Fibroblast Growth Factor Receptors sculpt Excitatory and Inhibitory

Synapses in the Hippocampal Neural Circuit

by

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CHAPTER I

Synapse Development

The word "synapse" (συν + απτειν, or clasping together) was first coined in the late 19th century, amidst an explosion of anatomical studies into the structure of neurons in the brain (Hortsch, 2009). The etymology reflects its structure and function – the tight apposition of an axon onto its postsynaptic target (in the brain, usually a dendrite) is required for the synapse to become a portal of communication between the neurons of the brain. Neurons in the central nervous system form an extraordinary number of synapses with each other: for example, in visual cortex of an adult human, there are approximately 300 million synapses in one cubic millimeter and an individual cortical neuron can receive on the order of ten thousand synapses (Huttenlocher, 1990). The inputs a neuron receives are diverse, with unique electrophysiological characteristics, and the neuron sends outputs onto many distinct cells within the neural circuitry. In some ways, the formation of a synapse between two neurons is akin to meeting up with a friend in Grand Central at rush hour – it requires careful communication on time and location to achieve that desired "clasping together" of a handshake. And this coordination must occur amidst numerous neurons throughout the brain simultaneously. Synapse development is caught between discrete and broad demands – one, to assemble a fully functional synapse between correct synaptic partners, and two, to simultaneously ensure coordinated synapse formation throughout the brain. As an illustration, the hippocampal neural circuit (which will be the focus of my work in the following chapters) demonstrates many of these principles of coordination (Figure 1.1): coordination of specific synapses onto one dendrite, such as excitatory versus inhibitory synapses to ensure proper excitatory-inhibitory balance (Figure 1.1 B), coordination of synapse development throughout a region, for example clonally-related excitatory neurons preferentially receive input from the same presynaptic cell (Xu et al., 2014) (Figure 1.1 C), and coordination of

A
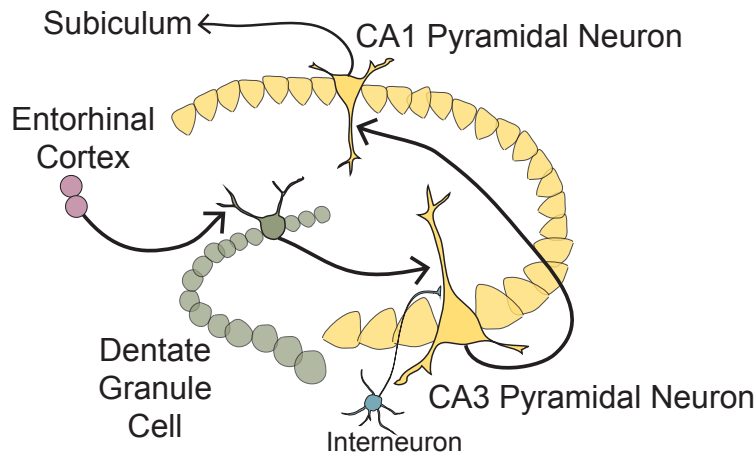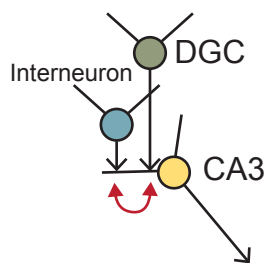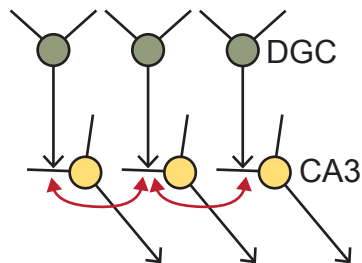
B   **C**   **D**
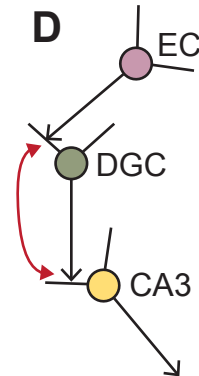
Figure 1.1. Coordination of synapse development in the brain.
A) An example of a neural circuit: Major excitatory projections in the hippocampal formation. Inputs from Entorhinal cortex (EC) to dentate granule cells (DGC) in dentate gyrus to CA3 Pyramidal Neurons to CA1 Pyramidal Neurons out to subiculum. Local interneurons provide inhibitory tone.
B) An example of synapse coordination between different synapses onto the same neuron: Balance of inhibitory synapses from interneurons and excitatory synapses from the dentate gyrus (DG) onto CA3 pyramidal neuron.
C) An example of synapse coordination in parallel: development of synapses between DGCs and CA3 throughout the hippocampus.
D) An example of synapse coordination across regions: synapses formed between entorhinal cortex (EC) and dentate gyrus (DG), in balance with synapses between DG and CA3.

synapses between regions, such as coordination of the inputs and outputs across a neuron (Figure 1.1 D). In this chapter, I will discuss basic events of synapse development, delve into the underlying molecular mechanisms, and finally outline current evidence in support of abnormal synapse development leading to neurological disease in humans. I will use this as a backdrop to explain the role of two synaptogenic molecules, FGF22 and FGF7, in inducing excitatory and inhibitory presynaptic differentiation in area CA3 of the hippocampus. What are the mechanisms through which the synaptogenic FGFs coordinate synapse development? This is a question I will address in the remainder of my thesis.

Synapse development occurs during late gestational and early postnatal development, and encompasses a phase of remarkable neural network emergence en route to a mature brain. Careful observation of postmortem human brain of various ages using electron microscopy by Patricia Goldman-Rakic and Pasko Rakic, among others, demonstrated that the first synapses appear at 5 weeks gestation and continue to increase exponentially in infancy until they peak in number at 2 years of age – in visual cortex at nearly 200% of mature brain count – and then steadily decrease until they finally achieve the "mature" number of synapses in the third decade of life (de Graaf-Peters and Hadders-Algra, 2006; Tau and Peterson, 2010). In terms of absolute numbers, synapse development can be divided into stages (Bourgeouis, 1997): First, synapses appear at a slow rate and occupy a low-density of neural tissue, at a time when neurogenesis (the birth of new neurons) is still ongoing. Next, a phenomenal acceleration of synapse formation occurs, coinciding with the end of neurogenesis and overlapping but extending past axon and dendrite elaboration. In humans, this huge addition of synapses starts in the second trimester of pregnancy and continues until the 2nd year of age, when synapse number peaks. These synapses are then slowly pruned away over the next decade of human life, through puberty, and then until the third decade of life, when the "mature" state is finally achieved (Petanjek et al., 2011). The rate of synapse formation and elimination does not occur simultaneously in different brain regions: even within the cerebral cortex of humans, the dynamics of synapse development vary, with areas of cortex for sensory input developing first and prefrontal areas last (Huttenlocher and Dabholkar, 1997). The general pattern of cortical synapse development is conserved among many species – for example in rodents, the exponential phase of synapse addition extends for two weeks starting after birth, peaks at postnatal day 28 (P28), and stabilizes at around P40 (Bourgeois, 1997; Bhatt et al., 2009); furthermore, many of the molecules involved in synapse

formation and function are highly conserved across species (Ryan and Grant, 2009). Thus, rodents provide important experimental vertebrate models for understanding normal and pathological synapse development (Nestler and Hyman, 2010).

Zooming in on individual synapses reveals that synapses are highly specialized structures. Simplistically, the activated presynaptic neuron releases neurotransmitter into the synaptic cleft, which is then received by the postsynaptic neuron: hence, at the presynaptic side there are neurotransmitter-laden synaptic vesicles and the machinery required for activity dependent release at the axon terminal, and on the postsynaptic side are the receptors that respond to the neurotransmitter and the associated machinery important for modulating the response in the dendrite. But synapses in the brain are structurally, molecularly, and physiologically diverse (Walmsley et al., 1998). *Presynaptically*, neurons produce distinct neurotransmitter, which instruct its role in the neural circuit: excitatory neurons produce glutamate, which activates the postsynaptic neuron, inhibitory neurons produce GABA, which suppresses the postsynaptic neuron, and neuromodulatory neurons produce acetylcholine, dopamine, or serotonin, depending on the identity, which modulate transmission at excitatory and inhibitory synapses (in great simplification). Beyond neurotransmitter identity, diverse synaptic boutons throughout the nervous system contain different stereotypic numbers of synaptic vesicles, from two hundred synaptic vesicles at an excitatory synapse in CA1 to over twenty thousand vesicles at mossy fiber boutons, the synapses between dentate granule cells and CA3 pyramidal neurons (Harris and Sultan, 1995; Rollenhagen et al., 2007). The active zone contains protein machinery that is important for docking synaptic vesicles and releasing them in response to an increase in calcium (usually elicited by an action potential), including RIM, Bassoon, Munc13, and SNARE proteins, as well as voltage-gated calcium channels (Dresbach, 2015; Imig et al., 2014). *Postsynaptically*, dendrites must segregate neurotransmitter receptors to the appropriate synapses – glutamatergic receptors, such as NMDA-type or AMPA-type receptors to excitatory synapses, GABAergic receptors to inhibitory synapses. The morphology of excitatory postsynaptic sites is often further specialized into a dendritic spine – a protuberance from the shaft of the dendrite that serves in part to electrically isolate individual excitatory synapses on a dendrite (Yuste, 2013). Excitatory and inhibitory poststsynaptic densities have numerous proteins in the postsynaptic density that are important for distinct function – for example postsynaptic density protein PSD95 is found at excitatory synapses, gephyrin is at

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inhibitory synapses, as well as numerous metabotropic receptors, potassium channels, and even elements of local translational machinery (Sheng and Kim, 2011). Synapses are complex and highly specialized structures; it is not surprising that their assembly should require many levels of control.

Time-lapse studies of assembly of individual synapses *in vitro* and *in vivo* have revealed that it is a multi-step and highly dynamic process (Figure 1.2). In neuronal culture, synapses appear to assemble within hours of initial contact, with the presynaptic components typically preceding the postsynaptic components (Friedman et al., 2000). Presynaptic components are trafficked along the axon as transport packets, containing a number of synaptic vesicles or active zone proteins forming a complex (Ahmari et al., 2000) and accumulate at the nascent synapse. Synaptic vesicle transport packets pause along the axon at fixed sites, in a synapsin-, calcium-, and actin-dependent manner, even in the absence of postsynaptic contact, where the synaptic vesicles release neurotransmitter, suggesting an intrinsic predisposition to form hemisynapses within the axon (Sabo et al., 2006). However, in the presence of appropriate postsynaptic contacts, synaptic vesicles accumulate at nascent synapses and outcompete inappropriate sites lacking postsynaptic contact, preventing ectopic synapse formation (Allen et al., 2008). Synaptic vesicles and active zone proteins are trafficked separately; elements of the active zone can recruit other presynaptic proteins, for example the active zone protein Bassoon directly recruits voltage-gated calcium channels (Davydova et al., 2014). Not all the components of the presynaptic terminal assemble simultaneously at the nascent synapse: first synaptic vesicles assemble, then active zone proteins, followed by components of the synaptic vesicle release machinery, such as synapsin, whose recruitment is controlled by Cdk5 (Easley-Neal et al., 2013), suggesting that distinct stages of presynaptic assembly can be regulated by distinct signals. The elements on the postsynaptic side also arrive at the nascent synapse independently. Clusters of NMDA-type glutamate receptors are transported in packets (akin to the synaptic vesicle and active zone packets presynaptically), bound to postsynaptic density components such as EEA1 or SAP102 (Washbourne et al., 2002). The recruitment of NMDA receptor components can be independent of recruitment of PSD95 (Washbourne et al., 2002), suggesting that there may be numerous and simultaneous processes of postsynaptic protein recruitment. In contrast, the postsynaptic density protein, PSD95, is not only transported along the dendrite as a packet, but also accumulates at the synapse from a cytosolic pool; the postsynaptic density either forms de novo from the
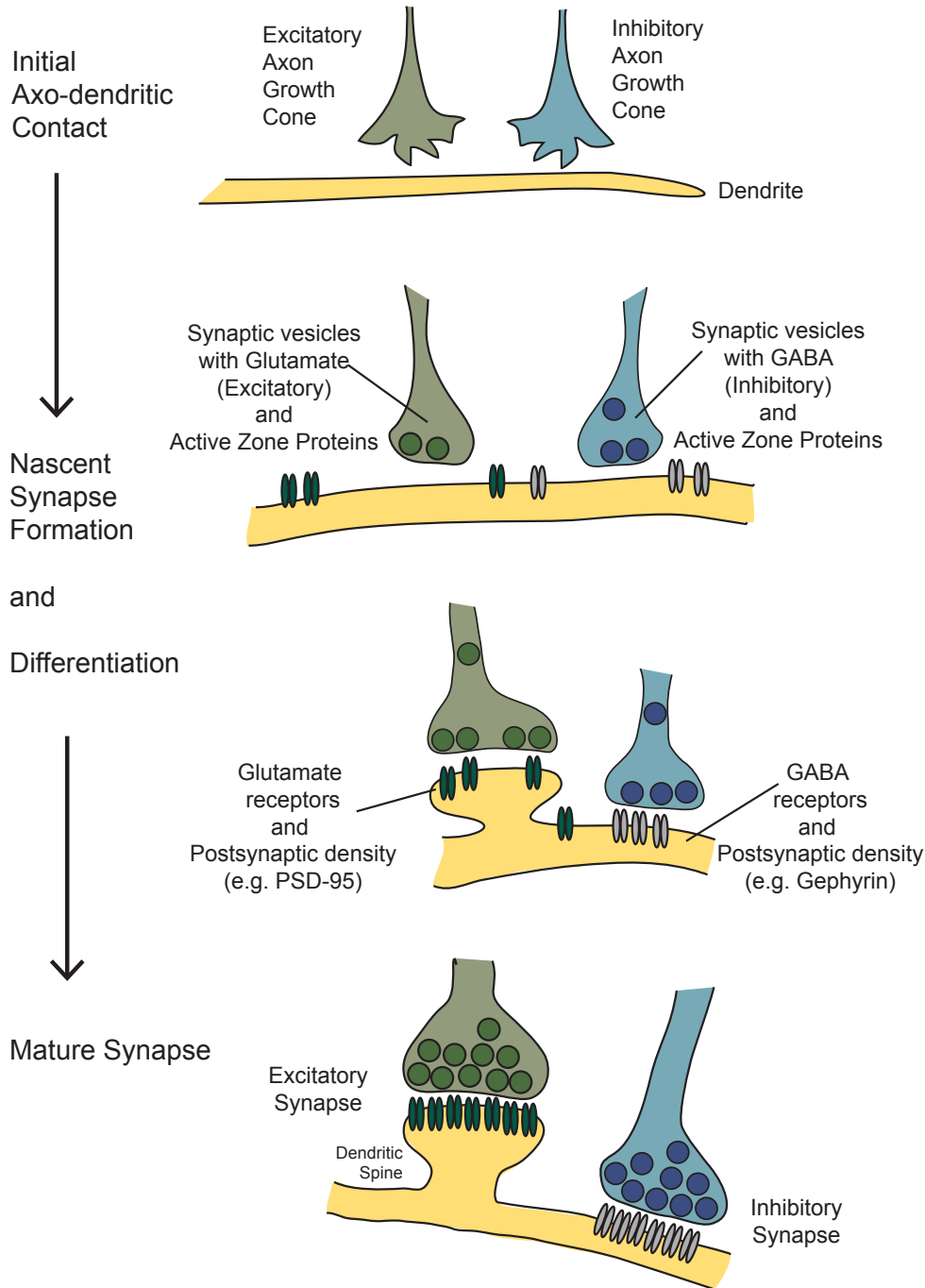
Figure 1.2. Schematic of excitatory and inhibitory synapse development.
Synapse development begins with axo-dendritic contact. Synaptic vesicle and active zone proteins accumulate in active zone terminal presynaptically. Excitatory synaptic vesicles contain glutamate, inhibitory synaptic vesicles contain GABA. Postsynaptically, glutamate receptors and excitatory postsynaptic density proteins (for example PSD-95) accumulate at nascent excitatory synapses in the dendrite; GABA receptors and inhibitory postsynaptic density proteins (such as Gephyrin) at inhibitory nascent synapses. Nascent synapses continue to exchange molecular cues and differentiate into mature synapses.

cytosolic pool or by splitting from pre-existing postsynaptic densities that are apposed to a presynaptic terminal (Bresler et al., 2001). Dynamics of inhibitory synapse formation in culture are similar to excitatory synapse formation although the dynamics may be slower (Dobie and Craig, 2011) – *de novo* synapses appear over 8 hours, with presynaptic protein accumulation preceding postsynaptic protein accumulation by 2 hours; and synapses are often mobile as a presynaptic-postsynaptic unit (Dobie and Craig, 2011). Again, components of the synaptic densities can themselves recruit other proteins: Gephyrin, the postsynaptic density protein at inhibitory synapses, is required for clustering of GABA receptors at inhibitory postsynaptic densities (Jacob et al 2005). The fact that presynaptic components assemble before postsynaptic components (at least *in vitro*) and that they spontaneously exocytose neurotransmitter at pause sites suggests that the released neurotransmitter could facilitate the accumulation of the appropriate receptors (glutamatergic receptors to glutamate, GABAergic receptors to GABA) at the nascent synapse on the dendrite. Indeed, NMDA-type glutamate receptors that are trafficked along dendrites are routinely exocytosed to sample the extracellular space (Washbourne et al., 2004). As further support, blocking synaptic vesicle release in retinal ganglion cells leads to fewer synapses in the retina (Kerschensteiner et al., 2009): a contributing mechanism to the loss of synapses could be a failure to appropriately recruit synaptic machinery. Furthermore, synapses are highly dynamic both *in vitro* and *in vivo*, and immature synapses tend to be more dynamic than mature synapses (Koleske, 2013). Live imaging in vivo in the cortex of developing 11-day-old (P11) mice revealed that dendritic spines can form and retract within two hours (Lendvai et al., 2000). Younger animals have higher dynamics of spine formation and retraction than older animals, although turnover does persist into the adult brain (Lendvai et al., 2000; Bhatt et al., 2009), supporting the notion that elevated dynamics of synaptic structure turnover are an important part of development. In fact, filopodia-like spines have a higher motility and are more likely to form transient contacts with presynaptic boutons, compared to spines with more "mature" structure, that are less likely to be motile and more likely to form more stable contacts with presynaptic boutons (Konur and Yuster, 2004). Presynaptic boutons turn over as well: in mature hippocampus, when there is no net change in synapse number, presynaptic boutons turn over on a scale of one to three days (De Paola et al., 2003). Dynamic changes in synapse structure and molecular composition are both an inherent feature of synapses, and an important aspect of synapse development.

Synapse development occurs on a dynamic backdrop. *Firstly*, synapse formation occurs concurrently with other major events of brain development, including neurogenesis and neurite elongation (Tau and Peterson, 2010). In some cases, synaptic partners are born at different times, and one mechanism of ensuring their ultimate connectivity is for earlier born neurons to synapse onto temporary synaptic partners to await their final postsynaptic targets, for example preplate neurons in the cortical plate provide a synaptic target for thalamic input in the time before layer 4 pyramidal neurons are born (Chao et al., 2009). Axons and dendrites are still elaborating as synapses are formed (Becker et al., 1984). Cajal-Retzius cells provide a synaptic target for entorhinal cortical input into the hippocampus in the time before pyramidal dendrites have elaborated into the stratum lacunosum moleculare (Chao et al., 2009). The relationship between synapse formation and the growing nervous system is bidirectional. Synapse formation reciprocally affects neurogenesis: immature synapses from parvalbumin-positive interneurons are an important survival cue for proliferating dentate gyrus neurons (Song et al., 2013). Synapse formation and activation also stabilizes developing axons and dendrites as they grow and branch, preventing their retraction (Alsina et al., 2001; Meyer and Smith, 2006; Rajan and Cline, 1998; Rajan et al., 1999; Li et al., 2000; Niell et al., 2004; Sin et al., 2002). Thus, there is a reciprocal relationship between "earlier" stages of development, such as neurogenesis and neurite growth, and synaptogenesis.

