the erbB4 receptor. The astrocyte-specific dominant-negative mutation of erbB4 in the DNerbB4 mice leads to a decrease in SynCAM mRNA and protein expression in the hypothalamic astrocytes of these cells. The mRNA decrease appears to be accounted for almost entirely by SynCAM isoform 2, a form that appears to contain O-linked glycosylation that is not present in the other isoform expressed by astrocytes, isoform 4. Since glycosylation is heavily implicated in the adhesive properties of proteins, the reduction in this isoform could mean a loss of SynCAM adhesiveness. A reduction in the physical adherence between LHRH neurons and astrocytes may be a factor in the plasticity necessary for pubertal activation. It has been shown already that the area of the LHRH neurons covered by astrocytes is reduced as rats enter estrus [45], a period of high estrogen levels which appear to directly modify the astrocytes morphology [229]. Little is known about astrocytic plasticity during the onset of puberty, but the current study indicates that SynCAM's presence and association with erbB4 makes it a likely factor in such plasticity.

The mRNA for the three isoforms of SynCAM that are expressed in the mouse preoptic area as whole, isoforms 2, 4 and 6, appear to change in relative abundance during the first few weeks of life. At the same time, the glycosylation pattern of the SynCAM protein in this brain area changes from higher molecular weight modifications to lower molecular weight. The further discovery that SynCAM may contain polysialic acid, a modification found to increase plasticity [159], lends evidence to the hypothesis that SynCAM engages in a dynamic, modifiable adhesion / signaling system that is intrinsic to the development of the central nervous system.

The expression of SynCAM also changes during development [30]. Other work in our lab shows that, in the primate hypothalamus, overall levels of SynCAM increase at the time of puberty (ref). Our results in the mouse show that the mRNA of isoform 2 of SynCAM reaches a peak around day 12 postnatally in the preoptic area, a time of major synaptogenesis and neural organization in the hypothalamus. Surprisingly, although we see the protein levels of SynCAM overall reduced at day 30 in the DNerbB4 preoptic area, the mRNA for isoform 2 is elevated in this area in the mutant animals at each age examined. We know that isoform 2 is specifically reduced

in DNerbB4 astrocytes, it then follows that cells surrounding those astrocytes containing isoform 2 could be upregulating the expression of this specific form in the mutant due to lack of feedback from binding to astrocytic isoform 2.

Activation of ErbB4 leads to signaling though various pathways, such as those effecting cell proliferation, survival, chemotaxis, and differentiation [230]. ErbB4 may also associate with other cell surface molecules that could influence its trafficking or signaling properties - including MUC1 [231] and CD44 [232]. Thus, there are a number of mechanisms by which erbB4 signaling could affect SynCAM gene expression. ErbB4 can signal through the PI3K (CYT1 isoform only, [86,233]) pathway to induce cell survival and migration, through the Shc and Grb2 signaling pathway [91] to influence cell proliferation and through MAP kinase to stimulate cell differentiation [234]. One isoform of erbB4, erbB4-JMa, can be cleaved extracellularly in a PKC-dependent manner by TACE to produce a membrane-bound intracellular portion with unique properties [86,88]. This 80kD erbB4 fragment can be degraded by proteosome activity following polyubiquitination [235]. Alternatively, the 80kD erbB4 fragment can be cleaved close to the cell membrane by γ-secretase [236,237] and translocated to the nucleus [236] where it may interact with proteins like STAT5A [94,95] and YAP65 [90] to influence gene transcription. Further studies will be needed to determine what pathway is responsible for the effect of erbB4 activation upon SynCAM gene expression.

Studies in several tumor types where SynCAM is suppressed demonstrate that the SynCAM promoter can be hypermethylated [238-240]. Our results show that culturing DNerbB4 astrocytes in the presence of 5-aza-cytidine, a demethylating reagent [241,242], increases SynCAM isoform 2 expression. Furthermore, in vitro methylation of the SynCAM promoter greatly diminishes its activity in the luciferase assay. However, upon closer examination of wildtype and DNerbB4 genomic DNA via methylation-specific PCR and bisulfite sequencing, it does not appear that changes in erbB4 activity impact greatly the methylation status of the SynCAM promoter. In fact, the SynCAM promoter is surprisingly hypomethylated in hypothalamic tissues and cultures astrocytes, at least compared to published results from studies examining different tumor types [216,225]. This lends support to the idea that SynCAM is fairly active in normal tissues, and its silencing by methylation is a hallmark of neoplastic conditions. Thus, although the SynCAM promoter can be regulated via methylation, the stimulatory effects of erbB4 activity must be directly through another pathway.

Why then does 5-aza-C treatment of DNerbB4 astrocytes lead to a modest increase in SynCAM isoform 2 mRNA? One possibility is that the seven-day treatment at 10-20μM 5-aza-cytidine lead to a toxicity response, perhaps upregulating SynCAM mRNA as part of an apoptotic pathway. The 7-day duration of 5-aza treatment used in the current experiment was derived from work from other labs using 5-aza-C in astrocyte cultures [211]. Nonquantitative microscopic examination of living cultures did not reveal any obvious differences in cell morphology that would indicate a toxic effect of the treatment. In addition, we see no effect of 5-aza-C treatment on

wildtype astrocytes. This could be studied in further detail with apoptosis assay kits. A more likely possibility is that the treatment led to the demethylation and activation of genes whose expression increased either the promoter activity of SynCAM or the stability of its mRNA transcripts, and that this did not affect the already active promoter in the wildtype astrocytes. One known binding partner of SynCAM, DAL-1/Protein 4.1B [243] is, like SynCAM, hypermethylated on its promoter in non small-cell lung cancer and, like SynCAM, this methylation is associated with poor prognosis [244]. Further investigation would be worthwhile to determine whether the presence of DAL-1/4.1B may activate the SynCAM promoter, and if the promoter of DAL-1/4.1B is, unlike that of SynCAM, methylated in hypothalamic astrocytes. In addition, simple RT-PCR and Western blotting could determine whether the expression of DAL-1/4.1B is altered in the DNerbB4 animals. The current data should be viewed with caution, nevertheless, because of the rather modest effect of the 5-aza-C on SynCAM mRNA and the relatively long time period of exposure.