Secondly, the electrophysiological properties of the neural circuit change, which affects how developing synapses function within the network. In the mature brain, GABAergic signaling is inhibitory, but during early postnatal development, GABAergic signaling is excitatory, because of a shift in the intracellular chloride concentration at the end of the first week postnatally (Ben-Ari et al., 1997; Kerschensteiner, 2013). Another change in the developing neural network is in the distribution of glutamate receptor types at excitatory synapses: over the course of development there is an increase in the ratio of AMPA-type to NMDA-type glutamate receptors and within the subunits of the NDMA-type glutamate receptors themselves there is a gradual replacement of GluN2B subunits (which contains a C terminus CaMKII binding region) by GluN2A subunits (which do not have this C terminus region). The change in NMDA receptor subunit composition is critical to synapse development: prolonged overexpression of GluN2B prolongs spine motility, while premature expression of GluN2A decreases spine density and volume, and decreases spine motility (Gambrill and Barria, 2011). Interestingly, GluN2B has

opposite effects in synapse formation in excitatory and inhibitory neurons: GluN2B delays the formation of glutamatergic synapses onto excitatory neurons, but promotes excitatory synapse formation onto inhibitory synapses (Kelsch et al., 2014). The switch in NMDA-type glutamate receptor subunits may be an important mechanism contributing to balanced formation of excitatory and inhibitory tone in the neural network. The shift in GluN2B and GluN2A subunits also does not happen in all regions of the brain, but it is correlated with a shift in NMDA-type glutamate receptor coagonist: in the hippocampus, the switch occurs in the synapses between CA3 and CA1 pyramidal neurons, but not at the synapses from entorhinal cortical neurons onto dentate granule cells; there is a concurrent shift in NMDA-type glutamate receptor coagonists released in these regions – serine (preferentially used by GluN2A) starts to be expressed at the time of the subunit switch in the CA1, but glycine (preferentially used by GluN2B) remains released in the dentate gyrus (Le Bail et al., 2015). This suggests that there is co-regulation of presynaptic and postsynaptic aspects of the physiological change at glutamatergic synapses. Thus, the electrophysiological properties of synapses undergo important shifts during development that directly affect synapse development.

Thirdly, the neural network requires constant neural activity for its survival and appropriate development from its inception (Kerchenstein, 2013; West and Greenberg, 2011). Neural activity is generated by a combination of spontaneous waves of activity and sensory input into the neural circuit. Activity is important in driving many aspects of neural development. Deletion of a SNARE protein that is required for the exocytosis of synaptic vesicles, Munc18-1, does not impair the initial formation of brain circuits, but without neurotransmitter release, the neurons then progressively degenerated through apoptosis, suggesting that the maintenance of a functional brain requires constant activity and/or that neurotransmitters themselves are trophic (Verhage et al., 2000). Neuron depolarization controls gene expression: depolarization of dendrites induces an increase in intracellular calcium, which regulates the transcription factors NFAT, CREB, and NPAS4, and these in turn control both synapse formation and excitatory versus inhibitory synapse balance (West et al., 2002; West and Greenberg, 2011). Activity of the circuit and molecular control of synapse formation form a reciprocal relationship in controlling synapse development.

Thus, synaptogenesis is happening on the backdrop of a dynamically developing nervous system, with changes in the architecture of the neural circuit, the electrophysiological properties

of the circuit, and constant neural activity running through the circuit. Given the complexity of the process and of the final outcome, synapse formation requires strict molecular control to ensure appropriate assembly of synaptic machinery at the correct locations (Craig and Boudin, 2001; Shen and Cowan, 2010; Dabrowski and Umemori, 2011). Molecules exchanged between pre- and postsynaptic partners serve to promote the formation of synapses between appropriate synaptic partners and with appropriate synaptic machinery, ultimately leading to unique, specialized synapses in the brain. Molecules that are able to induce presynaptic or postsynaptic differentiation are called synaptic organizers, and simplistically can be categorized into cell adhesion molecules and secreted molecules.

Synaptogenic cell adhesion molecules include neurexins/neuroligins, SynCAMs, leucine-rich repeat proteins (LRRs), and some cadherins (reviewed in de Wit and Ghosh, 2014; Missler et al., 2012; Craig and Kang, 2007; Arikkath and Reichardt, 2008). The synaptogenic cell adhesion molecules are transmembrane proteins, with relatively short intracellular domains compared to their long, extracellular domains containing many "sticky" protein motifs. A general principal for their mechanism of action is transsynaptic binding – either homophilic (the same molecule is expressed both presynaptically and postsynaptically and binds to itself across the synapse, for example SynCAM1 or cadherins) or heterophilic (different molecules are expressed presynaptically and postsynaptically, for example neurexins, which are presynaptic, bind to neuroligins, which are postsynaptic). Transsynaptic binding allows recognition of synaptic partners: for example, specific sets of LRRs are expressed at distinct synapses in the hippocampus, such as excitatory projections from entorinal cortex versus CA3 onto CA1 pyramidal neurons or different subsets of inhibitory synapses in CA1 (de Wit and Ghosh, 2014; Tomioka et al., 2014). Cell adhesion molecules promote synapse maturation and stabilization: The cell adhesion molecule IgSF9/Dasm1 regulates inhibitory synapse development in the CA1 of the hippocampus, and does so purely through transsynaptic binding, independent of its cytosolic domain (Mishra et al., 2014). Postsynaptic calsyntenin3 (which belongs to the cadherin family) interacts transsynaptically with neurexin in interneurons to induce inhibitory synapses (Lu et al., 2014; Um et al., 2014), while calsyntenin1 is required postsynaptically at excitatory synapses to stabilize developing dendritic spines in CA1 (Ster et al., 2014). Loss of NCAM destabilizes dendritic spines (de Paola et al., 2003). Postsynaptic LAR-RPTP binds transsynaptically to Slitrk, which then clusters laterally within the presynaptic terminal to induce

presynaptic differentiation (Um et al., 2014), suggesting that one mechanisms of synapse differentiation driven by adhesion molecules is to form homomeric clusters. Cell adhesion molecules and synaptic density proteins reciprocally affect each other's synaptic localization – the postsynaptic density protein Dlg5 is required for dendritic spine formation by delivering N-Cadherin to the surface (Wang et al., 2014). Expression of synaptogenic cell-adhesion molecules drives distinct aspects of synapse maturation. The regulation of N-Cadherin expression is coordinated with synapse maturation by co-transport of N-Cadherin together with AMPA-type glutamate receptors through the scaffolding protein GRIP1, which promotes dendritic spine growth in hippocampal cultures (Heisler et al., 2014). Calsyntenin1 promotes the NMDA-type glutamate subunit switch from GluN2B to GluN2A in CA1 (Ster et al., 2014). Although many cell adhesion molecules promote synapse development through direct recruitment of synaptic proteins via protein-protein interactions, some transsynaptic cell adhesion molecules promote synapse differentiation through downstream signaling, for example Eph/Ephrin signaling, where transsynaptic binding of the Eph receptors activates their tyrosine kinase activity (Hruska and Dalva, 2012). Synaptogenic cell adhesion molecules are important for organizing presynaptic and postsynaptic densities, and for recognition of synaptic partners via direct binding in the extracellular matrix.

Secreted synaptic organizers include fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), Wnts, and Sonic hedgehog (Shh) (Dabrowski and Umemori, 2011; Johnson-Venkatesh and Umemori, 2010; Williams and Umemori, 2014, Harwell et al., 2012). Secreted molecules function through activating specific receptors that then induce presynaptic and/or postsynaptic assembly through signaling pathways. Secreted molecules can have both an autocrine (onto the same neuron it was secreted from) and paracrine (onto the neuron across the synaptic cleft) effect; secreted molecules can also function through numerous receptors to induce distinct aspects of synapse differentiation. Wnts (Wnt5a and Wnt7a) are important for both presynaptic and postsynaptic differentiation (Salinas, 2012). *In vivo*, in the cerebellum, Wnt7a is a retrograde signal required for presynaptic differentiation (including both synaptic vesicle accumulation and synaptic vesicle recycling) onto cerebellar granule cells; in the hippocampus Wnt7a induces dendritic spines in areas CA3 and CA1 (Hall et al., 2000; Ahmad-Annuar et al., 2006; Cerpa et al., 2008). In cultured hippocampal neurons, Wnt7a induces both presynaptic and postsynaptic differentiation at excitatory synapses through distinct mechanisms, and Wnt5a

induces excitatory presynaptic differentiation and both excitatory and inhibitory postsynaptic differentiation. Presynaptically, Wnts act through three receptors - two Frizzled receptors (which are G-protein coupled receptors) and Ror (a receptor tyrosine kinase receptor). The mechanisms of presynaptic differentiation (*in vitro* both synaptic vesicles and active zone proteins accumulate) are transcription-independent, and involve Dishevelled signaling (downstream of Frizzled receptors) and inhibition of Gsk3 signaling, although further mechanisms of synaptic vesicle and active zone recruitment are not yet known (Davis et al., 2008; Varela-Nallar et al., 2009; Sahores et al., 2010; Paganoni et al., 2010). Postsynaptically, Wnts utilize distinct signaling pathways from presynaptic pathways, and distinct signaling between excitatory and inhibitory postsynaptic differentiation: both Wnts induce calcium signaling in the dendrite, and additionally Wnt7a induces PSD-95 clustering at excitatory synapses through a mechanism involving Dishevelled and CaMKII; Wnt5a induces PSD-95 clustering through a mechanism involving JNK, but GABA receptor clustering at inhibitory synapses through CaMKII (Cuitino et al., 2010; Ciani et al., 2011; Farias et al., 2009). Thus, Wnts have a complex role in synapse development through utilization of many distinct signaling pathways. Another retrograde signal that acts through a still different set of signals to induce presynaptic differentiation is Shh, which is secreted by layer 5 cortical neurons to induce presynaptic differentiation at synapses from layer 2/3 cortical neurons (Harwell, 2012). Here, presynaptic differentiation does not require canonical Shh-induced Gli-dependent transcription, but instead the Shh co-receptor Boc is required, possibly utilizing Src kinase signaling.

Finally, FGFs, which will be the focus of the remaining chapters of this dissertation, induce synaptic vesicle accumulation in axon terminals (Umemori et al., 2004; Terauchi et al., 2010; Dabrowski and Umemori, 2011). There are twenty-two FGFs in mice and men, although only eighteen are secreted, these bind to seven FGF receptors (FGFRs), which are receptor tyrosine kinases (Ornitz and Itoh, 2015). Among the secreted FGFs, only a subset induces synaptic vesicle clustering *in vitro* in cultured neurons (Umemori et al., 2004), and of these the roles of FGF22 and FGF7 have been best demonstrated *in vivo*. FGF7, FGF10, and FGF22 were first identified as a presynaptic organizing molecule in the cerebellum and neuromuscular junction (Umemori et al., 2004, Fox et al., 2007). Interestingly, in the CA3 region of the hippocampus, FGF22 and FGF7 are both secreted from CA3 pyramidal neurons (Terauchi et al., 2010; Terauchi et al., 2015) but function at distinct axon terminals: FGF22 induces synaptic

vesicle accumulation at excitatory synapses, but FGF7 acts as a presynaptic organizer at inhibitory synapses (Terauchi et al., 2010). Thus, FGF22 and FGF7 provide synapse-specific differentiation cues (Figure 1.3). However, the mechanisms through which FGF22 and FGF7 induce specific presynaptic differentiation were unknown. Thus, there is still much to be learned about the distinct signaling pathways that induce presynaptic differentiation.

In order to limit synapse formation and prevent ectopic, oversized, and/or supernumerary synapses, there must also be negative regulation. Negative regulators during synapse development limit synapse formation or growth; additionally molecules that promote synapse elimination during later stages of brain development will prune away already formed synapses: loss of either negative regulators or molecules promoting synapse elimination will result in supernumerary or oversized synapses in the mature brain. One example of a negative regulator is a secreted semaphorin, Sema3F (Tran et al., 2009), which acts through a complex of the receptors neuropilin-2 and plexin-A3 (Tran et al., 2009), and the adhesion protein NrCAM (Demyanenko et al., 2014), to repress dendritic spine formation in subsets of neurons in the visual cortex and in the dentate gyrus. A transmembrane semaphorin, Sema5A, also negatively regulates dendritic spine density on dentate granule cells, cell-autonomously via binding the receptor plexin-A2 in *cis* (Duan et al., 2014). Similarly, the three mammalian Nogo receptors cooperate to repress dendritic spine density in hippocampal CA3 and CA1 *in vivo* (Wills et al., 2012). Slit1a negatively regulates synaptic vesicle accumulation, in part through its receptor Robo2, within axons of retinal ganglion cells in zebrafish (Campbell et al., 2007). Protocadherin 17 negatively regulates presynaptic differentiation in striatum at synapses from cortical neurons (Hoshina et al., 2013). Thus, multiple levels of negative regulation in a synapse-specific manner provide crucial signals for synapse development.

In addition to molecular cues exchanged between the presynaptic and postsynaptic neurons, the balance between specific synapse formation and broad coordination of synapse formation throughout the brain is also informed by cues from astrocytes and microglia. Because of their ability to sample broadly across populations of neurons by forming extensive contacts with thousands of neurons, astrocytes and microglia may provide an important mechanism of coordination of synapse development in parallel (Figure 1.1 C). The role of glia in synaptogenesis was first demonstrated by Barres and colleagues, when they demonstrated that neuronal media in which glia were first grown had a very strong synaptogenic effect in vitro
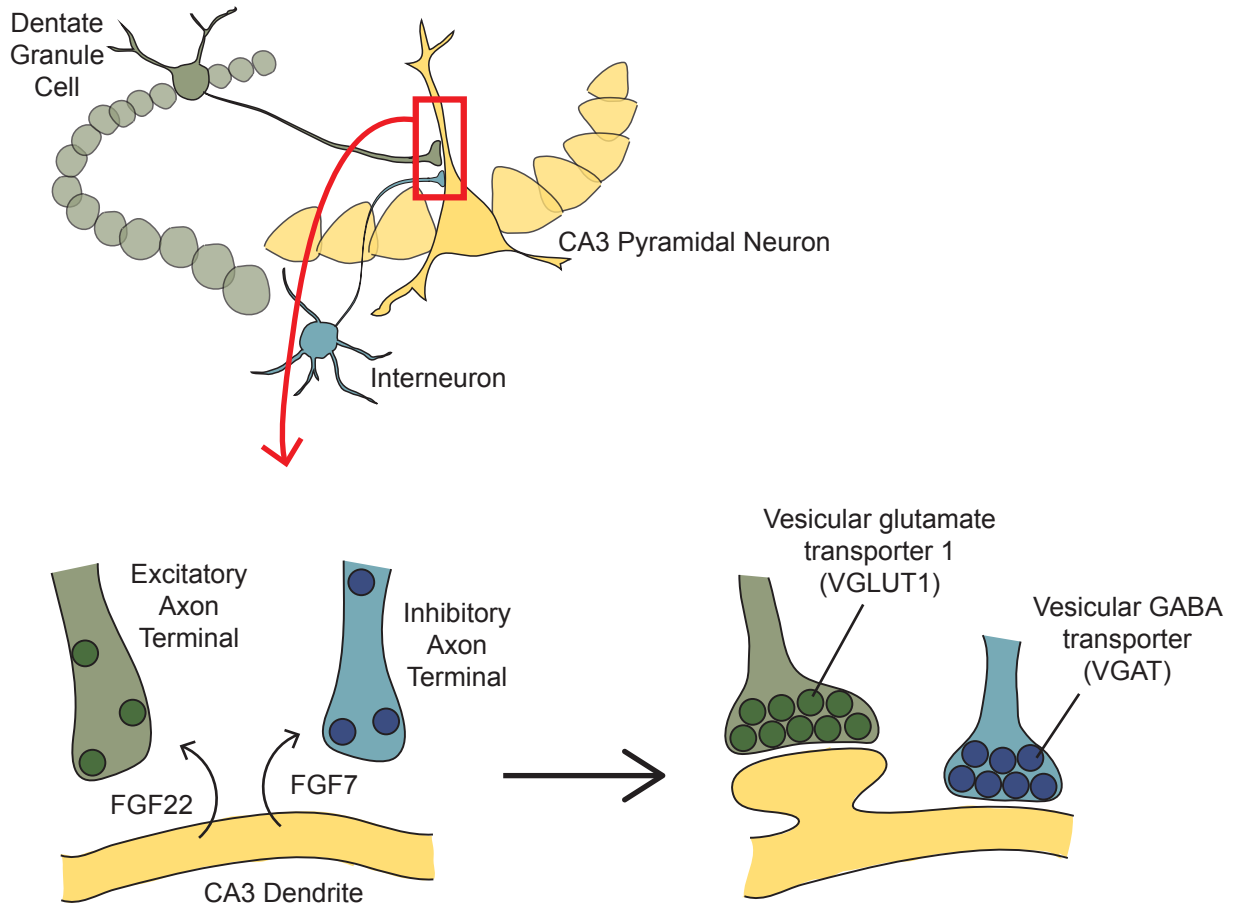
Figure 1.3. The role of FGF22 and FGF7 in presynaptic differentiation.
FGF22, secreted from CA3 dendrites, induces excitatory presynaptic differentiation. Excitatory synaptic vesicles can be visualized by detecing vesicular glutamate transporter 1 (VGLUT1). FGF7, secreted from CA3 dendrites, induces inhibitory presynaptic differentiation. Inhibitory synaptic vesicles can be visualized by detecting vesicular GABA transporter (VGAT). (Terauchi et al., 2010)

(Ullian et al., 2001). Since then, the role of astrocytes and microglia has been increasingly appreciated.

Astrocytes closely contact synapses, poised for their many roles in modulating synapse formation and function (Chung et al., 2015). Astrocytes promote both excitatory and inhibitory synaptogenesis through direct contact via the γ-protocadherins – loss of astrocytic expression of γ-protocadherin leads to lower excitatory and inhibitory synapse number embryonically that recovers in early postnatal development (Garrett and Weiner, 2009). Astrocytes also secrete numerous factors to induce synaptogenesis. Thrombospondins are secreted during early synapse development and induce excitatory synapse formation (Christopherson et al., 2005) through binding to the calcium channel subunit Cacna2d1 (Eroglu et al., 2009), although the synapses induced by thrombospondins are silent, and require further signaling for full maturation. TGF-β secreted by astrocytes onto nascent synapses induces increased excitatory synapse number through upregulating D-serine expression, which is a coagonist of NMDA receptors (Diniz et al., 2012). During later stages of development, between P14 and P25, hevin and SPARC are secreted from astrocytes to modulate excitatory synaptogenesis (Kucukdereli et al., 2011; Risher et al., 2014). Interestingly, hevin induces growth of excitatory presynaptic boutons, but SPARC directly antagonizes it: how relative hevin and SPARC expression is controlled is unknown. Furthermore, in cortical layer 2/3, hevin specifically promotes presynaptic maturation of inputs from the thalamus (VGLUT2-positive) over inputs from cortex (VGLUT1-positive) (Risher et al., 2014), suggesting a role of astrocytes in controlling synapse specificity amidst a population of neurons. Thus, astrocytes can use numerous mechanisms to coordinate synaptogenesis throughout development across a larger population of neurons that are in parallel neural circuits but not necessarily in direct contact with each other.

Microglia, once thought of as merely the macrophages of the central nervous system, also have important roles in synapse formation (reviewed in Salter and Beggs, 2014; Ueno and Yamashita, 2014). Microglia directly contact synapses in a highly dynamic manner (Tremblay et al., 2010). Microglia express trophic signals such as IGF-1 (Ueno et al., 2013) that may have important instructive roles in synapse maturation. Indeed, loss of CX3CR1 receptor in microglia leads to delayed maturation of excitatory thalamocortical synapse maturation in barrel cortex – the synapses have a delay in the increase of AMPA-type to NMDA-type glutamate receptor ratio and GluN2B to NR2A subunit switch (Hoshiko et al., 2012). Similarly, loss of the

transmembrane polypeptide DAP12 in microglia causes a failure in the functional glutamate receptor rearrangement in maturing synapses in hippocampus (Roumier et al., 2004). Furthermore, microglia have an important role in later stages of excitatory synapse maturation, indirectly, through controlling synapse elimination by direct engulfment of eliminated synapses through a mechanism co-opting the immune system complement signaling (Stevens et al., 2007; Paolicelli et al., 2011; Bialas and Stevens, 2013; Chu et al., 2010). Thus microglia, like astrocytes, have the ability to coordinate synapse formation across populations of neurons. Whereas astrocytes appear to control synapse formation throughout development, and especially in early stages of synaptogenesis, microglia appear to have a role predominantly in coordinating later steps of synapse maturation, such as functional glutamate receptor rearrangements and synapse elimination.

Synapses are essential to how information is processed in the brain; therefore it is intuitively obvious that defective synapse formation leads to neuropsychiatric disease (Williams and Umemori, 2014; Melom and Littleton, 2011; Spronsen and Hoogenraad, 2010; Blanpied and Ehlers, 2004; Zoghbi, 2003). There are many ways in which failure of synapse development can lead to disease: failure to form connections between the correct synaptic partners (or formation of connections between inappropriate synaptic partners) can lead to an alteration in the areas of the brain that are connected resulting in altered information processing (which is thought to underlie both schizophrenia and autism); similarly, failure to form fully functional synapses can cause the right connections to form but with inappropriate synaptic weight, leading to failures in information propagation (which can underlie schizophrenia, autism, mental retardation or epilepsy); failure of synapses to acquire the ability to have changes in synaptic plasticity can lead to altered learning (which is thought to be a contributing factor to addiction); and failure of synapses to maintain healthy structure throughout life can lead to neurodegeneration (for example in Alzheimer's disease). Many of the neurodevelopmental disorders, especially mental retardation, autism, epilepsy, and schizophrenia, appear to have overlapping etiologies, and sometimes even co-occur in patients (Guilmatre et al., 2009). Autism incidence overlaps with both mental retardation and epilepsy, especially in certain syndromic causes of autism, such as Tuberous Sclerosis, Fragile X Syndrome, or Rett Syndrome (Philips and Pozzo-Miller, 2015; Tuchman and Rapin, 2002). Schizophrenia can overlap with epilepsy, both due to common circuits being engaged in epilepsy-associated psychosis, but also due to overlapping genetic

causes (Cascella et al., 2009). The broad disease phenotypes, the overlaps between the neuropsychiatric diseases, and the multiple genetic and developmental routes underlying neuropsychiatric diseases reflect the wonderful complexity and terrifying delicacy of neural circuit formation.

There are four lines of evidence for defects in synapse pathology leading to neuropsychiatric disorders: 1) disease time course; 2) synaptic changes found in human patients; 3) genetic studies identifying synapse-related genes in human patients; and 4) animal models with mutated or deleted synaptic genes. The time course of emergence of such diseases as autism or schizophrenia in humans coincides with important events in synapse development (Andersen, 2003; Rubenstein, 2011). Autism is a developmental disorder characterized by impaired social skills and repetitive behaviors and typically manifests around two years of age after overtly normal infantile development, at the time corresponding to the peak of synapse number. In fact, children with autistic brains appear to have initially smaller brains at birth that then grow to be larger than average (Courchesne et al., 2003). Schizophrenia, a psychiatric disease that includes symptoms such as delusions, hallucinations, and disorganized thinking, typically manifests in late adolescence and early adulthood. In a theory first put forth by Feinberg (Feinberg, 1983), the onset of schizophrenia is thought to be linked to aberrant pruning of synapses and aberrant neural circuit refinement. Anatomical findings in post-mortem tissues from affected human patients also point to defects in synapses. Using dendritic spines (the excitatory postsynaptic dendritic protrusion) as a morphological readout, investigators found changes both in autistic and schizophrenic brains. In syndromes linked to autism, there are changes in dendritic spine morphology and density, but the changes differ between syndromes – in Tuberous Sclerosis there is an increased density in dendritic spines, in Fragile-X spine density is not changed but the synapses stay thin and filopodia persist without maturing, and in Rett and Angelman syndromes spine density is decreased and spines are less mature (Phillips and Pozzo-Miller, 2015). In schizophrenic brains, density of dendritic spines appears to decrease specifically on cortical layer 3 pyramidal neurons, suggesting defective connectivity within a subset of circuits (Moyer et al., 2014). Numerous studies have probed the genetic underpinning of neuropsychiatric diseases and found that networks of numerous synaptic proteins and proteins that are important for synapse formation are perturbed in autism (De Rubeis et al., 2014), schizophrenia (Mirnics et al., 2000; Mirnics et al., 2001; Nascimento and Martins-de-Souza, 2015), and epilepsy (Noebels, 2015).

Variations in FGFR2, which will be discussed in the remaining chapters, have been linked to autism, major depressive disorder, and Pfeiffer syndrome, which includes intellectual delay and seizures (De Rubeis et al., 2014; Williams and Umemori, 2014). Behavioral and morphological phenotypes in animal in which synapse-formation genes are knocked out lend further support to the importance of synapse formation in appropriate brain function (Williams and Umemori, 2014; Grone and Baraban, 2015; Nestler and Hyman, 2010). Mice lacking FGF7 or FGF22 have disrupted excitatory and inhibitory balance leading to altered susceptibility to seizure induction, which will be discussed in later chapters. Thus, there is strong evidence that aberrant synapse development can lead to neuropsychiatric disease. Targeting synaptic function and the overall neural circuitry has potential for therapeutic benefits in neuropsychiatric disease. Indeed, many neurological and psychiatric drugs target the synapse (Miyamoto et al., 2012; Rudolph and Konflach, 2011; Schousboe et al., 2014). The future holds great promise of new therapies, such as genetically reprogramming stem cells derived from patients and reintroducing them into diseased brain in extreme cases of otherwise unmanageable neurological disease (Parent and Anderson, 2015), and there is still a great need for more precise and effective therapies.

In this thesis, I will describe my work on elucidating the mechanisms through which FGF22 and FGF7 form a balanced hippocampal network. I will first demonstrate the receptors through which FGF22 induces excitatory presynaptic differentiation and FGF7 inhibitory presynaptic differentiation (Chapter 2); I will then describe the functional localization of the receptors to the presynaptic neuron and the signaling pathways through which FGFRs induce presynaptic differention (Chapter 3). Finally, in Chapter 4, I will describe a new line of experiments addressing FGF-induced gene expression and the potential role of synaptogenic FGFs in coordinating synapse development across a neuron (see Figure 1.1 D). This is an important contribution to understanding the mechanisms synapse formation, with implications for understanding how synapse development goes amiss leading to neurological disease.

CHAPTER II

Identification of Synaptogenic FGF Receptors

The following work is in press for publication in the journal Development *as: Dabrowki, A., Terauchi, A., Strong, C., and Umemori, H. (2015) Distinct sets of FGF receptors sculpt excitatory and inhibitory synaptogenesis.*

Introduction.

Neurons of the developing brain must form a network of excitatory and inhibitory synapses in a highly organized fashion, which requires differential molecular control between each synapse type (Chia et al., 2013; Dabrowski and Umemori, 2011; Jin and Garner, 2008; Johnson-Venkatesh and Umemori, 2010; McAllister, 2007; Sanes and Yamagata, 2009; Shen and Scheiffele, 2010; Siddiqui and Craig, 2011; Williams and Umemori, 2014; Williams et al., 2010). An imbalance between excitatory and inhibitory drive in the brain may lead to various neurological and psychiatric diseases including epilepsy, autism, and schizophrenia (Lisman, 2012; Southwell et al., 2014). Thus, the components of molecular signaling mediating synapse formation are key factors in the vulnerability or resilience to such disorders. Hippocampal circuits are critical to memory formation, emotional processing, and social behavior. The hippocampal circuits consist of highly organized synaptic connections, in which excitatory projections relay from the entorhinal cortex (EC) to the dentate gyrus (DG) to CA3 to CA1 to the EC, and are balanced by inhibitory inputs from local interneurons. In the hippocampus, two

fibroblast growth factors (FGFs), FGF22 and FGF7, are secreted from dendrites of CA3 pyramidal neurons and promote presynaptic differentiation of excitatory and inhibitory synapses, respectively (Terauchi et al., 2010). That this FGF-dependent regulation of excitatory and inhibitory presynaptic differentiation is important for proper brain development is evidenced by altered seizure susceptibility of knockout (KO) animals: Fgf22-KO mice have decreased, and Fgf7-KO mice increased susceptibility to epileptic seizures (Terauchi et al., 2010; Lee et al., 2012; Lee and Umemori, 2013). However, the signaling mechanisms underlying FGF-dependent synapse formation are not understood. Understanding the mechanisms through which FGF22 and FGF7 specifically promote excitatory and inhibitory presynaptic differentiation will give an important insight into how molecular signaling tunes discrete synapse formation.

FGFs regulate a variety of processes, including cell proliferation, migration, differentiation, tissue repair, and response to injury in almost all organs (Thisse and Thisse, 2005; Turner and Grose, 2010; Ornitz and Itoh, 2001). In the nervous system, FGF signaling is important in various steps of development including neural induction, patterning, proliferation, axon guidance, and synapse formation (Guillemot and Zimmer, 2011; Jones and Basson, 2010; Mason, 2007; Stevens et al., 2010a; Umemori, 2009). For the eighteen secreted FGFs, there are four FGF receptors (FGFRs), which, through b and c splice variants, encode seven distinct receptor tyrosine kinases (Chellaiah et al., 1994; Johnson et al., 1991; Ornitz et al., 1996; Belov and Mohammadi, 2013; Powers et al., 2000). Once FGFRs are activated, they recruit and/or phosphorylate a number of signaling molecules including fibroblast growth factor receptor substrate 2 (FRS2), phosphoinositide 3-kinase (PI3K), and phospholipase C γ (PLCγ), which result in the activation of downstream pathways (Mohammadi et al., 1996; Furdui et al., 2006; Bae et al., 2009; Lemmon and Schlessinger, 2010). Therefore, the cellular responses to FGFs are regulated at multiple levels by a number of different factors. What is remarkable in FGF-dependent synapse formation is that FGF22 and FGF7 are secreted from the same CA3 pyramidal neurons but specifically organize excitatory or inhibitory presynaptic differentiation. Important questions to ask regarding their specific effects include the identity of the receptors, location of their action, and the signaling pathways involved. Understanding the precise FGF signaling mechanisms during excitatory and inhibitory synapse development will not only

further our understanding of synaptogenesis, but will also yield more insight into how FGFs dictate cellular outcomes with such remarkable specificity.

In this chapter, I will address the following important questions regarding the mechanisms of FGF-induced excitatory and inhibitory synapse formation: What are the receptors through which FGF22 and FGF7 mediate their synaptogenic effects? Using multiple null and conditional knockout mice and primary cultures prepared from them, I found that distinct but overlapping sets of FGFRs mediate excitatory versus inhibitory synapse differentiation. My work uncovers mechanisms of FGF-induced specific presynaptic differentiation and contributes to a profound understanding of how appropriate neural networks are established during development in the mammalian brain.

<u>Methods</u>.

Mouse strains.

Fgfr1b mutant mice (Partanen et al., 1998) were from Juha Partanen. Fgfr2b mutant mice (Fox et al., 2007), Fgfr2$^{flox/flox}$ mice (Yu et al. 2003; Umemori et al., 2004), Fgfr1$^{flox/flox}$ mice (Hoch and Soriano, 2006) and Actin-CreER mice (Guo et al., 2002; Umemori et al., 2004) were described previously. All mice were on a C57BL/6 background, except Fgfr1b-/- mice, which were on a mixed 129sv/CD-1 background. WT mice used were either C57BL/6 or ICR/CD-1. All animal care and use was in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital and University of Michigan.

Neuronal culture.

For FGF responsiveness assays, hippocampi were dissected from P0 pups or E18-E19 embryos, dissociated with 0.5% Trypsin, and grown in culture media [B27 (Gibco), 2 mM L-glutamine, 1x Penicillin-Streptomycin in Neurobasal (Gibco)] at a density of 36,000 cells/poly-D-lysine-coated coverslip. FGF22 (R&D) and FGF7 (PeproTech) were dissolved to 2 nM for responsiveness experiments.

Immunostaining.

P8 animals were sacrificed by decapitation (Figs 1-2) or perfusion with 4%PFA (Fig. 3). Brains were fixed in 4% PFA overnight. 20 μm sagittal sections were cryosectioned. Cultured neurons were fixed with 100% methanol, -20°C for 5 minutes. Antibodies and dilutions: VGLUT1 (1:4000, Millipore), VGAT (1:4000, Synaptic Systems), Py (1:25, gift from M. Webb and P.L. Woodhams). Mounting media: glycerol with p-phenylenediamine or n-propyl gallate.

Image acquisition and analysis.

12-bit images were acquired with epifluorescence microscopes (Olympus) using 20x and 40x lenses with F-View II CCD (Soft Imaging System) and XM10 (Olympus) cameras at 1,376 x 1,032 pixels resolution. Section data were analyzed using MetaMorph software. For each image, a threshold was chosen to exclude signal from background, based on intensity of the fimbria, a myelinated tract of axons exiting CA3 medially. For analysis of VGLUT1 or VGAT puncta on Py-positive dendrites, images were merged using ImageJ and Adobe Photoshop in 16-bit, and masks were drawn in Adobe Photoshop over Py-positive dendrites. Image thresholds were chosen to subtract background staining and separate each punctum. The threshold was then subtracted from the intensity measurement. Dendritic lengths were measured manually by tracing the length in MetaMorph. The statistical tests performed were two-tailed Student's *t*-test. All data are expressed as mean ± s.e.m.

Results.

Excitatory presynaptic differentiation is impaired in Fgfr2b and Fgfr1b mutant mice

I first sought to identify the FGF receptors (FGFRs) involved in presynaptic differentiation by investigating whether knockout (KO) mice of candidate FGFRs show similar synaptic deficits observed in Fgf22-KO or Fgf7-KO mice. I focused on FGFR2b and FGFR1b because in mitogenesis assays with FGFR-expressing BaF3 cells, FGF22 activates FGFR2b and FGFR1b, and FGF7 activates FGFR2b (Zhang et al., 2006). Fgfr2 and Fgfr1b are expressed in excitatory neurons (Terauchi et al., 2010, Beer et al., 2000) and inhibitory interneurons in the hippocampus (Figure 2.1). To investigate excitatory presynaptic differentiation in mice lacking

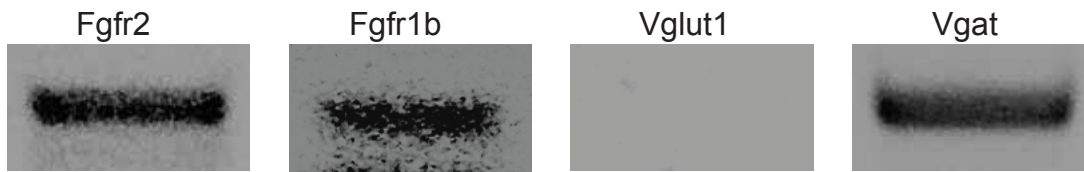**Figure 2.1. Expression of Fgfr2 and Fgfr1b in CA3 interneurons.** Interneurons were isolated using fluorescence-activated cell sorting from the CA3 area dissected from P14 Vgat-Venus mice (Wang et al., 2009), followed by mRNA isolation and cDNA synthesis. PCR amplification using specific primers demonstrates the presence of Fgfr2, Fgfr1b, and Slc32a1 (Vgat) transcripts, but not Slc17a7 (Vglut1) transcripts, in the CA3 interneurons.

Fgfr2b or Fgfr1b, I stained brain sections for vesicular glutamate transporter 1 (VGLUT1), a marker of excitatory synaptic vesicles that cluster at nerve terminals as excitatory synapses develop (Figure 2.2). (This work was done together with Cameron Strong.) Fgfr2b-/- mice (Fig. 1A) are not viable postnatally due to lung agenesis, while Fgfr2b+/- animals, though viable, have developmental defects such as stunted seminal vesicle development (Kuslak et al., 2007) and hypoplastic submandibular glands (Jaskoll et al., 2005). Hence, I investigated whether Fgfr2b haploinsufficiency affects excitatory presynaptic differentiation. I focused our analysis in the CA3 of the hippocampus, because Fgf22-KO mice show specific defects in excitatory presynaptic differentiation in the CA3 (Terauchi et al., 2010). I found that Fgfr2b+/- mice have decreased intensity of VGLUT1 immunoreactivity in the stratum radiatum (SR) and stratum lucidum (SL) of hippocampal CA3 at postnatal day 8 (P8), an early stage of synapse formation (Figure 2.2 B,C). Further analysis revealed that VGLUT1 puncta are smaller and sparser in Fgfr2b+/- mice compared to wild-type (WT) littermate (Figure 2.2 D). I next investigated Fgfr1b-/- mice (Figure 2.2 E) for excitatory presynaptic differentiation. I found that VGLUT1 intensity is decreased both in the SL and SR layers of the hippocampal CA3 (Figure 2.2 F, G) and that VGLUT1 puncta are smaller and sparser in Fgfr1b-/- animals (Figure 2.2 H). I did not observe appreciable changes in postsynaptic differentiation, assessed by PSD95 immunostaining (an excitatory postsynaptic marker), in either Fgfr2b+/- or Fgfr1b-/- mice (Figure 2.3). Furthermore, VGLUT1 immunostaining in lateral septal nuclei does not perceptibly change in Fgfr mutant mice, suggesting that loss of Fgfr2b or Fgfr1b is not globally decreasing VGLUT1 levels (Figure 2.4). These data suggest that both FGFR2b and FGFR1b are involved in excitatory presynaptic differentiation in the hippocampal CA3. To determine whether FGFR2b and FGFR1b compensate for each other's function for excitatory presynaptic differentiation, I investigated the effect of Fgfr1b inactivation on an Fgfr2b+/- background. I found that Fgfr2b+/- Fgfr1b+/- (double heterozygotes) do not augment the loss of VGLUT1 staining in Fgfr2b+/- mice (Figure 2.2 I, J), suggesting that there is no apparent redundancy between FGFR2b and FGFR1b on excitatory presynaptic differentiation.