Yeast-two hybrid studies have confirmed that the SynCAM protein and the erbB4 receptor interact directly. This interaction depends on an intact kinase domain in the erbB4 receptor, indicating that this binding is related to the erbB4 protein's kinase activity. We show, by co-immunoprecipitation, that stimulation of the erbB4 receptor in hypothalamic astrocytes leads to an increased association between the receptor and SynCAM. Since erbB4 stimulation leads to auto-phosphorylation, we can assume that this kinase activity of erbB4 is critical to its association with SynCAM. In addition, we have shown that the activated erbB4 receptor associated more with SynCAM than at the basal state. Which intracellular domain of SynCAM is responsible for this activity remains to be determined. In addition, SynCAM is associated with erbB2, the preferred binding partner of erbB4, and this association, measured by immunoprecipitation, is decreased in the DNerbB4 animal. This would be expected, as only the activated, thus SynCAM-binding, erbB4 dimerizes with erbB2. SynCAM contains only one intracellular potential phosphotyrosine, as predicted by the Ethos 2.0 Server (http://www.cbs.dtu.dk/services/ Ethos/). SynCAM also does not appear to contain traditional SH2 domains for phosphotyrosine binding, as are common with erbB4-binding proteins [245]. It remains to be examined exactly what the effects of this binding are upon SynCAM or erbB4 signaling.

The role of erbB1 (EGFR) in SynCAM signaling also remains to be thoroughly investigated. Previous studies have shown no changes in erbB1 expression or function in the DNerbB4 animals [198]. We also have found no difference in the protein expression of SynCAM in whole-brain astrocyte cultures from *wav-2* (erbB1 null [246]) mice (Mungenast unpublished results). Nevertheless, the closely interacting erbB network that operates in hypothalamic astrocytes necessitates further experiments to determine the specificity of SynCAM's interactions with erbB4. We found no effect of erbB1 stimulation on the promoter activity of SynCAM, however these experiments relied on the stimulation of endogenous erbB1 receptors, while the results from erbB4 stimulation were with transfected, thus overexpressed, erbB4 receptor. Further promoter assays are needed with transfected erbB1 to show that this receptor does not activate the

SynCAM promoter. In addition, it would be helpful to determine whether erbB1 co-immunoprecipitates with SynCAM in protein from hypothalamic astrocytes. It could be expected that erbB1 will co-immunoprecipitate, due to its association with erbB2, but, unlike erbB2 and erbB4, the amount of erbB1 pulled down should not vary in the DNerbB4 astrocytes. If an association between erbB1 and SynCAM were discovered, this would have exciting implications for both astrocyte and tanycytic signaling as well as for non small-cell lung cancer treatments where new therapies are targeting the erbB1 receptor. The relationship of SynCAM with erbB1 versus erbB4 could be examined with the existing transgenic models of the *wav-2* mouse and the DNerbB4 mouse.

Although erbB4 stimulation does not appear to affect the methylation status of the SynCAM promoter, there are many other mechanisms by which this promoter could be affected by erbB signaling. The cloned human SynCAM promoter which we describe here contains binding sites for several transcription factors whose activity have been linked to erbB signaling, such as AP-1 [247], AP-2 [248], Oct-2 [249] and PEA3 [250]. It is possible that erbB4 may influence the SynCAM promoter via one of these factors, through known erbB4/erbB2 signaling pathways such as the MAP kinase pathway.

The adhesion assays performed in this study demonstrate a functional consequence of SynCAM binding in hypothalamic astrocytes. As a homophilic adhesion molecule, the SynCAM on the astrocyte cell surface will bind to fusion proteins containing SynCAM's extracellular domain [210]. We show here that SynCAM binding in hypothalamic astrocytes results in a significantly greater degree of adherence in this assay. Further controls are needed to confirm these findings, because the wildtype astrocytes also showed a high degree of binding to the mutated SynCAM protein. This could result from either adhesion mediated by the intact spliced region which may carry O-linked glycosylation, or non-specific adhesion to the human Fc portion of the fusion protein. One way to dissect these possibilities is to include a further control containing only the human Fc portion of the fusion protein. Thus, cell counts from this condition could be regarded as truly "background" adhesion and we could hypothesize that any further increase in adhesion to the mutated SynCAM ECD could be mediated by O-linked glycosylation.

We also find that adherence to the SynCAM extracellular domain is reduced in the DNerbB4 hypothalamic astrocytes, indicating that the loss of a functional erbB4 receptor, and the subsequent downregulation of SynCAM expression, results in a decreased ability of these astrocytes to interact with SynCAM on surrounding cells. Although normalization using the cell counts outside the ring of fusion protein can control for the number of cells plated, we do not yet know if there are any subtle differences in the general adhesive abilities of DNerbB4 astrocytes vs. wildtypes. This is unlikely as, after two weeks in culture and re-plating in T150 flasks, DNerbB4 astrocytes reach confluency in the same time frame and yield equal numbers of cells per flask as wildtype astrocytes (data not shown). To address this issue it will be necessary to count and plate DNerbB4 and wildtype astrocytes from a number of different cultures onto culture

slides, stain with Hoescht or DiO, and compare the cell counts between the two genotypes. To specify that the defect in DNerbB4 adhesion is a result of loss of SynCAM due to loss of erbB4 signaling, rescue experiments should be performed involving the overexpression of transfected SynCAM or erbB4 in the DNerbB4 primary astrocytes, and analysis to determine if the cell adhesion matched wildtype levels in the transfected astrocytes.

The difference in the morphology of the DNerbB4 and wildtype astrocytes when in contact with SynCAM may indicate a role for this system in plastic cytoskeletal rearrangements. The Ojeda lab has shown recently that TGFα stimulation of hypothalamic tanycytes leads to cytoskeletal rearrangement [47]; neuregulin signaling could have a similar effect, mediated by SynCAM and its association to the cytoskeleton through DAL-1/4.1B, in hypothalamic astrocytes. Staining for the cytoskeleton with β-actin or GFAP could more closely examine this possibility. If SynCAM is indeed an binding partner between astrocytes and LHRH neurons that modulates physical proximity, then these results suggest that it is an interaction modulated by erbB4 signaling, and disrupted in the DNerbB4 mutant, perhaps contributing to the developmental defects seen in these animals.

This work describes a novel interaction between a growth factor receptor and an adhesion/signaling molecule that has far-reaching implications. Concerning puberty particularly, this association can help explain the reduction seen in LHRH neuronal output in the presence of disrupted astrocytic erbB4 signaling. Subtle changes in the SynCAM / erbB4 relationship, perhaps mediated by the differential glycosylation of particular SynCAM isoforms, can impact the physical association of the astrocyte to the LHRH neurons. It has been shown that this association is extremely plastic; herein we describe a candidate protein to mediate such plasticity. This is also an extremely dynamic association, as both erbB4 and SynCAM are endowed with a multiplicity of signaling capabilities.

CHAPTER FOUR

SUMMARY AND CONCLUSIONS

Chapter 4: Summary and Conclusions.

This work was undertaken to examine the hypothesis that the pubertal hypothalamus contains adhesion/signaling molecules important for the neuronal-neuronal and glial-neuronal signaling which underlies the timely onset of puberty in mammals. The proximity of astrocytes to LHRH neurons can affect the function of those neurons both through control of synaptogenesis and synaptic strength, as well as through the release of soluble factors such as growth factors, glutamate and PGE_2. The molecules described in this work promise to be modulators of the interactions between LHRH neurons and the surrounding astrocytes and other neural cells (**Figure 44**). The studies described were undertaken to examine the involvements of three families of adhesion/signaling molecules (cadherin-related, Ig-like and neurexins) in the interactions between neurons and glia in the hypothalamus. Specifically, we were interested in adhesive events between LHRH neurons, glia and other neuronal types that may influence the functions of the LHRH neurons. In Chapter 2, published work from these efforts in the rhesus macaque described the presence of the neurexin family of adhesion/signaling molecules in the primate hypothalamus, as well as the neuronal-glial Caspr/contactin/RPTPβ signaling complex and the protocadherin-α family. In Chapter 3, we then delved further into functional aspects of these types of signaling molecules relative to sexual maturation with a mouse model of disrupted growth-factor receptor signaling that displays a delayed onset of puberty. The interaction of SynCAM and the erbB4 tyrosine kinase receptor is shown to be one relevant to astrocytic-LHRH interactions.

The work in this dissertation provides evidence for a novel interaction in the brain between glial growth factor receptors and adhesion molecules. Many of these adhesion molecules have been found to play roles in communication between neurons, but few have been studied in the interactions between astrocytes and neurons. Our investigation of these families of adhesion molecules and their possible roles in astrocytes have led us to discover a functional relationship between an adhesion molecule previously characterized as a synaptic modulator, SynCAM, and the erbB4 tyrosine kinase growth factor receptor, which has been shown by this lab to be critical in glia to neuron communication during sexual development.

There are a few examples in the literature of central interactions between growth factor receptors and adhesion molecules. This is the first example we have found of an interaction in astrocytes between a tyrosine kinase growth factor receptor and an adhesion molecule of the immunoglobulin superfamily. There are precedents, however, for interactions between growth factors and both immunoglobulin and cadherin superfamily adhesion molecules. Studies have shown that EGF can induce tyrosine phosphorylation of the cadherin binding partner β-catenin in epithelial cells, suggesting a role in signal transduction [251]. One of the members of the tyrosine kinase neurotrophin receptor family, TrkB, is able to induce cell aggregation in fibroblasts when co-expressed with cadherin [252]. The neural cell adhesion molecule NCAM, which shares homology with SynCAM and is crucial in neural plasticity, shows heterophilic binding to the

fibroblast growth-factor receptor (FGFR, for review see [253]) as well as the glial-derived neurotrophic factor (GDNF) receptor [254]. In cultured human optic nerve head astrocytes, treatment with the growth factor TGFβ results in the upregulation of several extracellular matrix adhesion molecules including fibronectin [255]. The erbB4 receptor itself has been shown to modulate neuroblast migration and placement in the adult forebrain [256]. SynCAM has been postulated to play a role in neuronal migration due to its colocalization in the developing nervous system with adhesion molecules known to be necessary for migration such as NCAM, TAG-1 and L1 [257]. It is thus possible that these two proteins interact in a number of different systems.