Figure 2.2. *(Following page)* **Loss of Fgfr2b or Fgfr1b results in decreased excitatory presynaptic differentiation in hippocampal CA3 in vivo.**

(A) Schematic of Fgfr2b isoform-specific deletion: stop codon introduced into Fgfr2 exon 8 leads to loss of Fgfr2b, but not -c splice isoforms.

(B-D) Decreased excitatory presynaptic differentiation (VGLUT1 immunostaining) in hippocampal CA3 of Fgfr2b+/- mice compared to WT at P8. (B) Representative images. SL: stratum lucidum, SR: stratum radiatum. (C) Quantification of VGLUT1 intensity, normalized to WT littermates. (D) VGLUT1 puncta size and density in SL. (n=[sections, animals] 77, 5 Fgfr2b+/-; 55, 3 WT)

(E) Schematic of Fgfr1b isoform-specific deletion: stop codon introduced into Fgfr1 exon 6 leads to loss of FGFR1b, but not -c splice isoforms.

(F-H) Decreased excitatory presynaptic differentiation (VGLUT1 immunostaining) in hippocampal CA3 of Fgfr1b-/- mice compared to WT at P8. (F) Representative images. (G) Quantification of VGLUT1 intensity, normalized to WT littermates. (H) VGLUT1 puncta size and density in SL. (n=[sections, animals] 76, 7 Fgfr1b-/-; 87, 7 WT)

(I-J) Loss of one copy of Fgfr1b does not exacerbate excitatory presynaptic defects in Fgfr2b+/- mice. (I) Representative images. (J) VGLUT1 intensity in SR, relative to WT littermates. (n=[sections, animals] 15, 5 Fgfr2b+/-Fgfr1b+/-; 17, 5 Fgfr2b+/-; 20, 5 Fgfr1b+/-; 24, 6 WT)

***p<0.001,**p<0.01, *p<0.05, "ns" not significant by Student's t test. Scale bars, 15 μm.

A Fgfr2 — Exon 7 | b (8) | c (9) — STOP

B VGLUT1 — WT (SR/SL), Fgfr2b⁺/⁻ (SR/SL)

C VGLUT1 Intensity (% of WT) — SL *** , SR *** — WT, 2b +/-

D VGLUT1 Size (μm²) *** ; VGLUT1 Density (#/mm²) *** — WT, 2b +/-

E Fgfr1 — Exon 5 | b (6) | c (7) — STOP

F VGLUT1 — WT (SR/SL), Fgfr1b⁻/⁻ (SR/SL)

G VGLUT1 Intensity (% of WT) — SL *** , SR *** — WT, 1b -/-

H VGLUT1 Size (μm²) *** ; VGLUT1 Density (#/mm²) ** — WT, 1b -/-

I VGLUT1 — WT (SR), Fgfr1b⁺/⁻ (SR), Fgfr2b⁺/⁻ (SR), Fgfr2b⁺/⁻ Fgfr1b⁺/⁻ (SR)

J VGLUT Intensity in SR (% of WT) — ** ; ns ; ns — WT, 1b +/-, 2b +/-, 2b +/- 1b +/-

26
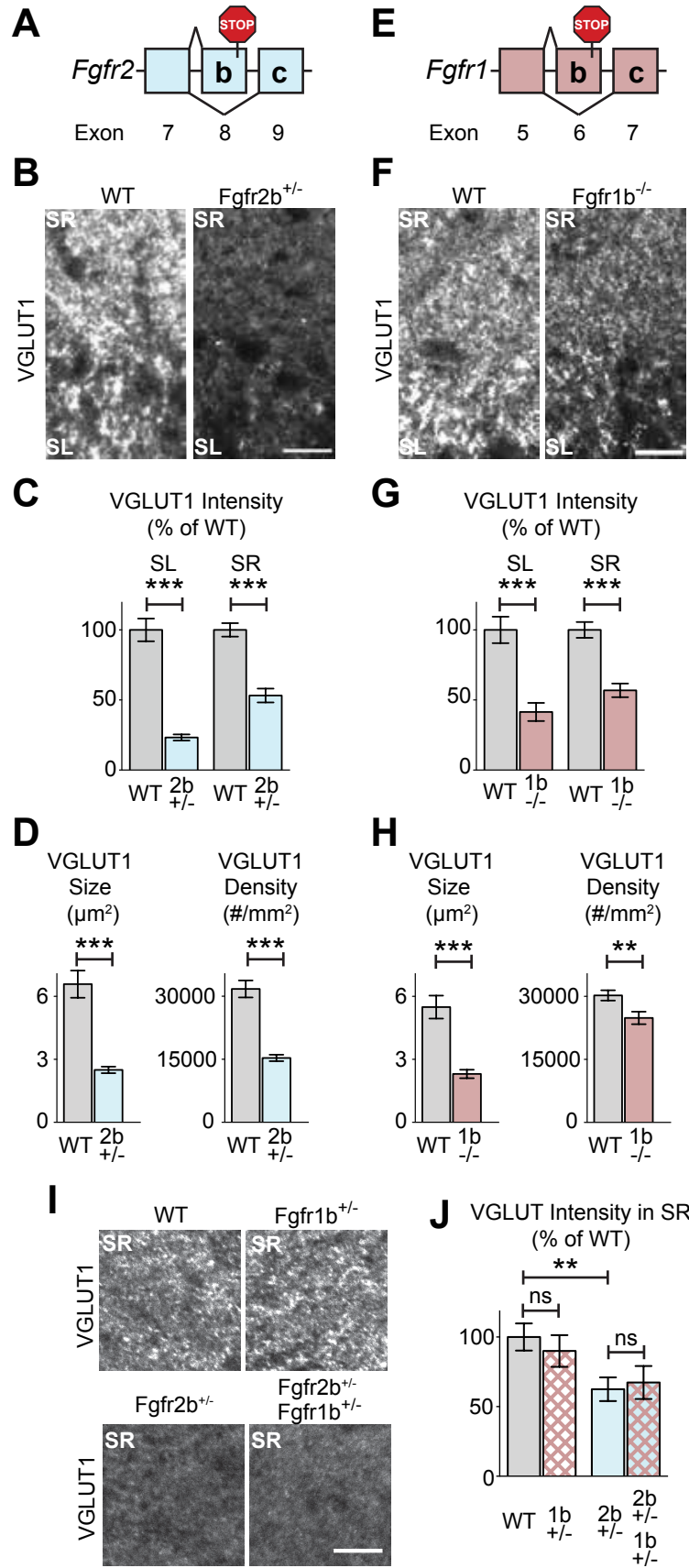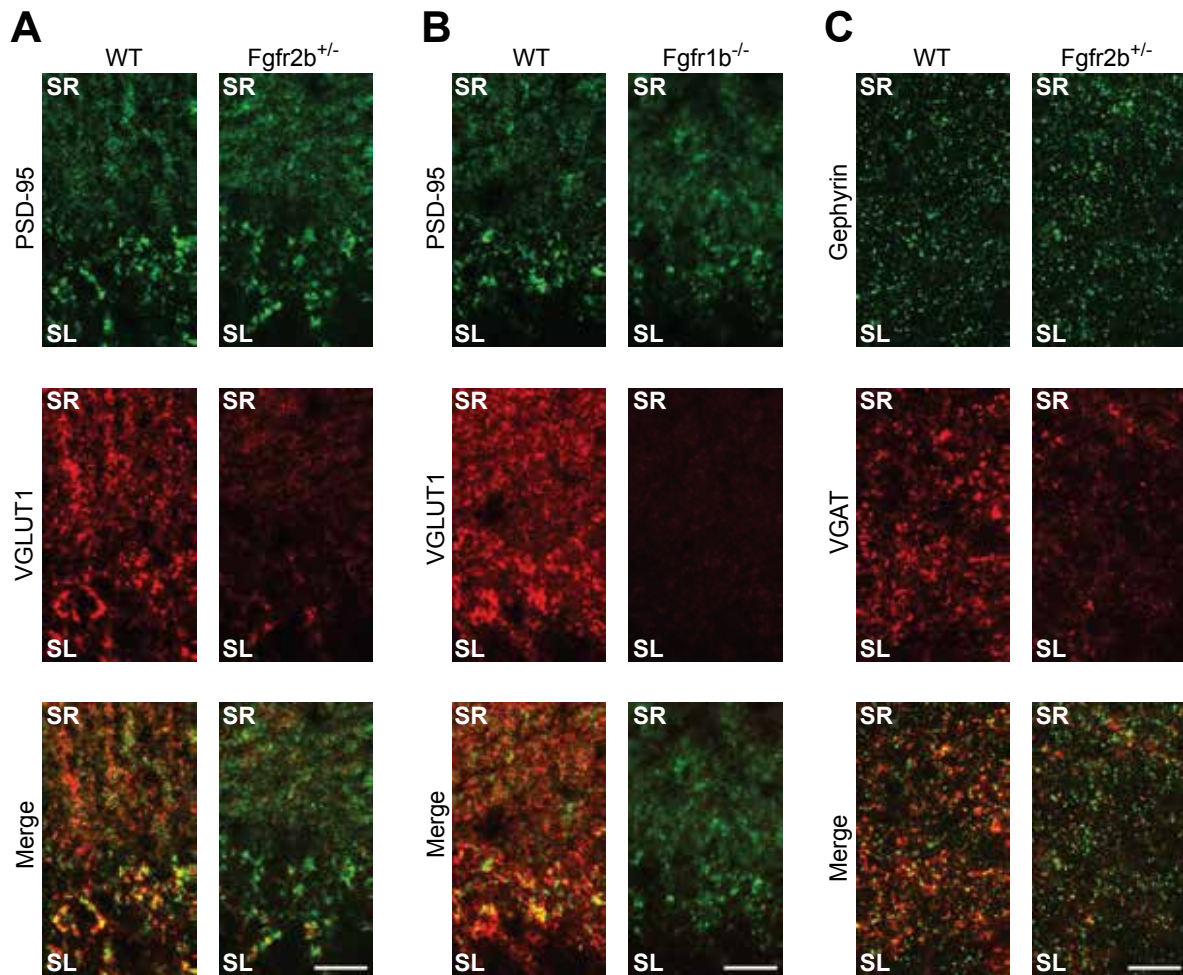
Figure 2.3. Postsynaptic differentiation is not affected by Fgfr deletion.
(A) Representative images showing that Fgfr2b+/- mice at P8 do not exhibit changes in the clustering of PSD-95, a postsynaptic scaffolding protein at excitatory synapses, despite a decrease in VGLUT1 signal. (B) Representative images showing that Fgfr1b-/- mice at P8 do not exhibit changes in PSD-95 clustering, despite decreased VGLUT1 signal.
(C) Representative images showing that Fgfr2b+/- mice do not exhibit changes in the clustering of Gephyrin, a postsynaptic scaffolding protein at inhibitory synapses, despite decreased VGAT signal. Scale bars, 15 µm.
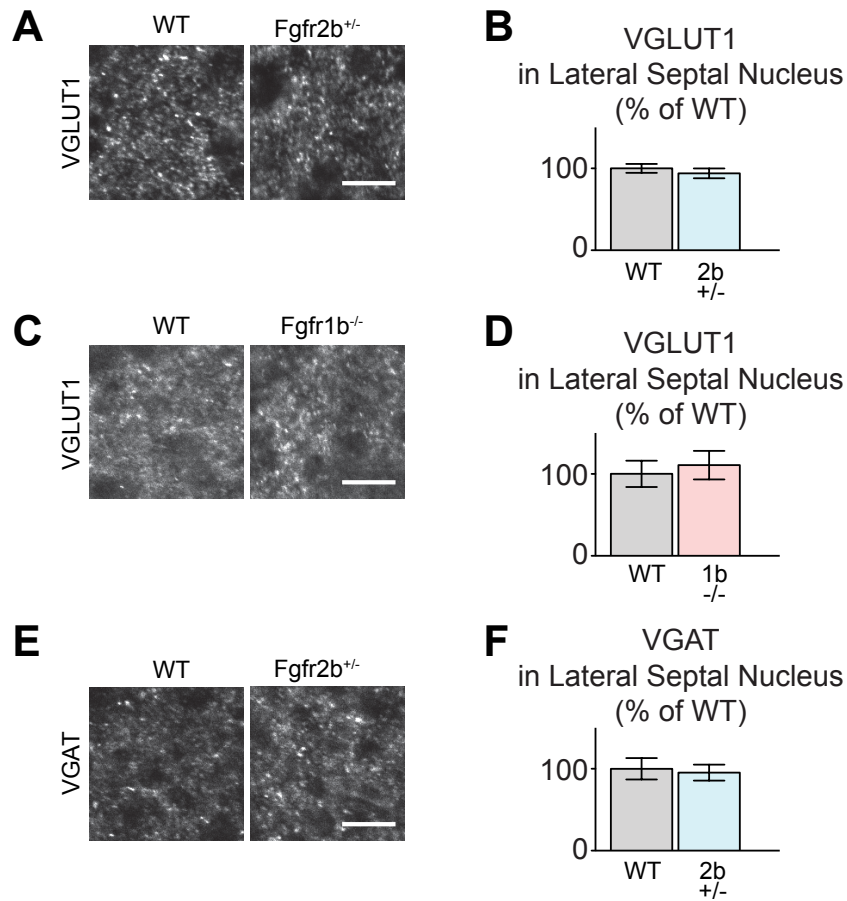
Figure 2.4. Presynaptic differentiation is not impaired in lateral septal nuclei of Fgfr-KO mice.
(A-B) Fgfr2b+/- mice at P8 have no change in the VGLUT1 staining intensity in the lateral septal nucleus: (A) representative images, (B) quantification of intensity normalized to WT littermates. (C-D) Fgfr1b-/- mice at P8 have no change in the VGLUT1 staining intensity in the lateral septal nucleus: (C) representative images, (D) quantification of intensity normalized to WT littermates. (E-F) Fgfr2b+/- mice at P8 have no change in the VGAT staining intensity in the lateral septal nucleus: (E) representative images, (F) quantification of intensity normalized to WT littermates. Data represents mean ± s.e.m. Scale bars, 15 µm.

Inhibitory presynaptic differentiation is impaired in Fgfr2b but not Fgfr1b mutants

I next investigated inhibitory presynaptic differentiation in Fgfr2b+/- and Fgfr1b-/- mice by analyzing vesicular GABA transporter (VGAT) immunostaining to detect the clustering of inhibitory synaptic vesicles (Figure 2.5). (This work was done together with Cameron Strong.) I found that Fgfr2b+/- mice have decreased VGAT intensity in SL and SR of hippocampal CA3 at P8 (Figure 2.5 A, B), resembling inhibitory presynaptic defects in Fgf7-KO mice (Terauchi et al., 2010). Further analysis showed that VGAT puncta in Fgfr2b+/- mice are smaller and sparser compared to WT littermates in SL (Figure 2.5 C). In contrast, Fgfr1b-/- animals do not have defects in inhibitory presynaptic differentiation measured by VGAT intensity, or puncta size or density (Figure 2.5 D-F). Even on an Fgfr2b+/- background, which has quantitatively lower levels of FGFR-mediated signaling, additional loss of a copy of Fgfr1b (double heterozygotes) does not change the level of inhibitory presynaptic defects relative to Fgfr2b+/- (Figure 2.5 G, H). This suggests that synapse formation is not simply regulated by the levels of FGFR signaling, but rather FGFR2b specifically is required for inhibitory presynaptic differentiation. I did not observe appreciable changes in Gephyrin immunostaining (an inhibitory postsynaptic marker) in Fgfr2b+/- mice (Figure 2.3). VGAT immunostaining in lateral septal nuclei does not perceptibly change in Fgfr2b+/- mice (Figure 2.4). These results suggest that FGFR2b, but not FGFR1b, contributes to inhibitory presynaptic differentiation in hippocampal CA3.

FGFRs are required during the postnatal stage of presynaptic differentiation *in vivo*

FGFR2 and FGFR1 also regulate embryonic neuronal development, including neuronal precursor specification and proliferation (Paek et al., 2009; Gutin et al., 2006; Stevens et al., 2010b). To exclude the influence of embryonic events to presynaptic defects in Fgfr2b+/- and Fgfr1b-/- mice, we utilized conditional Fgfr-KO mice and asked whether the receptors are necessary during the postnatal synaptogenic stage. (This work was done by Dr. Akiko Terauchi.) Fgfr2 or Fgfr1 was selectively deleted in a temporally controlled manner by crossing mice possessing tamoxifen-inducible Cre (CreER) under the Actin promoter (Guo et al., 2002) with mice with a conditional allele of Fgfr2 (Fgfr2^{flox}) (Figure 2.6 A; Yu et al., 2003) or Fgfr1 (Fgfr1^{flox}) (Figure 2.6 B; Hoch and Soriano, 2006). Tamoxifen injection (100 μg) efficiently

Figure 2-5. *(Following page)* **Loss of Fgfr2b, but not Fgfr1b, results in decreased inhibitory presynaptic differentiation in hippocampal CA3 in vivo.**

(A-C) Decreased inhibitory presynaptic differentiation (VGAT immunostaining) in CA3 of Fgfr2b+/- mice compared to WT at P8. (A) Representative images. (B) Quantification of VGAT intensity, normalized to WT littermates. (C) VGAT puncta size and density in SL. (n=[sections, animals] 77, 5 Fgfr2b+/-; 52, 3 WT)

(D-F) No change in inhibitory presynaptic differentiation (VGAT immunostaining) in hippocampal CA3 of Fgfr1b-/- mice compared to WT at P8. (D) Representative images. (E) Quantification of VGAT intensity, normalized to WT littermates. (F) VGAT size and density in SL. (n=[sections, animals] 71, 6 Fgfr1b-/-; 71, 6 WT)

(G-H) Loss of one copy of Fgfr1b does not exacerbate inhibitory presynaptic defects in Fgfr2b+/- mice. (G) Representative images. (H) VGAT intensity, relative to WT littermates. (n=[sections, animals] 15, 5 Fgfr2b+/-Fgfr1b+/-; 14, 5 Fgfr2b+/-; 12, 4 WT)

Scale bars, 15 μm.