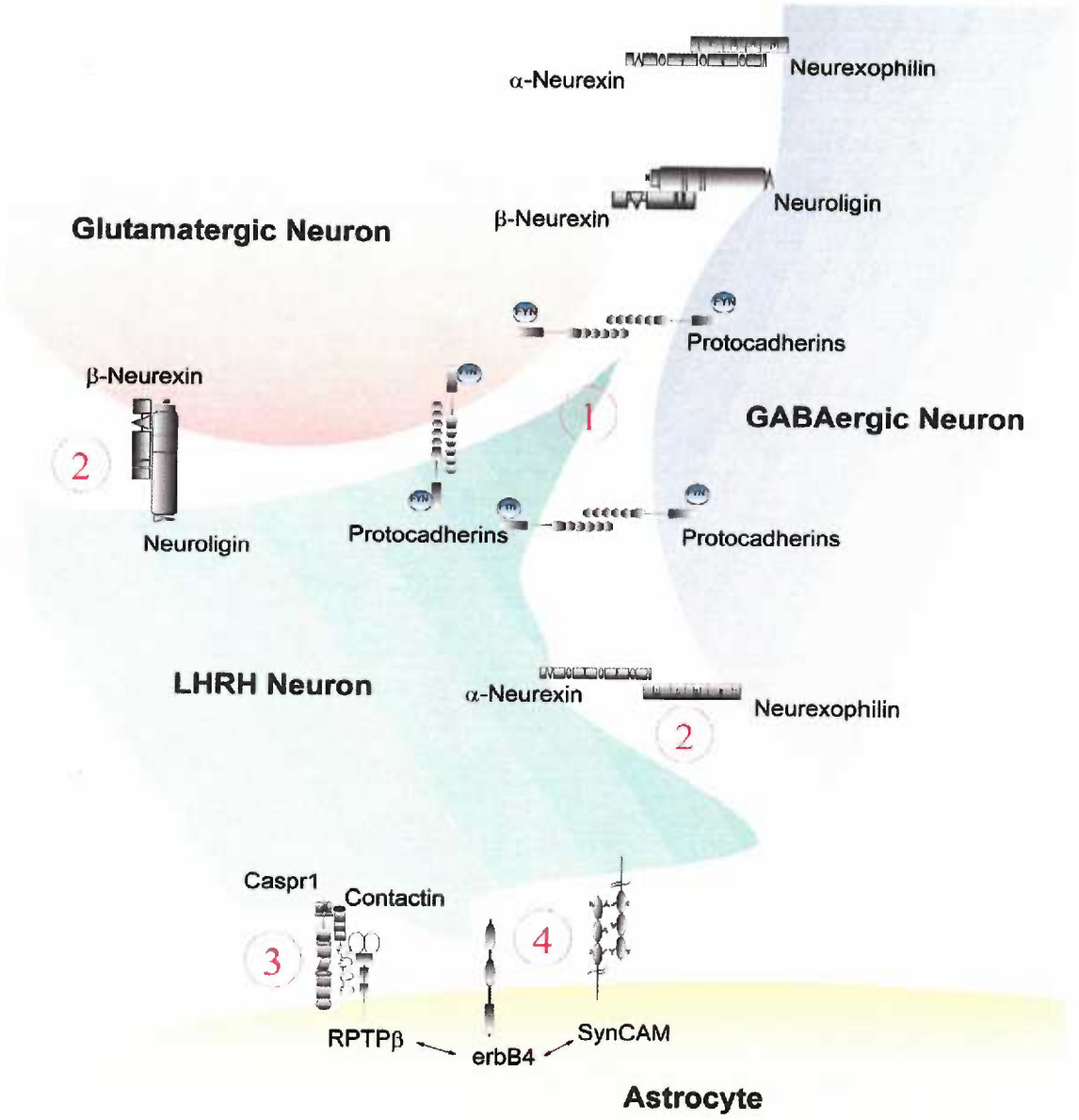


Figure 44. Adhesion/signaling molecule interactions in the LHRH neural network. 1. Protocadherins are expressed in both neurons and astrocytes and bind each other homophilically in *trans*. Changes in protocadherin expression may determine the physical proximity of both astrocytes and other neurons to the LHRH neuron. 2. Neuroligins determine the balance of inhibitory and excitatory synaptic inputs to the LHRH neuron. Alpha-neurexins on the LHRH neuronal membrane also can bind neurexophilins secreted by inhibitory neurons. LHRH neurons can also express β-neurexins (not shown) that may determine their efferent signaling to other neurons. 3. LHRH neurons express Caspr1 and contactin that bind to RPTPβ on neighboring astrocytes. The availability of RPTPβ may be determined by erbB4 activation. 4. SynCAM present in both the LHRH neurons and astrocytes binds homophilically, modulating the physical proximity of these two cell types. This connection may also be modulated by erbB4 activation.

Adhesion Molecules in the Brain; The cadherin and immunoglobulin superfamilies:

There have been a number of adhesion/signaling molecules described to have functions at the synapse described in the last several years (for review see [258]). These can be roughly divided into two superfamilies: the cadherins and the immunoglobulin (Ig)-like molecules. Most promising among the latter are proteins with immunoglobulin-like extracellular domains that participate in Ca^{2+}-independent adhesion to proteins on apposing cell membranes, such as the contactins [133,259], NCAM [260,261], nectins [262], nectin-like molecules/SynCAMs [263] and the sidekicks [264,265]. The cadherin superfamily has also generated much research in this area, as synaptic proteins such as N-cadherin and the protocadherin-α/CNRs have been localized to the synaptic membrane and implicated in the regulation of synaptic strength [37,42,123,266-268]. Other transmembrane proteins, displaying some homology to both these groups and which may interact with these adhesion molecules, have been shown recently to be critical for synapse formation and function, namely the neurexins and their ligands [269-271].

The mechanisms by which these adhesion molecules are regulated by the cell have not yet been fully described. Neurexins have been shown to bind to the presynaptic proteins synaptotagmin 1, CASK and Mintl [210,272-274], the transmembrane protein dystroglycan [275] and the Band 4.1 proteins implicated in synaptic clustering [276,277]. The neurexin binding partners neuroligin 1 and neuroligin 2 are localized to excitatory and inhibitory post-synaptic membranes, respectively [35]. The intracellular PDZ-binding domains of the neuroligins interact with PSD-95, SAP-102, PSD-93 [32] and S-SCAM [278]. The juxtamembrane domain of neuroligin 1 has also been shown to bind to Band 4.1 proteins [28].

Less is known about the partners with which the protocadherin-α/CNR (Pcdhα/CNR) proteins interact. The complex genomic organization of the Pcdh gene produces 15 separate family members from distinct promoters, which are all joined to the same intracellular domain [38,123,279]. The Pcdhαs display both homophilic and heterophilic Ca2+-dependent binding in trans through the extracellular ectodomains, and have been identified at the synapse [280]. Different neurons, even of the same phenotype, can express different Pcdhαs [37], thus these molecules provide a large variety of adhesive specialization and variety that single cells can express. Added variety may result from a high number of coding single-nucleotide polymorphisms found in the human Pcdhα genes [281].

Recent reports suggest that the cell-surface expression of Pcdhα is regulated by its relative, Pcdhγ [282], and that Pcdhα proteins are expressed in developing cortical axons and down-regulated during maturation of myelination [283]. These findings suggest that Pcdhα is more likely to be important for synaptic modification rather than synaptogenesis, and thus would play more of a modulatory role in the strength of established synapses within a neural network such as that involving LHRH neurons. The effect of Pcdhγ upon regulating the synaptic presence of Pcdhα in the hypothalamus remains to be examined.

Perhaps the most interesting, and least described, proteins at the synapse are the Ig-like molecules, which bind a number of different molecules in a Ca^{2+}-independent fashion and have both adhesion and signaling properties [218,263]. These molecules are implicated in a wide range of adhesion-mediated events, including polarization of epithelial cells (nectins [263]), neuronal migration (NCAM [284]) and axon guidance (Sidekick 1 and 2 [265]). NCAM in particular has been studied as a critical component of rapid plasticity within the hypothalamus [159]. Specifically at the synapse, the adhesion molecule SynCAM-1 (aka. NECL2, IGSF4, RA175, SgIGSF, TSLC1) plays a vital role both pre and post-synaptically in neurotransmission [26,30]. The functions of the other three SynCAM proteins are largely unknown, although a very recent study shows SynCAM-3 (Nectin-like Molecule 1, Necl1) to be present in both neurons and glia, to bind both homophilically and heterophilically to SynCAM-1, and to be increased at contact sites between neurons and glia [285].

Adhesion Molecules in Glia:

Apposition of neurons and astrocytes is common in the brain. A wide variety of neuronal influences of astrocytes have been described, from simple metabolic support to active control of neuronal excitability. The astrocyte has of later years become recognized as an essential component of synapse formation and function, outlines in the "tripartite synapse" concept which states that "synaptically associated astrocytes should be viewed as integral modulatory elements of synapses" [54]. Astrocytes influence synaptogenesis not only centrally, but in the peripheral nervous system as well [286-291]. Astrocytes play an well-established role in glutamate uptake [292], but also release glutamate themselves that is able to affect neighboring neurons and other astrocytes. Neuronal-astrocyte contacts are also sites of Kv2.1 potassium channel clustering [293], which can change the dynamics of depolarization and repolarization in the neurons [191].

Our lab has shown that LHRH neurons are modulated by astrocytes through secreted signaling proteins such as PGE_2 [58]. Other work has shown that soluble astrocytic factors, such as cholesterol [294,295] and thrombospondins [296], are critical for increasing synapse formation and activity [109,297,298] in a number of neuronal preparations, including those from the hypothalamus [299]. Although astrocytes have been shown to play all these roles in the modulation of neuronal function, the mechanisms by which neurons are maintained in contact with nearby astrocytes, and how these contacts are modulated by hormones, growth factors and neurotransmitters, are still unclear. In the current work, we give evidence that SynCAM has a dynamic relationship with erbB4 and is may increase astrocytic adhesion in a manner modulated by erbB4 signaling. Binding between SynCAMs on LHRH neurons and astrocytes could change the excitability of these neurons, via effects of cell proximity on ion concentrations and the availability of juxtacrine signaling partners.