A

WT   Fgfr2b$^{+/-}$

D

WT   Fgfr1b$^{-/-}$

B
VGAT Intensity
(% of WT)

E
VGAT Intensity
(% of WT)

C
VGAT
Size
(µm^2)   VGAT
Density
(#/mm^2)

F
VGAT
Size
(µm^2)   VGAT
Density
(#/mm^2)

G
Fgfr2b$^{+/-}$ Fgfr2b$^{+/-}$
Fgfr1b$^{+/-}$

H
VGAT Intensity
(% of WT)

31
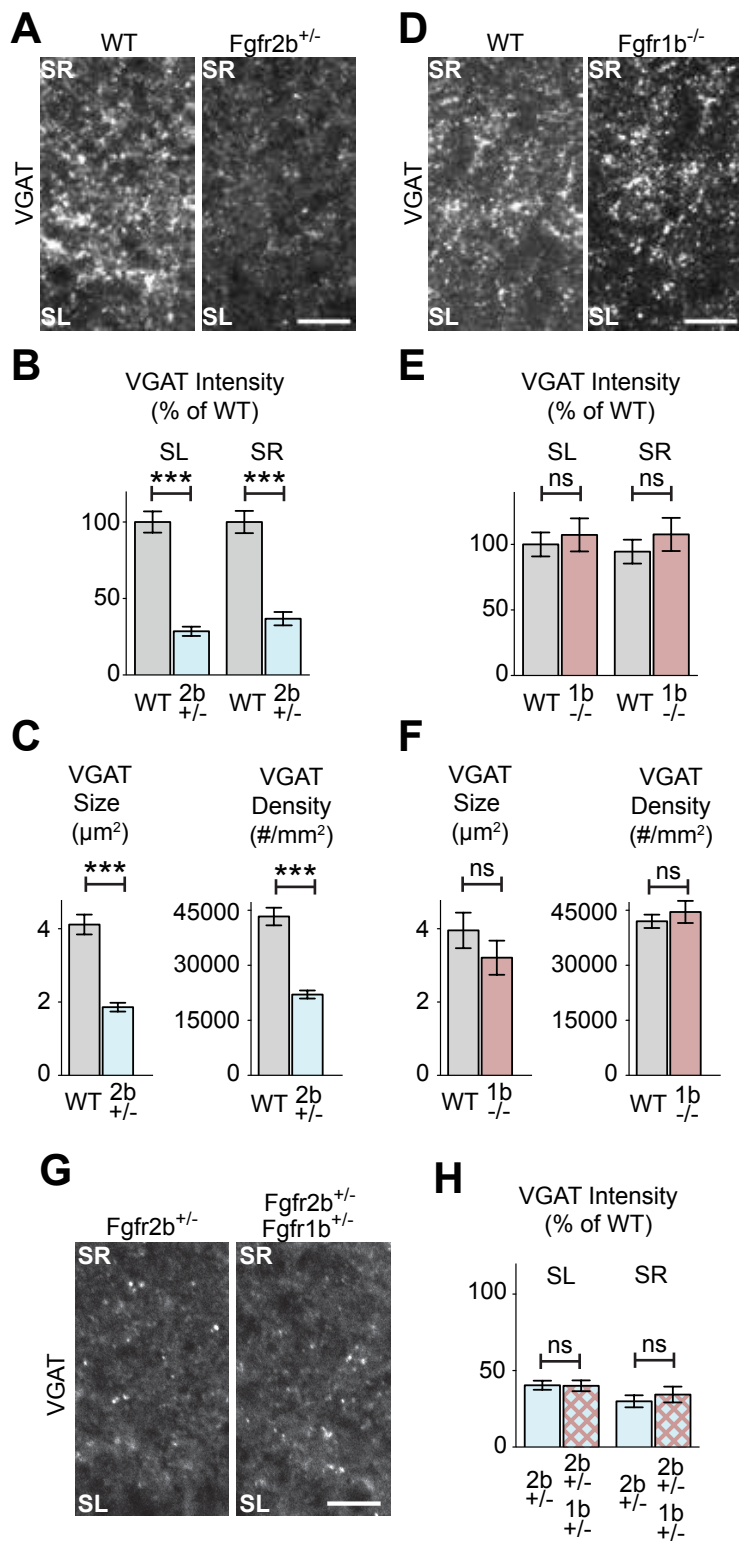
Figure 2.6. *(Following page)* **Temporally restricted loss of Fgfr2 and Fgfr1 during the synaptogenic period results in deficiencies in presynaptic differentiation in hippocampal CA3 in vivo.**

(A-B) Conditional Fgfr-knockout strategy: Mice carrying Actin-promoter-driven CreER were crossed with Fgfr2flox/flox or Fgfr1flox/flox mice. Tamoxifen injection at P0 induced Fgfr deletion (Fgfr2-KO and Ffgr1-KO, respectively) postnatally. Controls: Cre-negative, tamoxifen-injected littermates.

(C-E) Decreased excitatory presynaptic differentiation (VGLUT1 immunostaining) in CA3 of Fgfr2-KO and Fgfr1-KO at P8. (C) Representative images. (D) VGLUT1 intensity in SL, normalized to littermate controls. (E) VGLUT1 puncta size and density in SL. (n=[sections, animals] 28, 4 Fgfr2-KO; 21, 3 Fgfr2-Control; 24, 4 Fgfr1-KO; 24, 4 Fgfr1-Control)

(F-H) Decreased inhibitory presynaptic differentiation (VGAT immunostaining) in CA3 of Fgfr2-KO but not Fgfr1-KO at P8. (F) Representative images. (G) VGAT intensity in SL, normalized to littermate controls. (H) VGAT puncta size and density in SL. (n=[sections, animals] 34, 5 Fgfr2-KO; 35, 5 Fgfr2-Control; 18, 3 Fgfr1-KO; 18, 3 Fgfr1-Control)

Scale bars, 15 μm.

A

Actin — CreER

X

Fgfr2 — [7] ▶loxP [8] [9] [10] Neo ▶loxP [11]

Exon 7 8 9 10 11

B

Actin — CreER

X

Fgfr1 — [3] ▶loxP [4] Neo ▶loxP [5]

Exon 3 4 5

C

VGLUT1

Control | Fgfr2-KO | Control | Fgfr1-KO

D VGLUT1 Intensity (% of Control)

E VGLUT1 Size (μm²)

VGLUT1 Density (#/mm²)

F

VGAT

Control | Fgfr2-KO | Control | Fgfr1-KO

G VGAT Intensity (% of Control)

H VGAT Size (μm²)

VGAT Density (#/mm²)
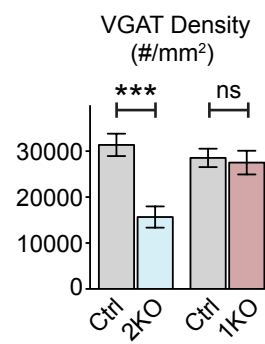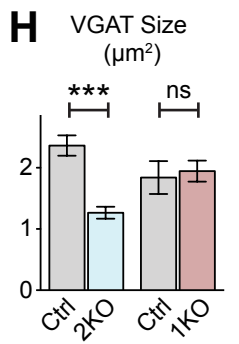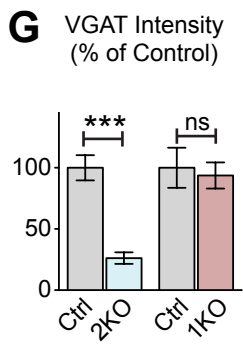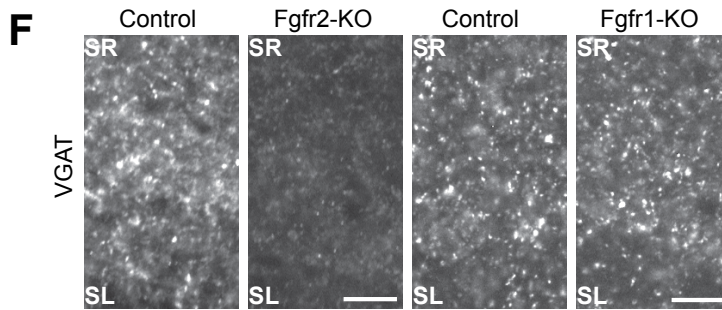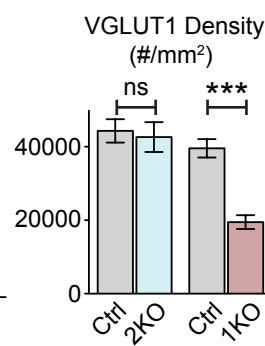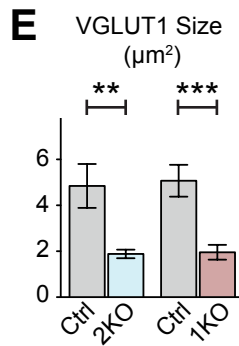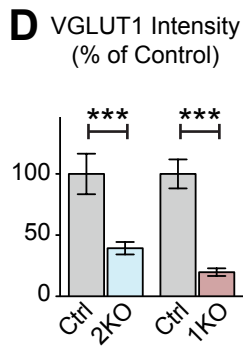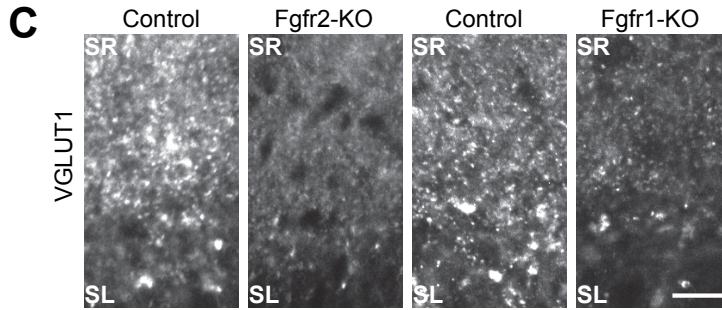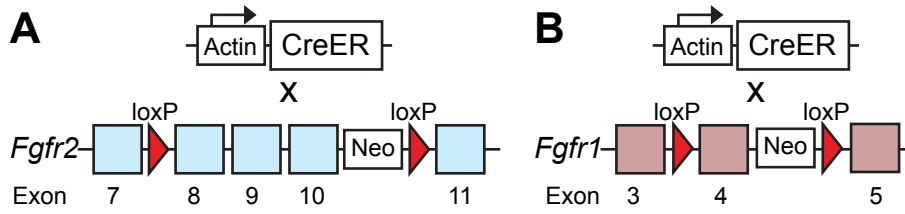
induces CreER-mediated excision of floxed genes (Figure 2.7). Tamoxifen was injected into Actin-CreER::Fgfr2$^{flox/flox}$ or Actin-CreER::Fgfr1$^{flox/flox}$ pups at P0, resulting in Fgfr2-KO or Fgfr1-KO animals, in which both b and c receptor splice forms are deleted postnatally. Both Fgfr2-KO and Fgfr1-KO animals have decreased excitatory presynaptic differentiation in hippocampal CA3 at P8, as measured by VGLUT1 immunoreactivity, compared to their respective controls (Figure 2.6 C-E). VGLUT1 puncta are smaller in Fgfr2-KO animals, and both smaller and sparser in Fgfr1-KO animals (Figure 2.6 E). On the other hand, only Fgfr2-KO, but not Fgfr1-KO animals have decreased inhibitory presynaptic differentiation at P8, measured by VGAT immunoreactivity (Figure 2.6 F-H). In Fgfr2-KO, but not Fgfr1-KO animals, VGAT puncta are smaller and sparser (Figure 2.6 H). Both the excitatory and inhibitory presynaptic defects in Fgfr-KO mice persisted at P14 (Figure 2.8), the end of initial synapse formation in the hippocampus, suggesting that Fgfr inactivation does not simply delay presynaptic differentiation. Thus, during the postnatal synaptogenic stage, excitatory presynaptic differentiation in hippocampal CA3 requires both FGFR2 and FGFR1, and inhibitory presynaptic differentiation requires FGFR2 but not FGFR1.

FGF22 requires FGFR2b and FGFR1b to induce excitatory presynaptic differentiation

I have shown that FGFR2b and FGFR1b are necessary for excitatory presynaptic differentiation *in vivo* (Figures 2.2 and 2.6). I next tested whether FGFR2b and FGFR1b are receptors for FGF22 to organize excitatory presynaptic terminals in neurons. I cultured hippocampal neurons from Fgfr2b-/- embryos, Fgfr1b-/- P0 pups, and their respective WT littermates, treated with FGF22 on day two *in vitro* (DIV2), and examined response to FGF22 by VGLUT1 staining on DIV14 (Figure 2.9 A). FGF22 acts on inputs synapsing onto CA3 pyramidal neurons (Terauchi et al., 2010), so I focused my analysis on synapses forming onto CA3 pyramidal neurons. CA3 neurons were identified by staining with the antibody Py (Woodhams et al., 1989), and VGLUT1 puncta contacting Py-positive dendrites were quantified. WT neurons respond to FGF22 with a two-fold increase in VGLUT1 signal along CA3 dendrites; however, Fgfr2b-/- neurons do not respond to FGF22 (Figure 2.9 B, C). Closer analysis revealed that the response to FGF22 in WT, but not Fgfr2b-/- neurons comprises increases in VGLUT1 puncta density along CA3 dendrites (Figure 2.9 D) and VGLUT1 puncta
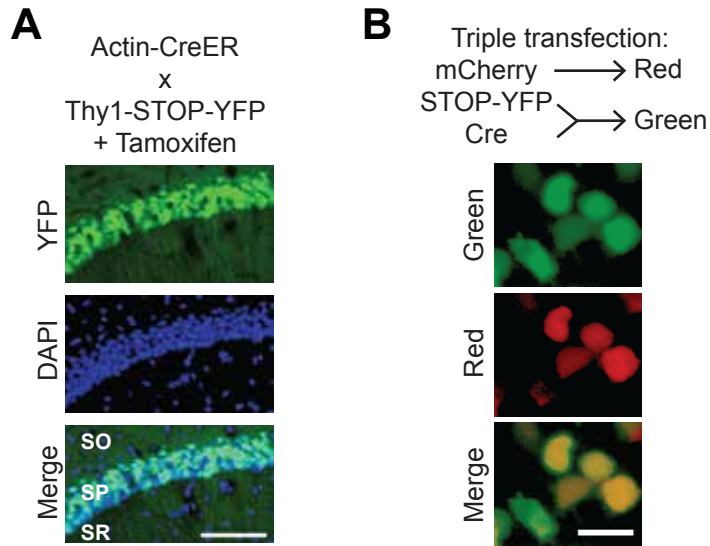
Figure 2.7. Demonstration of efficient Cre-mediated excision of floxed genes *in vivo* and *in vitro*. (A) Mice carrying the tamoxifen-sensitive Cre (CreER) under a CAG promoter (human CMV virus enhancer and chicken β-actin promoter) (Actin-CreER; Guo et al., 2002) were crossed with mice carrying EYFP preceded by a loxP-flanked STOP sequence under a Thy1 promoter (Thy1-STOP-YFP; Buffelli et al., 2003). Tamoxifen was injected at P0, activating CreER and leading to expression of EYFP in neurons, with an efficiency of >85%. Representative image of CA1 pyramidal neurons. SO – stratum oriens; SP – stratum pyra-midale; SR – stratum radiatum. Scale bar, 100 μm. (B) A Cre expression plasmid was co-transfected with a loxP-STOP-loxP-YFP plasmid and an mCherry plasmid into HEK cells using calcium-phosphate transfection method. >90% of transfected cells (red) showed Cre-mediated excision of the STOP cassette (green). Scale bar, 50 μm.
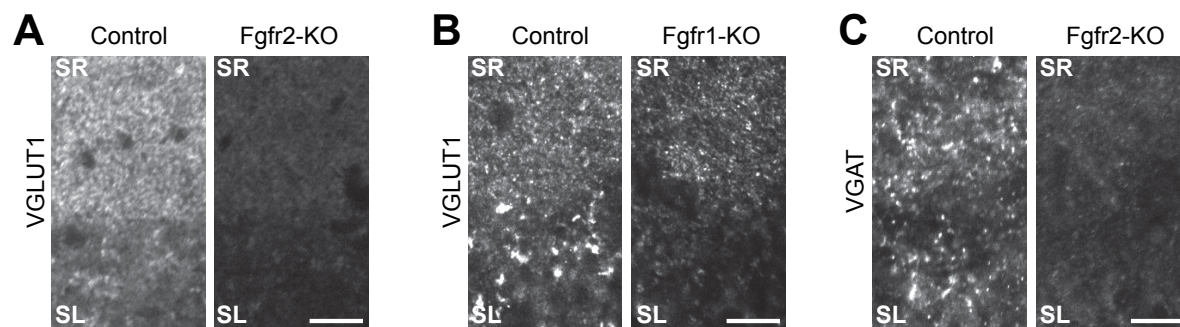
Figure 2.8. Presynaptic defects persist in the hippocampus of Fgfr-KO mice at P14.
(A) Representative images showing that defects in VGLUT1 clustering persist at P14 in
Fgfr2-KO mice. (B) Representative images showing that defects in VGLUT1 clustering
persist at P14 in Fgfr1-KO mice. (C) Representative images showing that defects in VGAT
clustering persist at P14 in Fgfr2-KO mice. Scale bars, 15 µm.
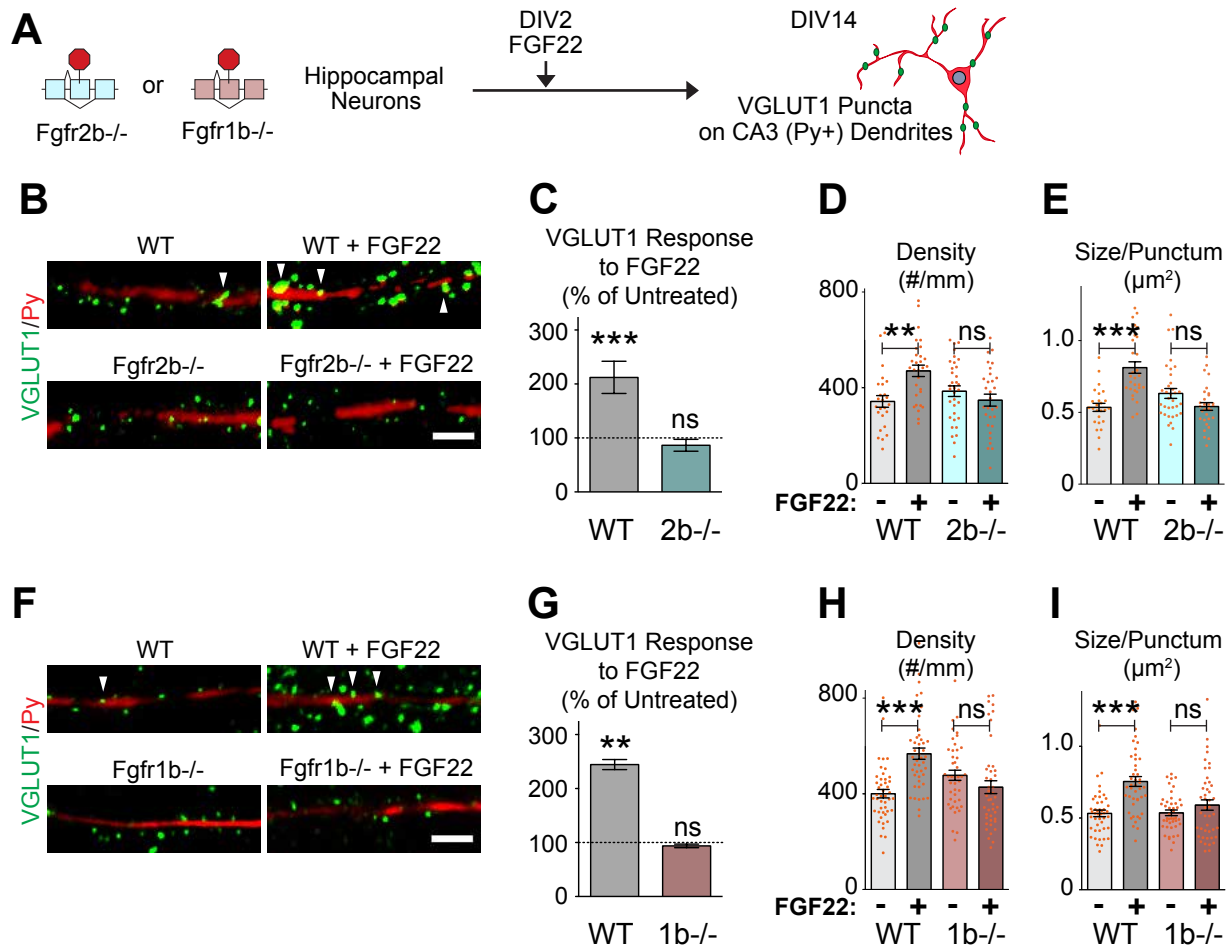
Figure 2.9. Fgfr2b-/- or Fgfr1b-/- neurons do not respond to FGF22 in culture.
(A) Experimental scheme. Hippocampal neurons were cultured from Fgfr2b-/- embryos or
Fgfr1b-/- pups and WT littermates. 2 nM FGF22 was applied on DIV2. Neurons were stained at
DIV14 for VGLUT1 and Py, a marker of CA3 pyramidal neurons. VGLUT1 puncta contacting
CA3 dendrites were analyzed.
(B-E) Fgfr2b-/- neurons do not respond to FGF22. (B) Representative images of VGLUT1
puncta (green) contacting CA3 dendrites (red). Contacting puncta are indicated in WT condition
with arrowheads. (C) Quantification of FGF22 responsiveness. For each genotype, total
intensity of VGLUT1 on CA3 dendrite of FGF22-treated neurons was divided by that of
untreated neurons. (D) VGLUT1 density and (E) size along CA3 dendrites. (n=[neurons,
experiments] 25, 3 WT; 30, 3 WT+FGF22; 34, 3 Fgfr2b-/-; 30, 3 Fgfr2b-/-+FGF22)
(F-I) Fgfr1b-/- neurons do not respond to FGF22. (F) Representative images. (G) VGLUT1
response to FGF22, quantified as in (C). (H) VGLUT1 density and (I) size along CA3 dendrites.
(n=[neurons, experiments] 45, 6 WT; 45-, 6 WT+FGF22; 44, 6 Fgfr1b-/-; 44, 6 Fgfr1b-/-+FGF22)
In scatter plots, each dot represents average value from an individual neuron analyzed. Scale
bar, 5 µm.