There are several other adhesion / signaling molecules that show promise as glial modulators of neuronal function. Among these, the Ig-like molecule contactin has a role in

neuronal-glial communication. Although most of the research done on the Contactin/Caspr/RPTPβ complex has been aimed towards elucidating its function in the paranodal region of the nodes of Ranvier [132,133], this complex may also operate in the neuroendocrine brain, as suggested by the increased contactin expression observed in actively secreting hypothalamic magnocellular neurons [134]. We have shown, moreover, that contactin and Caspr are present in LHRH neurons, and RPTPβ is present in hypothalamic astrocytes. Ongoing studies in the laboratory are yielding evidence that RPTPβ associates with the erbB4 receptor in the astrocyte, and this association may influence the function of associated LHRH neurons [188].

Growth-factor signaling in the brain, erbB receptors and puberty:

Astroglial cells regulate LHRH secretion via two mechanisms: by induction of plastic rearrangements within the median eminence and by activating specific glia-to-glia and glia-to-neuron signaling pathways (reviewed in [77]). The work in this dissertation provides evidence for the involvement of adhesion molecules in both these mechanisms. In regard to the second pathway, previous work in the Ojeda lab has shown that tyrosine kinase growth factor receptors of the erbB (or EGFR) family are critical to the timely onset of puberty (for review see [77]). Disruption of erbB4 signaling specifically in hypothalamic astrocytes leads to a delayed onset of puberty and an attenuation of reproductive function in mice [198]. This is due to a decreased LHRH output of the LHRH neurons which, in part may result from a loss of prostaglandin E_2 release from the astrocytes [198].

Astrocytes have been shown to contain glutamate receptors and to response to glutamate stimulation with the production of bioactive molecules such as PGE_2 [69] and glutamate itself [69,300]. Our lab has shown that the functional capability of astrocytic erbB signaling molecules is enhanced by the combined activation of ionotropic and metabotropic glutamate receptors on the astrocytes [56,301]. These glutamate receptors are associated, as in the neuron, with their respective clustering/interacting proteins PICK-1 and Homer. These results described the functional presence of a number of signaling molecules that had before been implicated mostly in neuronal, not glial, function. The ability of glutamate to upregulate both erbB function and glutamate release from hypothalamic astrocytes demonstrates that these growth factor receptors are important to glial-to-neuronal communication, but little is known about what other associated synaptic or glial proteins might be involved.

It appears clear that selective disruption of astrocytic erbB-4 function results in delayed puberty, and that this delay is at least in part due to an impaired ability of NRGs to initiate PGE_2-mediated glia-to-neuron signaling [56]. Nothing is known, however, about additional, and perhaps even more relevant, cell-cell communication pathways that might be affected by the astrocytic defect in erbB-4 function. In the studies described here, we have demonstrated a dynamic association between erbB4 receptors and the synaptic adhesion molecule SynCAM that promises

to further explain the bi-directional communication pathways between astroglial cells and LHRH neurons.

Neuronal-Glial Communication: further roles of SynCAM.

The presence and association of these signaling/adhesion molecules may outline a functional glial-neuronal complex responsible for the morphological association between astrocytes and LHRH neurons, and capable of being modulated by growth factor signaling. Such a complex could consist of SynCAM, RPTPβ, erbB4 and neuroligin colocalizing in the astrocyte membrane and associating with their respective binding partners in the apposing LHRH neurons, namely: SynCAM, Caspr1/contactin, neuregulin and β-neurexin. Chapter 3 of this thesis describes evidence that SynCAM and erbB4 interact directly. Also, at least one neuroligin has been shown to be present in astrocytes [302]. SynCAM co-precipitates with neuroligin-1 [210], although it is not known if this is a direct association, or perhaps one mediated by their common ability to bind Protein 4.1B [303]. We show that SynCAM also immunoprecipitates with erbB2, supposedly through its direct association with erbB4. As erbB2 also binds protein 4.1B, SynCAM and neuroligin could be brought together through this indirect association. Through neuronal SynCAM, erbB signaling could also affect the neuroligin / neurexin connection and further assist in the modulation of synaptic connectivity and strength. Future studies should help to elucidate possible interactions between the erbB receptor family and the neuroligins, and if signaling through erbB receptors might affect synapse strength by influencing neuroligins and SynCAM.

Work in the peripheral nervous system has shown that neuregulin-erbB signaling is critical to the myelination of axons, as the conditional knockout of either neuregulin or erbB2 in myelinating Schwann cells results in hypomyelination of the nerve fibers [304]. SynCAM may also be present and able to interact with erbB receptors in and around the nodes of Ranvier in myelinated axons. SynCAM binds DAL-1/Protein 4.1B [243] and may interact with RPTPβ through erbB4 [188]; this is worth investigating further, as Protein 4.1B and the RPTPβ ligands Caspr/Contactin are critically important to the glial-axonal interactions underlying the formations of the nodes of Ranvier in myelinated axons [276,305-307]. In the peripheral nervous system, recent studies have found SynCAM3/Necl-1 to be concentrated at the contact sites of the cellular processes of Schwann cells at the nodes of Ranvier as well as in the axonal membrane [285]. We do not yet know if the SynCAM described in the present work, SynCAM1, is present at this location, nor how the functions of the four SynCAM genes may overlap. This nodal region containing SynCAM3 also has present erbB2 in the Schwann cells, but not erbB4, caspr1 or 4.1B. If the roles of the different SynCAM genes are non-overlapping, perhaps as we see SynCAM3 at the node of Ranvier, we may well expect SynCAM1 to be at the paranode or juxtaparanodal regions, where the enwrapping Schwann cells express caspr, contactin, 4.1B and caspr2, 4.1B, respectively (for review see [132]). As we hypothesize SynCAM to be important in the erbB-

mediated astrocytic coverage of LHRH neurons, this function of SynCAM could be also important in a more general scheme of astrocyte-neuronal plasticity found in other areas of the brain.

Additionally, as a tumor suppressor protein found to be reduced in a number of cancers including lung cancer [216,244,303,308], pancreatic cancer [239,309] and meningeoma [303,310], SynCAM could have a role in the genesis of schwannomas (in which erbB receptors may be involved [311]) and other glial malignancies. The interaction of SynCAM with the erbB receptor system could have particular significance to cancer biologists, as we already know that SynCAM and erbB1, as well as DAL-1/4.1B, are important proteins in the etiology of non-small-cell lung cancer [225,309,312,313]. As a modulator of glial-neuronal proximity, SynCAM could also be involved in spinal cord regeneration and the formation of glial "scars" following injury to nerve tissue. Immunohistochemistry to colocalize the SynCAM1 protein with nodal, paranodal or juxtaparanodal proteins in myelinated axons would thus be a worthwhile endeavor.