size (Figure 2.9 E). Fgfr1b-/- neurons also do not respond to FGF22 (Figure 2.9 F-I): there was no FGF22-induced increase in density (Figure 2.9 H) or size (Figure 2.9 I) of VGLUT1 puncta. In control experiments examining neuronal cell fate, dendritic morphology of CA3 neurons and their excitatory postsynaptic differentiation, I found no apparent differences between WT neurons, Fgfr2b-/- neurons, or Fgfr1b-/- neurons in the presence or absence of FGF22 (Figures 2.10 and 2.11), suggesting that FGFR2b and FGFR1b primarily affect presynaptic differentiation. Together, these results indicate that both FGFR2b and FGFR1b are required for FGF22-dependent induction of excitatory presynaptic differentiation.

FGFRs are necessary during the synaptogenic stage for FGF22-dependent excitatory presynaptic differentiation

To determine whether FGFRs are required specifically during the synaptogenic stage *in vitro*, I temporally restricted Fgfr2 and Fgfr1 deletion. Hippocampal neurons were cultured from Actin-CreER::Fgfr2$^{flox/flox}$ or Actin-CreER::Fgfr1$^{flox/flox}$ mice. On DIV1, 10 nM 4-hydroxytamoxifen (4-OHT) was applied to cultures, generating Fgfr2-KO or Fgfr1-KO neurons. We found this dose efficiently inactivates Fgfrs (Figure 2.12). FGF22 was applied on DIV2, and the neurons were assessed on DIV14 for VGLUT1 clustering on Py-positive dendrites (Figure 2.13 A). Control neurons respond to FGF22 with a three-fold increase in VGLUT1 immunoreactivity along CA3 dendrites, while Fgfr2-KO neurons do not respond (Figure 2.13 B,C). There are no FGF22 responses in VGLUT1 puncta density (Figure 2.13 D) and size (Figure 2.13 E) in Fgfr2-KO neurons. Conversely, Fgfr1-KO neurons have an attenuated, but still present response to FGF22 (Figure 2.13 F, G). Where control neurons respond to FGF22 with a three-fold increase in VGLUT1 signal along CA3 dendrites, Fgfr1-KO neurons respond with a two-fold increase (Figure 2.13 G), with significant increases in VGLUT1 density (Figure 2.13 H) and size (Figure 2.13 I). Therefore, I conclude that FGFR2 is required during the synaptogenic stage to respond to FGF22 to induce excitatory presynaptic differentiation, while FGFR1 is not absolutely required, but is involved during this stage.
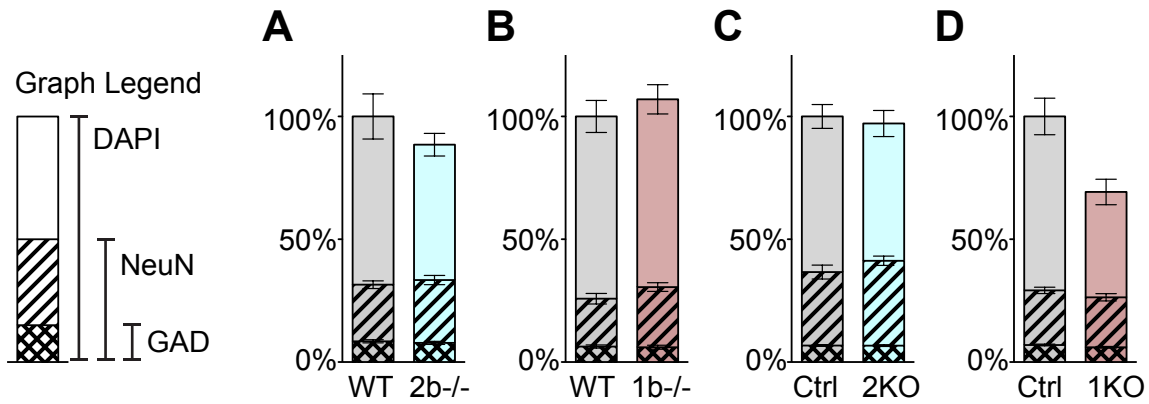
Figure 2.10. Cell numbers and fates of cultured hippocampal neurons *in vitro*.
Cultured hippocampal neurons from WT and Fgfr2b-/- mice (A), WT and Fgfr1b-/- mice (B),
Control and Fgfr2-KO mice (C), and Control and Fgfr1-KO mice (D) were stained with DAPI
to count total cell number, NeuN (1:500, Millipore) to detect mature neurons, and GAD65/67
(1:500, Millipore) to detect inhibitory neurons. Cells were counted from 15 fields (A, B) or 12
fields (C, D) per genotype, each from 3 experiments. No significant changes in the number
of total cells, total neurons, or inhibitory neurons were detected between Fgfr2b-/-, Fgfr1b-/-,
or Fgfr2-KO cultures and their respective controls (A, B, C). A significant (p<0.01) decrease
in the total number of cells, but not total number of neurons or inhibitory neurons, in
Fgfr1-KO cultures compared to control (D). Cell counts for each set of experiments were
normalized to WT or Ctrl DAPI count.
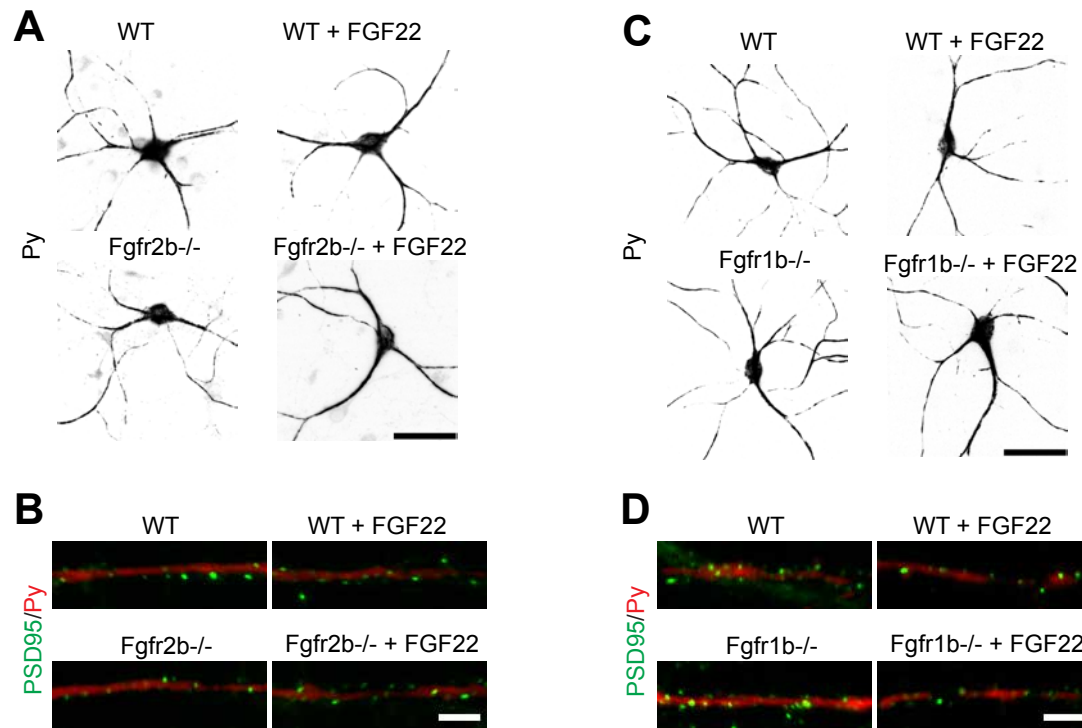
Figure 2.11. Loss of Fgfr2b or Fgfr1b as well as application of FGF22 do not lead to apparent changes in dendritic morphology or excitatory postsynaptic differentiation of CA3 neurons. (A) Representative images of WT and Fgfr2b-/- neurons with and without FGF22 treatment exhibit similar dendritic morphology, as observed by Py staining in culture. (B) WT and Fgfr2b-/- neurons with and without FGF22 treatment do not demonstrate changes in PSD95 (1:500, NeuroMab) staining on Py-positive dendrites. (C) Representative images of WT and Fgfr1b-/- neurons with and without FGF22 treatment exhibit similar dendritic morphology, as observed by Py staining in culture. (D) WT and Fgfr1b-/- neurons with and without FGF22 treatment do not demonstrate changes in PSD95 staining on Py-positive dendrites. Scale bars in (A, C) are 50 µm. Scale bars in (B, D) are 5 µm.

A

RT-PCR

Fgfr1b

Actin

1 **2** **3**
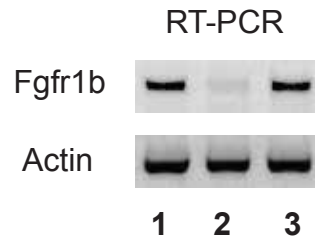
1: Actin-CreER::Fgfr1[flox/flox] hippocampal neurons

2: Actin-CreER::Fgfr1[flox/flox] hippocampal neurons + 10 nM 4-OHT (KO)

3: (No Cre) Fgfr1[flox/flox] hippocampal neurons + 10 nM 4-OHT

B

Fgfr1b mRNA Levels
RT-PCR Quantification
(% of Controls)

100

0

Ctrl KO   Ctrl KO
Normalized   Normalized
to no 4-OHT   to no Cre

C

FGFR1 Protein Levels
Western Blot Quantification
(% of Controls)

100

0

Ctrl KO   Ctrl KO
Normalized   Normalized
to no 4-OHT   to no Cre
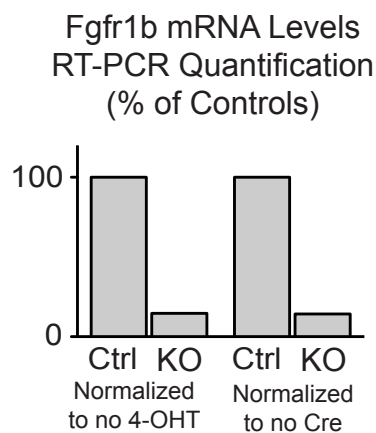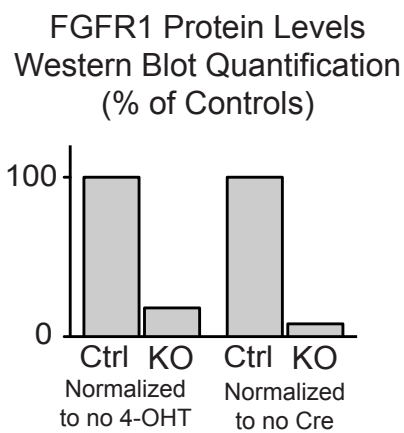
Figure 2.12. Efficient inactivation of floxed Fgfr genes by 4-OH tamoxifen treatment *in vitro*. Hippocampal neuron cultures were prepared from Actin-CreER::Fgfr1[flox/flox] and Fgfr1[flox/flox] (no Cre) littermates. Cultures were treated with 10 nM 4-OH tamoxifen (4-OHT). (A) mRNA was isolated and cDNA synthesized from Actin-CreER::Fgfr1[flox/flox] not treated with 4-OHT (no 4-OHT control), Actin-CreER::Fgfr1[flox/flox] treated with 10 nM 4-OHT at DIV1 (KO), and Fgfr1[flox/flox] treated with 10 nM 4-OHT (no Cre control). RT-PCR using primers specific for Fgfr1b revealed a stark decrease in Fgfr1b transcripts in KO neurons. (B) Quantification of Fgfr1b mRNA levels in KO neurons relative to no 4-OHT and no Cre controls. (C) Quantification of FGFR1 protein levels. KO neurons had a pronounced decrease in the amount of FGFR1 protein compared to no 4-OHT and no Cre controls. Protein lysate was prepared from cultured hippocampal neurons using 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40 lysis buffer supplemented with Protease Inhibitor Cocktail (Roche). Western blot for FGFR1 was performed with the anti-FGFR1 antibody (1:100, Sigma, Cat# F5421). The intensity of FGFR1 bands was quantified with ImageJ.
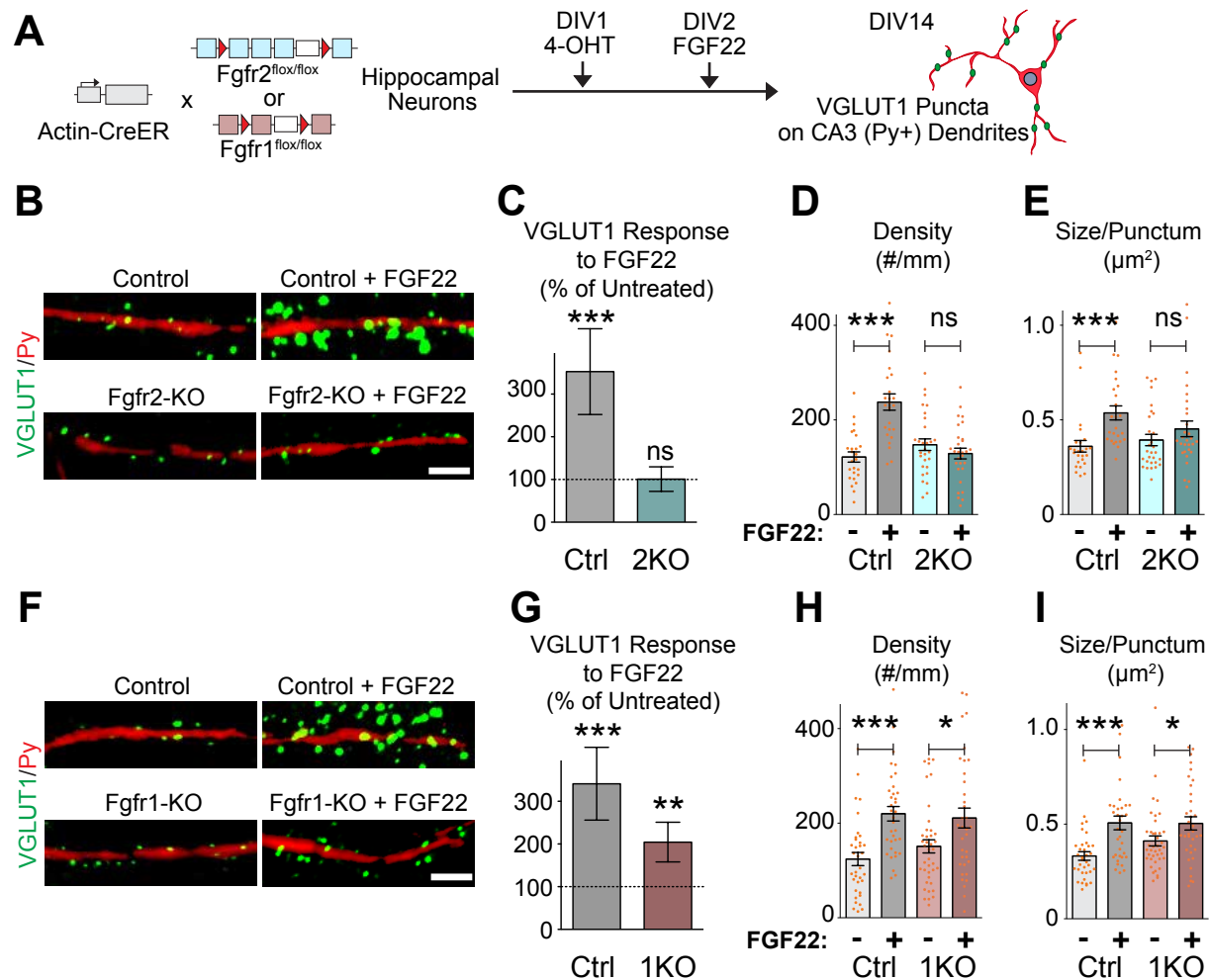
Figure 2.13. Fgfr2-KO neurons, in which receptor deletion is temporally restricted to synaptogenic period, do not respond to FGF22 in culture.
(A) Experimental scheme. Hippocampal neurons were cultured from Actin-CreER::Fgfr2flox/flox or Actin-CreER::Fgfr1flox/flox pups and littermate controls lacking CreER. 10 nM 4-OHT was applied at DIV1 to induce receptor deletion, and FGF22 was applied at DIV2. Neurons were stained at DIV14 for VGLUT1 and Py. VGLUT1 puncta contacting CA3 dendrites were analyzed.
(B-E) Fgfr2-KO neurons do not respond to FGF22. (B) Representative images of VGLUT1 puncta (green) contacting CA3 dendrites (red). (C) VGLUT1 response to FGF22, quantified as in Fig. 4C. (D) VGLUT1 density and (E) size along CA3 dendrites. (n=[neurons, experiments] 25, 3 Control; 25, 3 Control+FGF22; 29, 3 Fgfr2-KO; 29, 3 Fgfr2-KO+FGF22).
(F-I) Fgfr1-KO neurons partially respond to FGF22. (F) Representative images. (G) VGLUT1 response to FGF22, quantified as in Fig. 4C. (H) VGLUT1 density and (I) size along CA3 dendrites. (n=[neurons, experiments] 35, 4 Control; 35, 4 Control+FGF22; 40, 4 Fgfr1-KO; 35, 4 Fgfr1-KO+FGF22)
Scale bars, 5 µm.