CHAPTER FIVE

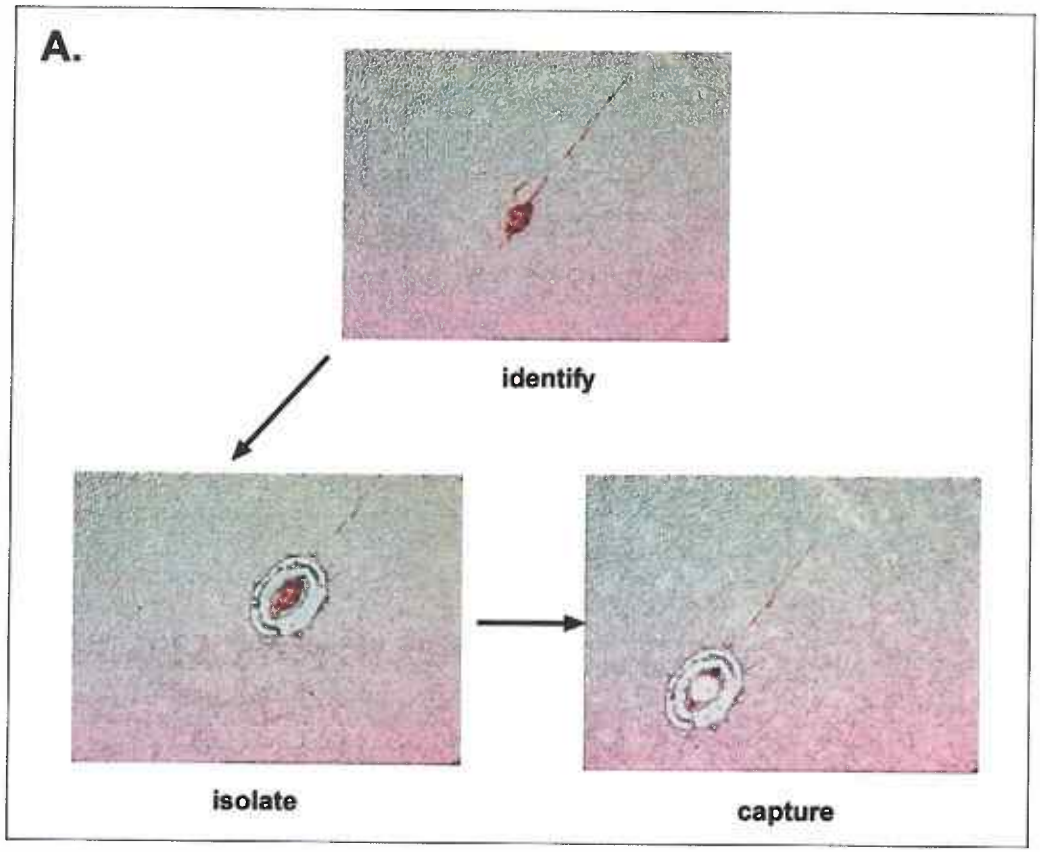
FUTURE DIRECTIONS

Chapter 5: Future Directions

Adhesion molecules in the pubertal rhesus hypothalamus:

The published work [314] described in this manuscript has provided evidence that the juvenile primate hypothalamus contains many members of the neurexin, protocadherin and Caspr/contactin/RPTPβ adhesion/signaling molecule families. The adhesion molecule SynCAM is also present in the primate hypothalamus, and increases in mRNA expression at puberty (unpublished results). However, these preliminary studies tell us nothing about the relevant cell types in which these molecules are expressed. In addition, these studies do not describe how these molecules may change in expression and function in relation to the onset of puberty. We are, of course, presently running a number of microarray studies examining gene expression in the primate hypothalamus relative to pubertal status, and these have been fruitful. However, in a tissue as heterogeneous as the hypothalamus, array studies like these will miss changes in molecules expressed only in a small subset of cells, or molecules whose expression changes in opposite manners in different cell types.

To address this problem, we have developed a system which will allow us to isolate phenotypically identified single hypothalamic cells and subject them to microarray analysis. This is accomplished by the process of laser-capture of immunostained neurons followed by RNA amplification (**Figure 45**). With this powerful tool, we can examine changes in a discrete set of genes within individual neurons of different phenotypes, as well as individual glia, underlying the onset of puberty in the primate. Unfortunately, as this work is extremely laborious, time-consuming and expensive, the potential of these promising experiments has not yet been realized. We are confident that, with time and technical expertise, the cell-by-cell examination of relevant molecules in the pubertal hypothalamus will soon be possible.



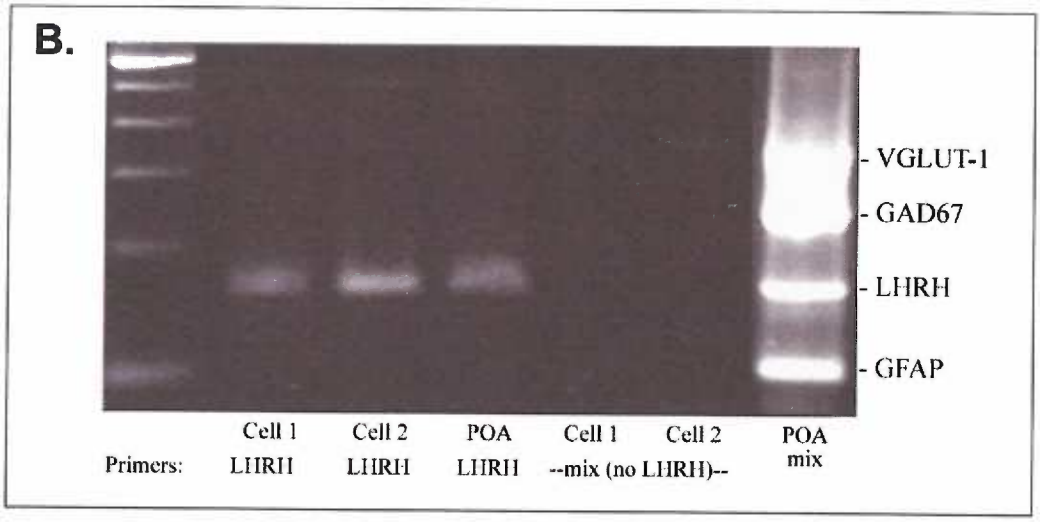


Figure 45. Laser-capturing single LHRH neurons. A. Single immunostained LHRH neurons are located in the rhesus monkey medial basal hypothalamus. The laser is used to isolate the LHRH neuron by ablation of surrounding cells, reducing contamination. A single laser catapulting pulse then ejects the cell from the surface of the slide to be captured a microfuge tube cap containing lysis buffer. B. Molecular characterization of single laser-captured LHRH neurons. Left lanes labeled Cell 1 and 2 represent LHRH mRNA detected in 2 individual cells laser-captured from the monkey preoptic area. LHRH mRNA was amplified by two rounds of nested RT-PCR. The lane labeled monkey POA shows an LHRH PCR product amplified from total POA RNA after one round of PCR amplification using the outer LHRH primers only. The next two lanes labeled Cell 1 and Cell 2 demonstrate that the captured LHRH neurons are not contaminated with glutamatergic neurons (i.e. they are devoid of VGLUT-1 mRNA) or GABAergic neurons (i.e., they are devoid of GAD-67 mRNA). In addition, they are free of astrocytic contamination (i.e., they lack GFAP). The lane labeled POA shows the detection of all four cell types in the POA using the complete set of diagnostic primers.

SynCAM and erbB4 Signaling:

We have shown in this work that the stimulation of erbB4 receptors increases the promoter activity and gene expression of SynCAM, as well as the physical association between these two membrane proteins. We still know very little about how SynCAM may be interacting with the complex signaling capabilities of the erbB4 receptor. For instance, we can see maximal increases in both promoter activity and mRNA expression of SynCAM after about 8 hours of erbB4 stimulation, and these levels fall by 12 hours. This would indicate a possible negative feedback loop by which erbB4 is regulating SynCAM expression. Thus, after a certain amount of time, the stimulation of erbB4 is either inhibitory to SynCAM expression, or does not have any influence at all. Further time points in these experiments could tell us if, indeed, erbB4 stimulation may have a negative effect on SynCAM expression compared to control conditions.

There are a number of ways in which erbB4 can signal through well-known pathways, described earlier in this manuscript. One of the most obvious differences that can be measured is that of the erbB4 isoform involved. As reviewed in [315], there are four possible combinations of erbB4 isoforms, existing of molecules carrying either the JM-a or JM-b, and the CYT-1 or CYT-2, combinations. The brain expresses each one of these splice variants. As shown in **Figure 46**, different combinations of these isoforms endow different signaling capabilities upon the erbB4 receptor.

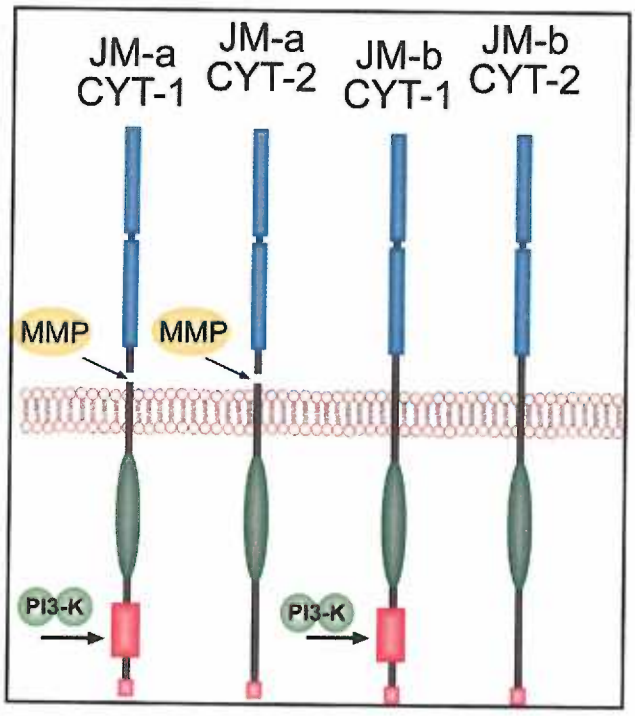


Figure 46. The erbB4 juxtamembrane and cytoplasmic isoforms. Four possible combinations of the alternative domains are generated in tissues. The erbB4 juxtamembrane (JM) isoforms may be differentially processed by matrix metalloproteases (MMP). The erbB4 cytoplasmic (CYT) isoforms differ in their coupling to phosphoinositide 3-kinase (PI3-K).

We have expression constructs that contain the JM-a form of erbB4 and either the CYT-1 or CYT-2 isoforms in pcDNA3. Using these in our promoter assay, we can at least establish whether or not PI3-K signaling is involved in the erbB4-dependent activation of the SynCAM promoter. ErbB4 can also signal through the MAPK/JAK-STAT pathways. We have antibodies that can differentiate between the phosphorylated and unphosphorylated forms of MAPK. Co-transfection of wildtype SynCAM or a mutant form with erbB4 in a suitable cell line, followed by Western blotting for MAPK, could shed some light on whether or not erbB4's interaction with SynCAM affects this signaling pathway.

We have shown in this work that the kinase domain of erbB4 is necessary for its binding to SynCAM. Does erbB4 phosphorylate SynCAM, or is SynCAM binding to the phosphotyrosines on erbB4? Our collaborators in the Corfas lab are working on this problem by incorporating point mutations in the kinase domain of erbB4 and testing these mutants for binding with SynCAM in a targeted yeast two-hybrid assay.

This work has focused on the neuregulin receptor erbB4. The other neuregulin receptor, erbB3, was not addressed in this work because we have previously found it to be absent in hypothalamic astrocytes [316]. However, erbB3 is present in the tanycytic glial cells that interact with LHRH fibers in the median eminence. ErbB3 is also the main receptor for neuregulin in the myelinating Schwann cell. Work in both these models would benefit from studies designed to test the hypothesis that one of the SynCAM genes interacts with glial erbB3.

Adhesive properties of SynCAM:

SynCAM has previously been shown to be an adhesion molecule that can bind itself homophilically [210] as well as be bound heterophilically by SynCAM-3 [285]. The published descriptions of SynCAM have all dealt with the isoform 2 of SynCAM (SynCAM-1, isoform 2). We also show that this isoform is selectively downregulated in the absence of erbB4 signaling. These studies lead to the question of what are the different capabilities and properties of the different isoforms?

We have shown in the current work that at least one difference may be that of O-linked glycosylation. The abundant N-linked glycosylation of SynCAM is located on the Ig domains of the extracellular region, which are identical in the different SynCAM isoforms. However, the spliced region of the extracellular domain is predicted to carry O-linked glycosylation. Furthermore, isoform 2 is predicted to have a unique pattern of O-linked glycosylation which is much more abundant than that predicted for isoform 4, the other form present in hypothalamic astrocytes. O-linked glycosylation, and especially the terminal sialic groups is may contain, can confer radically different adhesive properties to the different SynCAM isoforms.

It remains to be biochemically established that this region of SynCAM carries O-linked glycosylation. O-linked glycans are potentially much more complex than N-linked, and numerous

enzymes are needed to strip away the different groups to reveal the glycan backbone to the enzyme O-glycosidase. An easier approach may involve new kits, which can differentially stain the different sugar groups, such as DIG-labeled lectin binding assays (Pierce). The discovery of a small amount of polysialation on the SynCAM molecule is extremely exciting to anyone who has studied developmental plasticity and the critical importance of SynCAM's cousin, NCAM. The presence or absence of polysialic acid (PSA) on the SynCAM protein can only be firmly established via release of PSA with a rare endo-N-acetylneuraminidase enzyme [221].