FGF7 requires FGFR2b but not FGFR1b to induce inhibitory presynaptic differentiation

Fgfr2b+/- mice exhibit defects in inhibitory presynaptic differentiation (Figures 2.5 and 2.6), similar to Fgf7-KO mice (Terauchi et al., 2010). I next tested whether FGFR2b is a receptor for FGF7 to organize inhibitory presynaptic terminals. Hippocampal neurons from Fgfr2b-/- embryos, Fgfr1b-/- P0 pups, and WT littermates were treated on DIV2 with FGF7, and assessed at DIV14 for VGAT clustering on Py-positive dendrites (Figure 2.14 A). WT neurons respond to FGF7 with a two-fold increase in VGAT staining along CA3 dendrites, while Fgfr2b-/- neurons do not respond (Figure 2.14 B-E). In Fgfr2b-/- neurons, FGF7 does not increase VGAT puncta density (Figure 2.14 D) or size (Figure 2.14 E). Meanwhile, Fgfr1b-/- neurons respond equally to WT controls with a two-fold increase in VGAT intensity along CA3 dendrites (Figure 2.14 F, G) as well as increases in VGAT density (Figure 2.14 H) and size (Figure 2.14 I). I observed no apparent changes in dendritic morphology of CA3 neurons and inhibitory postsynaptic differentiation on CA3 dendrites between WT cultures, Fgfr2b-/- cultures, or Fgfr1b-/- cultures, with or without FGF7 (Figure 2.15). Together, these data suggest that FGFR2b, but not FGFR1b, is required for FGF7-induced inhibitory presynaptic differentiation.

FGFR2 is necessary during the synaptogenic stage in FGF7-dependent inhibitory presynaptic differentiation

To confirm that FGFR2 is required during the synaptogenic stage to respond to FGF7, we temporally restricted Fgfr2 deletion. Actin-CreER::Fgfr2$^{flox/flox}$ hippocampal cultures were treated with 10 nM 4-OHT on DIV1 to induce receptor deletion, FGF7 on DIV2, and assessed on DIV14 for VGAT clustering along Py-positive dendrites (Figure 2.16 A). In response to FGF7, control neurons have a three-fold increase in VGAT intensity along CA3 dendrites, while Fgfr2-KO neurons do not respond (Figure 2.16 B, C). Where control neurons respond to FGF7 with increases in VGAT puncta density (Figure 2.16 D) and size (Figure 2.16 E), Fgfr2-KO neurons do not. Thus, I conclude that Fgfr2 is required during the synaptogenic stage to respond to FGF7.
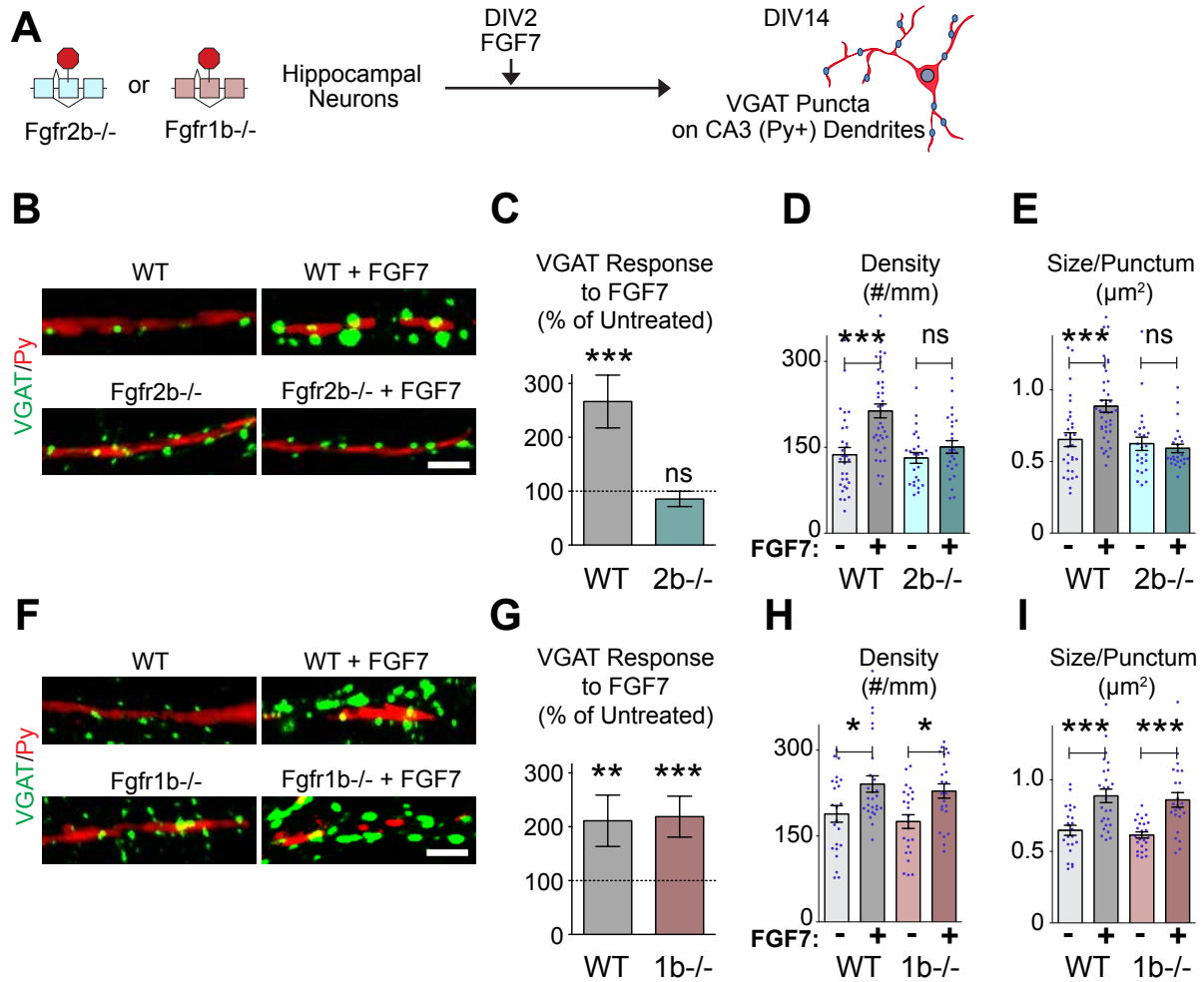
Figure 2.14. Fgfr2b-/- neurons do not, but Fgfr1b-/- neurons do respond to FGF7 in culture.

(A) Experimental scheme. Hippocampal neurons were cultured from Fgfr2b-/- embryos or Fgfr1b-/- pups and WT littermates. 2 nM FGF7 was applied on DIV2. Neurons were stained at DIV14 for VGAT and Py. VGAT puncta contacting CA3 dendrites were analyzed.

(B-E) Fgfr2b-/- neurons do not respond to FGF7. (B) Representative images of VGAT puncta (green) contacting CA3 dendrites (red). (C) Quantification of FGF7 responsiveness. For each genotype, total intensity of VGAT on CA3 dendrite of FGF7-treated neurons was divided by that of untreated neurons. (D) VGAT density and (E) size along CA3 dendrites. (n=[neurons, experiments] 30, 3 WT; 38, 3 WT+FGF7; 25, 3 Fgfr2b-/-; 25, 3 Fgfr2b-/-+FGF7)

(F-I) Fgfr1b-/- neurons respond to FGF7. (F) Representative images. (G) VGAT response to FGF7, quantified as in (C). (H) VGAT density and (I) size along CA3 dendrites. (n=[neurons, experiments]   23, 3 WT; 26, 3 WT+FGF7; 24, 3 Fgfr1b-/-; 23, 3 Fgfr1b-/-+FGF7)

Scale bars, 5 μm.
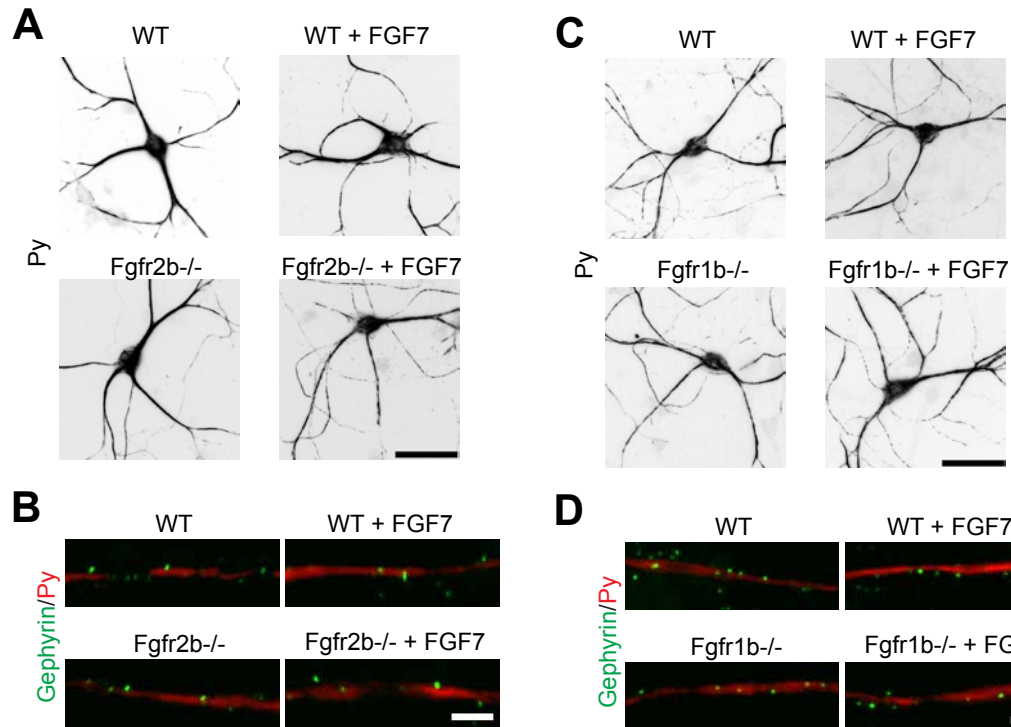
Figure 2.15. Loss of FGFR2b or FGFR1b as well as application of FGF7 do not lead to apparent changes in dendritic morphology or inhibitory postsynaptic differentiation of CA3 neurons. (A) Representative images of WT and Fgfr2b-/- neurons with and without FGF7 treatment exhibit similar dendritic morphology, as observed by Py staining in culture. (B) WT and Fgfr2b-/- neurons with and without FGF7 treatment do not demonstrate changes in Gephyrin (1:50, Synaptic Systems) staining on Py-positive dendrites. (C) Representative images of WT and Fgfr1b-/- neurons with and without FGF7 treatment exhibit similar dendritic morphology, as observed by Py staining in culture. (D) WT and Fgfr1b-/- neurons with and without FGF7 treatment do not demonstrate changes in Gephyrin staining on Py-positive dendrites. Scale bars in (A, C) are 50 μm. Scale bars in (B, D) are 5 μm.
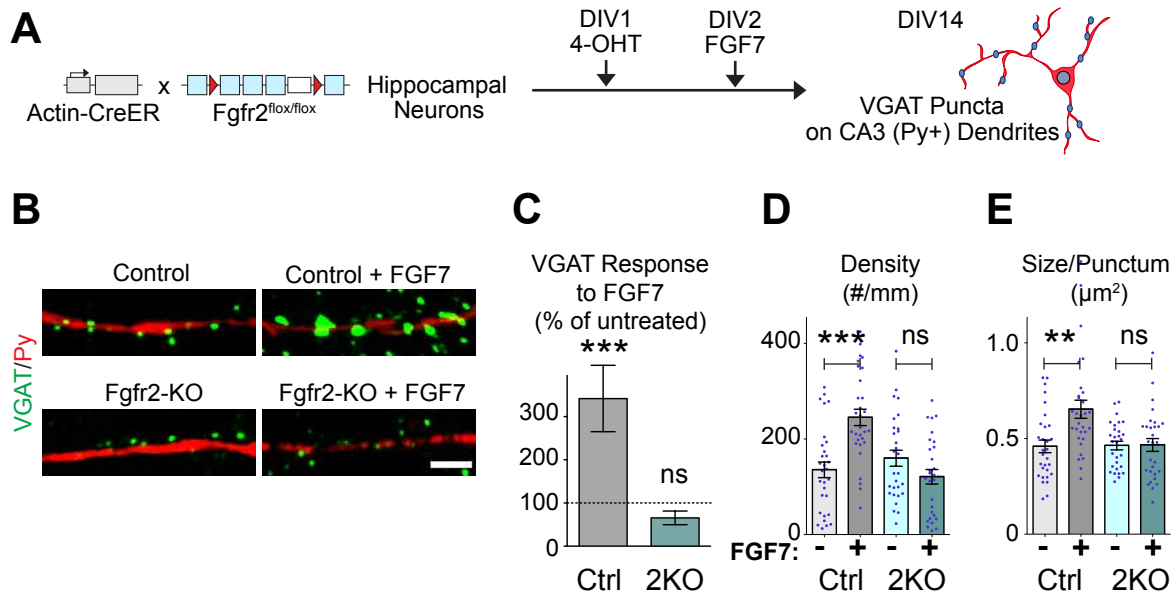
Figure 2.16. Fgfr2-KO neurons, in which receptor deletion is temporally restricted to synaptogenic period, do not respond to FGF7 in culture.

(A) Experimental scheme. Hippocampal neurons were cultured from Actin-CreER::Fgfr2flox/-flox pups and littermate controls lacking CreER. 4-OHT was applied at DIV1, and FGF7 was applied at DIV2. Neurons were stained for VGAT and Py at DIV14. VGAT puncta contacting CA3 dendrites were analyzed.

(B-E) Fgfr2-KO neurons do not respond to FGF7. (B) Representative images of VGAT puncta (green) contacting CA3 dendrites (red). (C) VGAT response to FGF7, quantified as in Fig. 6C. (D) VGAT density and (E) size along CA3 dendrites. (n= [neurons, experiments] 30, 3 Control; 30, 3 Control+FGF7; 29, 3 Fgfr2-KO; 29, 3 Fgfr2-KO+FGF7).

Scale bar, 5 µm.

<u>Discussion.</u>

In this chapter, I demonstrated, using both *in vivo* and *in vitro* evidence, that differential use of FGFRs by FGF22 and FGF7 contributes to their presynaptic effects on excitatory and inhibitory synapses in the CA3 region of the hippocampus.

The coordinated development of excitatory and inhibitory synapses in the hippocampal CA3 is guided by FGF22 and FGF7 with remarkable specificity. After the fate of excitatory and inhibitory neurons has been specified and as their axons approach CA3, FGF22 and FGF7 are secreted from CA3 dendrites at discrete excitatory and inhibitory postsynaptic sites, respectively, to promote distinct presynaptic differentiation (Terauchi et al., 2010). I found that, in addition to specific localization of ligand secretion, overlapping but distinct sets of FGFRs are required for excitatory versus inhibitory presynaptic differentiation, providing robustness to the system. FGF22 and FGF7 both activate FGFR2b, but we propose that FGFR1b plays an important role in coordinating specificity. FGF22, but not FGF7, activates FGFR1b in *in vitro* suspension cell proliferation assays (Zhang et al., 2006), and we found that in the hippocampus, FGFR1b is required for excitatory synapse formation in response to FGF22 (Fig. 4), but not for inhibitory synapse formation in response to FGF7 (Figure 2.14). Consistent with this specificity, Fgfr2b-lacking mice have excitatory and inhibitory presynaptic deficits, and Fgfr1b-lacking mice have only excitatory presynaptic deficits (Figure 2.2, 2.5, and 2.6). The receptor KO mice do not completely lose excitatory or inhibitory presynaptic differentiation, suggesting that FGFRs cooperate with other synaptogenic receptors for presynaptic differentiation.

What is the relationship between FGFR2b and FGFR1b in promoting excitatory presynaptic differentiation in response to FGF22? Both FGFR2b and FGFR1b appear to be necessary presynaptically for responsiveness to FGF22 *in vitro* (which I will show in Chapter 3); however, they do not appear to be redundant in terms of their effect on excitatory presynaptic differentiation (Figure 2.2). One possibility is that FGFR2b and FGFR1b form heterodimers. Under conditions of complete ligand absence, FGFR2 and FGFR1 can form heterodimers (Wang et al., 1997) and ligand can induce transphosphorylation (Bellot et al., 1991), although whether the native forms do so during development has not yet been demonstrated. Alternatively, they cooperate as independent homodimers to promote presynaptic differentiation, where FGFR2b is

the dominant receptor required for both excitatory and inhibitory presynaptic differentiation, while FGFR1b specifically modulates excitatory presynaptic differentiation in response to FGF22. Interestingly, FGFR1b appears to have a modulatory role in relation to FGFR2b in other systems. For example, FGF10, which, like FGF22, activates both FGFR2b and FGFR1b (Zhang et al., 2006), plays an important role in lung and submandibular gland development. In both systems, FGFR2b promotes glandular proliferation and elongation and induces FGFR1b expression at the end buds, while FGFR1b is important specifically for end bud expansion (Steinberg et al., 2005; Patel et al., 2008). Identification of the precise roles of FGFR1b in excitatory presynaptic differentiation is an important next question.

Unbalanced excitation-inhibition has behavioral consequences: Fgf22-KO and Fgf7-KO mice have altered epileptic seizure susceptibility (Terauchi et al., 2010; Lee et al., 2012; Lee and Umemori, 2013). Additionally, other FGFs have also been implicated in epilepsy (Paradiso et al., 2013). What are the behavioral, neurological, or psychiatric consequences of losing FGFR2b or FGFR1b signaling in humans? Dominant negative mutations and mutations leading to overactivation in FGFR2 and FGFR1 cause craniosynostosis syndromes, dominated by cranial defects, which also include seizures and intellectual disability (Agochukwu et al., 2012; Melville et al., 2010; Stevens et al., 2010a; Williams and Umemori, 2014). Analysis of single-nucleotide polymorphisms in human patients has linked mutations in FGFR2 to susceptibility to schizophrenia (O'Donovan et al., 2009) and bipolar disorder (Wang et al., 2012), and FGFR1 mutations to susceptibility to depression (Gaughran et al 2006) and schizophrenia (Shi et al., 2011). These analyses do not differentiate between the isoform-specific contributions of the receptors; however, all of this evidence supports the idea that FGFR signaling is crucial to proper neural development, and FGFRs may prove to be important druggable therapeutic targets in neurological diseases.

CHAPTER III

Synaptogenic FGFR Signaling

The following work is in press for publication in the journal Development *as: Dabrowki, A., Terauchi, A., Strong, C., and Umemori, H. (2015) Distinct sets of FGF receptors sculpt excitatory and inhibitory synaptogenesis.*

Introduction.