Once differential glycosylation amongst the SynCAM isoforms has been established, the need remains to determine the functional consequences of this glycosylation. Site-directed mutagenesis may be used to generate small deletions of the SynCAM spliced region, abolishing or lessening the load of O-linked glycans. The consequences of this can be determined in adhesion assays with primary cultures of astrocytes and neurons, as well as with neural cell lines carrying wildtype or altered isoforms of SynCAM. In addition, point mutations in the SynCAM extracellular domain of the fusion protein could be introduced to determine the most important parts of the protein for astrocyte adhesion. As the SynCAM-ΔECD-Fc fusion protein still has the spliced region intact, which may carry O-linked glycosylation, it is important to determine whether abolishing this region further reduces astrocyte binding.

The adhesion assays described in this work give us a physiological assay by which we can examine the functional consequences of the interaction between SynCAM and erbB4. The fusion proteins we've produced could also provide a handy tool for blocking SynCAM binding, by occupying the extracellular domain of SynCAM on the astrocyte surface. This allows us to test, for instance, whether blocking SynCAM on the astrocyte surface could diminish the well-established synaptogenic effect of astrocytes on co-cultured neurons [317].

Physiology of SynCAM:

We show in this work that an astrocyte-specific loss of the erbB4 receptor leads to a decrease in SynCAM protein and mRNA levels in astrocytes, resulting in a loss of adhesive properties of the mutant astrocytes. What are the consequences of the loss of SynCAM signaling on neural connectivity and, specifically, the timing of puberty? To answer this question, we propose to make a transgenic animal expressing an astrocyte-specific dominant-negative mutant of the SynCAM protein. This will be accomplished by cloning a fusion protein consisting of the transmembrane and extracellular portion of SynCAM isoform 2 joined to EGFP into an expression vector carrying a GFAP promoter [198]. This will result in a dominant-negative SynCAM mutant which can bind extracellular partners but which is unable to signal internally. Thus we can study the physiological consequences of the loss of SynCAM's association with erbB4 and other possible signaling pathways.

SynCAM and other adhesion molecules:

A role for the RPTPβ/Caspr1/Contactin complex in neuronal-glial plasticity has already been well established at the node of Ranvier [133]. Neuregulin signaling is also necessary for the proper development of the myelinated axons [132]. Evidence currently being gathered in our laboratory suggests that LHRH neurons adhere preferentially to wildtype RPTPβ in vitro, indicating that they express the functional Caspr1/Contactin complex [188]. RPTPβ also appears to interact with erbB4, and that this association is decreased upon neuregulin signaling. We have shown in the present work that SynCAM and erbB4 associate more closely after neuregulin stimulation. Thus these findings can incorporate both these glial adhesion molecules into a model of astrocyte-LHRH plasticity modulated by neuregulin signaling (**Figure 47**). In this model, hypothalamic astrocytes express RPTPβ and SynCAM, which bind to Caspr1/Contactin and SynCAM on the LHRH neuronal membrane, respectively. In an unstimulated state, erbB4 receptors are bound to RPTPβ and SynCAM is associated with the cytoskeleton through Protein 4.1B. Upon stimulation, the autophosphorylation of erbB4 repels RPTPβ and attracts SynCAM. SynCAM then dissociates from the cytoskeleton and is made available to SynCAM on the apposing cell. At the same time, RPTPβ is free to bind to the Caspr/Contactin complex, also on the apposing LHRH neuron. Simple co-localization experiments using immunohistochemistry in vivo on astrocytes and LHRH neurons should be the first step towards examining the relationships between these adhesion molecules and astrocytic-LHRH plasticity.

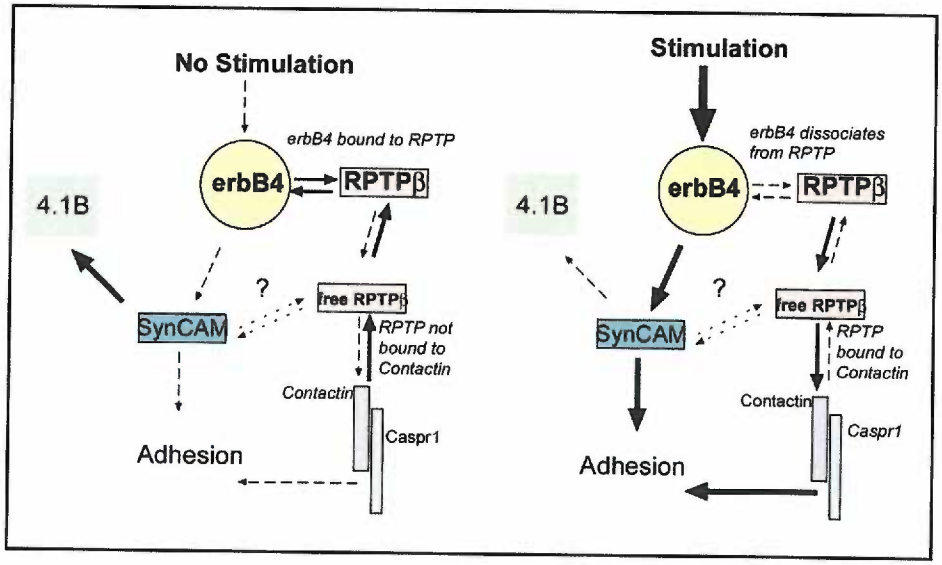


Figure 47. A working model of the interactions between erbB4, RPTPβ and SynCAM in the hypothalamus. **1.** Astrocytic erbB4 receptors are stimulated by ligand. **2.** Stimulated erbB4 receptors dissociate from RPTPβ. **3.** Free RPTPβ binds the Contactin-Caspr1 complex on the surface of LHRH neurons. **4.** Stimulated erbB4 receptors activate SynCAM. **5.** Both processes lead to increased adhesiveness between astroglial cells and LHRH neurons.

SynCAM in other models:

As discussed in the Conclusions section, one SynCAM gene, SynCAM3/Necl1, has been localized to the node of Ranvier in peripheral myelinated axons [285]. Neuregulin signaling is important to the generation of Schwann cells and their association with the nerve fibers [318]. We know from the present work that SynCAM interacts with erbB4/erbB2 in a functionally relevant manner. We could hypothesize that SynCAM may also interact with growth factor receptors at the node of Ranvier, and thus influence the physical relationships between the myelinating Schwann cells and the axonal membrane. A simple experiments would be to perform double immunohistochemistry in thin longitudinal sections of a mouse sciatic nerve, and to examine whether SynCAM colocalizes with nodal markers like NrCAM, paranodal Caspr, or juxtaparanodal Caspr2. A similar experiment with antibodies raised again SynCAM2 and SynCAM4 could also be helpful.

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