Compared to what is known about signaling pathways involved in postsynaptic development (Ebert and Greenberg, 2013; Hagenston and Bading, 2011; Mabb and Ehlers, 2010; Shen and Cowan, 2010; Stamatakou and Salinas, 2014; Tolias et al., 2011), less is known about signaling pathways guiding presynaptic differentiation. The presynaptic contribution of individual signaling pathways has been studied using broad inhibition or overactivation: PI3K/AKT (Martin-Peña et al., 2006; Cuesto et al., 2011), extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) (Li et al., 2002; Kushner et al., 2005; Nakata et al., 2005; Wairkar et al., 2009; Giachello et al., 2010), and PLCγ (Yoshida et al., 2009) are all proposed to be involved in presynaptic development. The contributions of different signaling pathways may reflect the molecular complexity of synapse formation, underscoring how crucial it is to understand the signaling pathways downstream of specific synaptogenic receptors to tease apart this complexity.

In the previous chapter, I showed that differential sets of FGF receptors are required for excitatory and inhibitory presynaptic differentiation in the hippocampal CA3. Specifically, I demonstrated that FGF22 requires FGFR2b and FGFR1b for excitatory presynaptic differentiation, while FGF7 requires FGFR2b for inhibitory presynaptic differentiation. In this chapter, I will address where the receptors function, and what the downstream intracellular signaling pathways required for presynaptic differentiation are. Where do the receptors act? What are the signaling pathways involved? Using conditional knockout mice and a series of *in vitro* experiments, I showed that FGFRs are required in the presynaptic neuron through a presynaptic mechanism that involves PI3K and FRS2 signaling.

<u>Methods</u>.

Mouse strains.

Fgfr2$^{\text{flox/flox}}$ mice (Yu et al. 2003; Umemori et al., 2004) and Fgfr1$^{\text{flox/flox}}$ mice (Hoch and Soriano, 2006) were described previously. All mice were on a C57BL/6 background. WT mice used were either C57BL/6 or ICR/CD-1. All animal care and use was in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital and University of Michigan.

Neuronal culture.

For DGC and interneuron transfections, dissociated DG and CA3 cells were plated at a density of 50,000 cells/coverslip, and culture media was supplemented with 5 mM KCl. 1 μg of plasmids per coverslip were transfected using CalPhos Transfection Kit (Clontech). FGF22 (R&D) and FGF7 (PeproTech) were dissolved to 3 nM for Synaptophysin-YFP experiments.

Plasmids.

To generate Fgfr1b-GFP, the coding region of Fgfr1b cDNA was PCR amplified from neonatal mouse skin cDNA and fused with GFP in pEGFP-N1 (Clontech). To generate Fgfr2b-EGFP, the coding region of Fgfr2b cDNA from IMAGE clone 5349249 (ATCC) was inserted into pcDNA3.1 with EGFP cDNA.

Fgfr2b mutant constructs were generated using PCR amplification and insertion into the pAP-TAG5 plasmid (GenHunter) using the following combinations of primers: 5'-ATACTAGTCATGGGATTACCGTCC-3' (primer-N) with 5'-ATGGGCCCTCATGTTTTAACACTGCCG-3' (primer-C) for WT Fgfr2b; primer-N with 5'-ATGTCGACTTCCAGTCAAGTGGATGGCTCC-3'and 5'-ATGTCGACCATTTGTGGTCTTTTTTTCTTCGTCTATGTTGTTG-3' with primer-C for Fgfr2b-KD; primer-N with 5'-ATCTGCAGAGTCCAGCTCCTCCATGAAC-3'and 5'-ATCTGCAGAAACCTGTCTCCGCAGGGGG-3' with primer-C for Fgfr2b-FRS2; primer-N with 5'-ATGAGCTAGCCATGATGATGAG-3'and 5'-TCATGGCTAGCTCATTGGTGC-3' with primer-C for Fgfr2b-PI3K; and primer-N with 5'-ATAAGCTTTGGATCTCACCCAGCCTC-3'and 5'-ATAAAGCTTCCTCATTGGTTGTGAGAG-3' with primer-C for Fgfr2b-PLCγ. All PCR products were verified with sequencing.

Immunostaining.

Cultured neurons were fixed with 100% methanol, -20°C for 5 minutes. Antibodies and dilutions: GFP (1:5000, Aves), Prox1 (1:500, Millipore), Py (1:25, gift from M. Webb and P.L. Woodhams). Mounting media: glycerol with n-propyl gallate.

Image acquisition and analysis.

12-bit images were acquired with epifluorescence microscopes (Olympus) using 20x and 40x lenses with F-View II CCD (Soft Imaging System) and XM10 (Olympus) cameras at 1,376 x 1,032 pixels resolution. Synaptophysin-YFP images were analyzed using Fiji. The entire axonal arbor was imaged and analyzed. Images were processed to exclude dendrites and converted to 8-bit. For each neuron, a threshold was chosen separately to capture the dimmest punctum and remove diffuse background axonal staining. Masks were applied to images and puncta size and count were calculated in Fiji. Axon length was traced manually in Fiji. The statistical tests performed were two-tailed Student's *t*-test. All data are expressed as mean ± s.e.m.

<u>Results</u>.

FGFR2 and FGFR1 localize and function presynaptically to respond to FGF22

Since FGF22 and FGF7 are secreted from postsynaptic sites and retrogradely induce differentiation at presynaptic terminals, I hypothesize that FGFR2b and FGFR1b are localized and function presynaptically in the axon. Previous studies reported that FGFR1 and FGFR2 are expressed in axons, dendrites and soma of cultured hippocampal neurons (Li et al., 2002); however, there are no adequate antibodies against FGFR2b or FGFR1b to directly test their endogenous localization. Thus, I used an alternative strategy of overexpressing fluorescently-tagged FGFR2b and FGFR1b in cultured neurons to follow their localization (Figure 3.1). FGFR2b-GFP and FGFR1b-GFP both localized throughout the cell including the axon, along which FGFRs accumulate in a punctate manner (Figure 3.1 A, B). In order to demonstrate their localization to presynaptic terminals within the axon, we co-transfected fluorescently-tagged Synaptophysin, which marks synaptic vesicles (Li and Murthy, 2001; De Paola et al., 2003; Umemori et al., 2004). The majority of FGFR2b-GFP colocalized with Synaptophysin-mCherry (Figure 3.1 C), and the majority of FGFR1b-GFP colocalized with Synaptophysin-mCherry (Figure 3.1 D). This indicates that FGFR2b and FGFR1b can indeed localize to axon terminals, consistent with a presynaptic role.

I then examined whether the receptors function presynaptically. FGF22 acts on excitatory axon terminals contacting CA3 pyramidal neurons, including those from dentate granule cells (DGCs). Therefore, I first focused on FGF22-induced excitatory presynaptic differentiation within the axons of DGCs. Neuronal cultures prepared from DG and CA3 from Fgfr2$^{flox/flox}$ or Fgfr1$^{flox/flox}$ animals were sparsely co-transfected at DIV1 with Cre, to induce cell-autonomous receptor deletion, and synaptophysin-YFP, to visualize synaptic vesicles. Co-transfection efficiency was nearly 100%, and efficiency of Cre-mediated excision of floxed genes was estimated to be ~90% (Figure 2.7). Thus, the Fgfr was deleted in transfected, Synaptophysin-YFP-positive cells, while untransfected cells remained WT. The transfection was very sparse to analyze single cells not contacting other transfected cells (Figure 3.2), ensuring a cell-autonomous effect. DGCs were identified by staining for the DGC marker Prox1 (Oliver et al.,
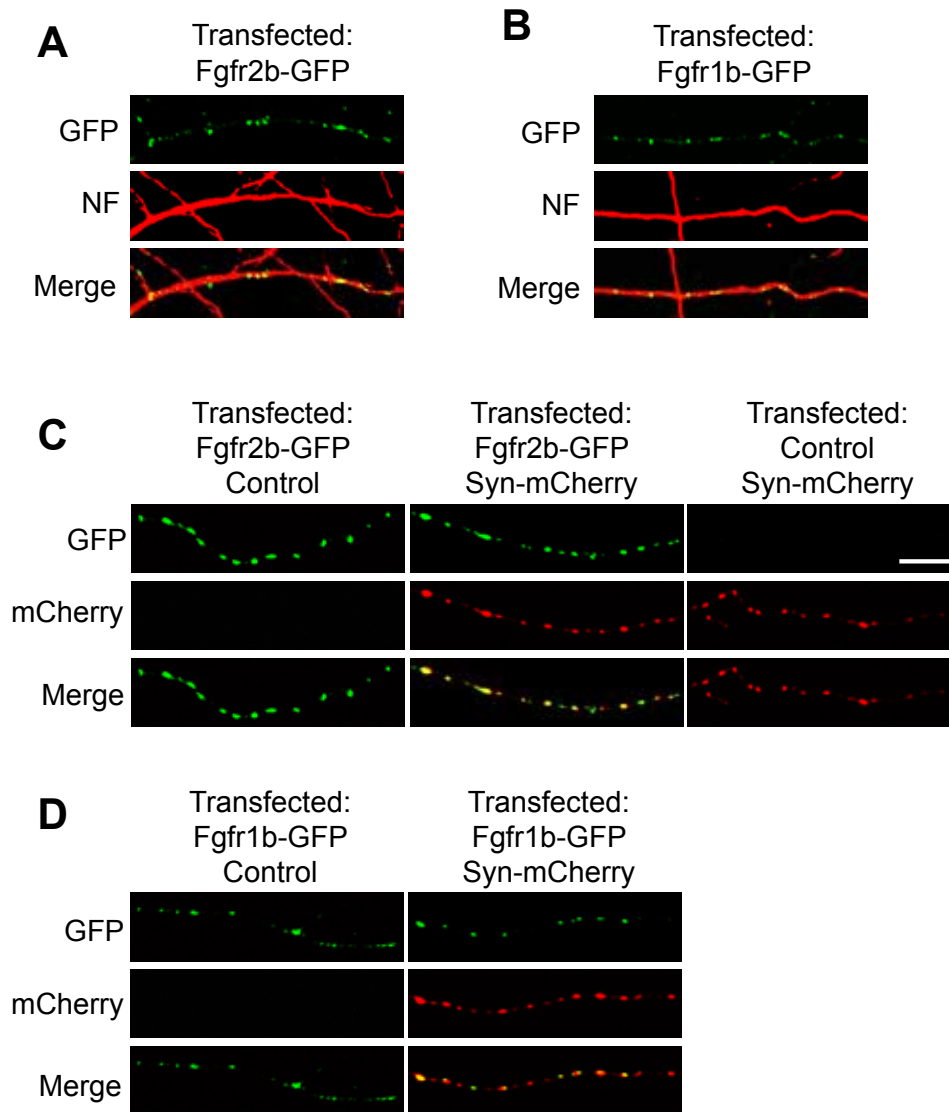
Figure 3.1. FGFR2b and FGFR1b localize to presynaptic terminals.
WT neurons in culture were transfected with GFP-tagged Fgfr constructs.
(A,B) FGFR2b-GFP (A) and FGFR1b-GFP (B) localize to neurofilament (NF)-positive axons.
(C,D) When co-transfected with Synaptophysin-mCherry, the majority of FGFR2b-GFP (C) and FGFR1b-GFP (D) puncta colocalize with Synaptophysin-mCherry.
Scale bar, 10 µm.

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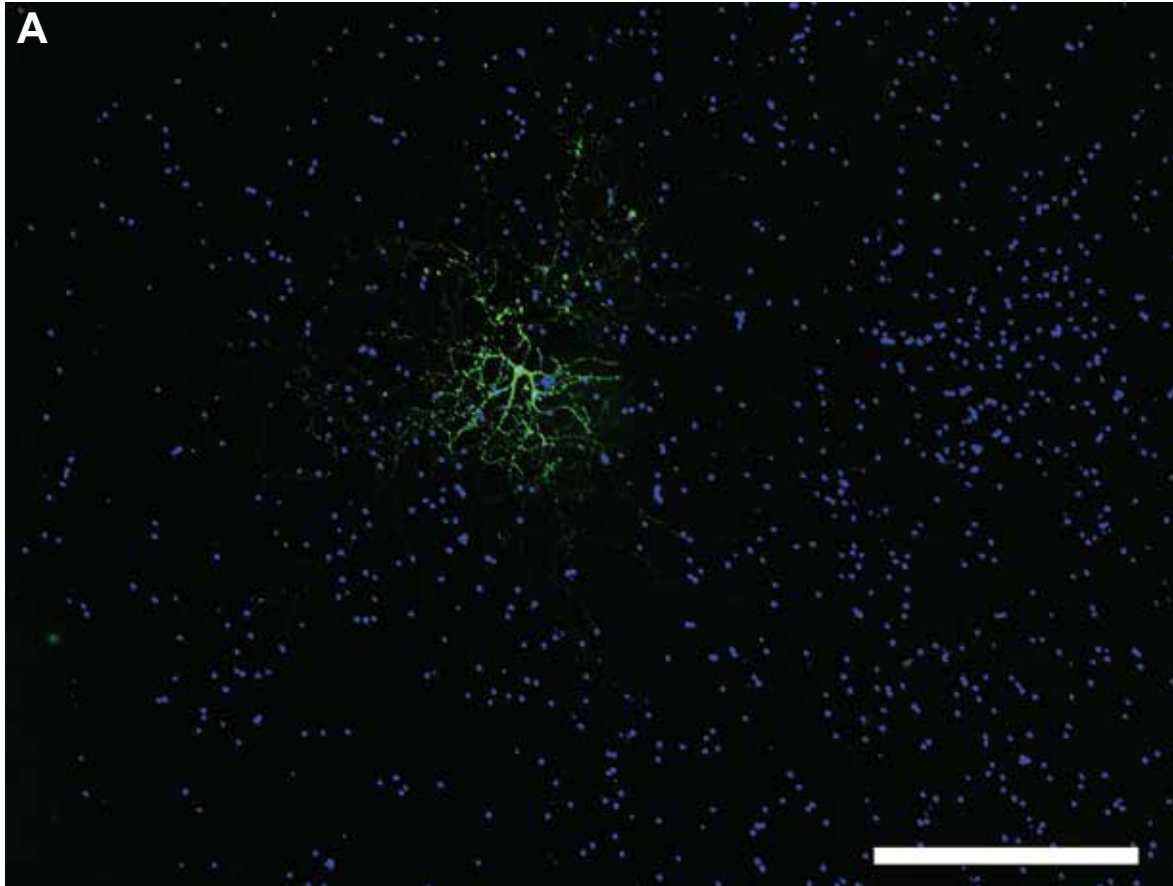
Figure 3.2. Sparse transfection of Synaptophysin-YFP into hippo-campal culture.

A) Representative image of a transfected neuron, with punctate Synaptophysin-YFP fluorescent labeling (green) of the entire axonal arbor. DAPI (blue) reveals the presence of other untransfected cells. To isolate the effect of transfection for a cell-autonomous effect and minimize the effect of other transfected cells, sparse transfections were performed in experiments described in Figures 3.4, 3.6, and 3.7. The expression plasmid carrying Synaptophysin-YFP under the CMV promoter, together with experimental plasmids (such as Cre expression or empty vector), were transfected into neuronal cultures. Of the approximately 50,000 cells plated on a 12-mm coverslip (113 mm^2), only about 50 cells were transfected per coverslip, thus there were fewer than 0.5 transfected cells per mm^2. Only transfected neurons not contacting other transfected cells were analyzed, to maximize the possibility of cell-autonomous effects. Scale bar, 500 µm.

B) Example image of a Synaptophysin-YFP transfected astrocyte. Most transfected cells were neurons and not astrocytes (less than 5% of transfected cells were astrocytes).
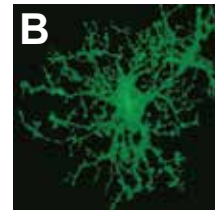
1993; Williams et al., 2011). Fgfr deletion did not obviously affect DGC axon length (Figure 3.3). The neurons were treated with FGF22 at DIV2 and fixed for visualization at DIV11 (Figure 3.4 A, B). Control DGCs (transfected with non-Cre plasmid) responded to FGF22 to induce Synaptophysin-YFP clustering, while both Fgfr2-deleted and Fgfr1-deleted DGCs failed to respond (Figure 3.4 C, D), both in terms of Synaptophysin-YFP puncta density within the axon (Figure 3.4 E) or size (Figure 3.4 F). These data suggest that both FGFR2 and FGFR1 are essential and function in the presynaptic neuron to respond to FGF22.

Next, I studied the functional presynaptic requirement of FGFR2 at inhibitory synapses in response to FGF7. To do this, we performed a similar experiment as described above, but treated with FGF7 and identified transfected interneurons using GABA immunoreactivity (Figure 3.5 A, B). We found that control interneurons respond to FGF7 to induce Synaptophysin-YFP clustering both in terms of density and size, while Fgfr2-KO interneurons fail to respond (Figure 3.5 C-F). These data suggests that Fgfr2 functions in the presynaptic interneuron to respond to FGF7.

Because each coverslip in my experiment contained more than one Cre/Synaptophysin-YFP transfected (i.e., Fgfr-KO) cell, I cannot completely exclude the possibility that other Fgfr-KO cells on the coverslip indirectly affected Synaptophysin-YFP clustering in the neuron I examined. However, because only ~0.1% of cells were transfected (Figure 3.2), untransfected WT cells would likely mask the indirect effect of sparse receptor deletion. Thus, my data suggest that FGFRs function directly in presynaptic neurons.

FGFR2b requires kinase activity, and FRS2 and PI3K binding to respond to FGF22

My experiments show that FGFR2 is required in the presynaptic neuron to respond to FGF22. We next asked whether kinase activity is required for and which intracellular signaling pathways are involved in FGF22-dependent presynaptic differentiation. FGFRs belong to the receptor tyrosine kinase family of proteins and have an intracellular kinase domain, which becomes activated after ligand-induced receptor dimerization, triggering a multi-step autophosphorylation cascade (Mohammadi et al., 1996; Furdui et al., 2006; Bae et al., 2009;

A DGC axon length

B Interneuron axon length
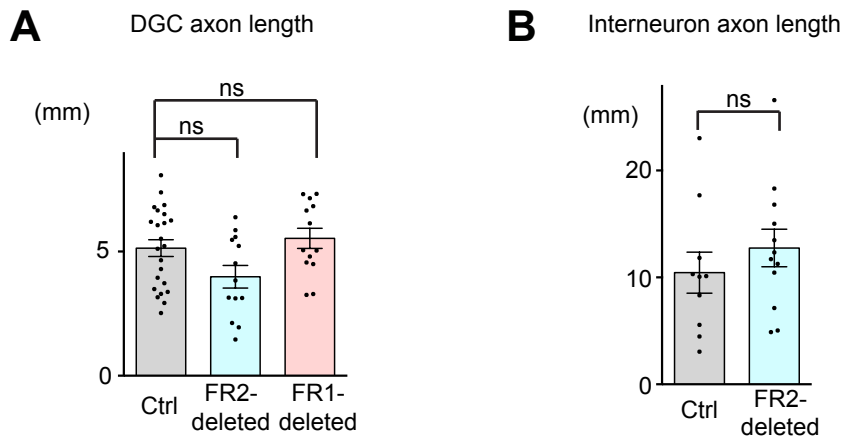
Figure 3.3. Axon length does not significantly change in Fgfr-deleted DGCs or interneurons. (A) Average axon length in Fgfr2-deleted or Fgfr1-deleted DGCs does not significantly change from control. (B) Average axon length in Fgfr2-deleted interneurons does not significantly change from control. Data represents mean ± s.e.m. DGC axon lengths were compared using a One-way ANOVA with a post hoc Tukey's test. Interneuron axon lengths were compared using a Student's t-test. "ns", not significant.
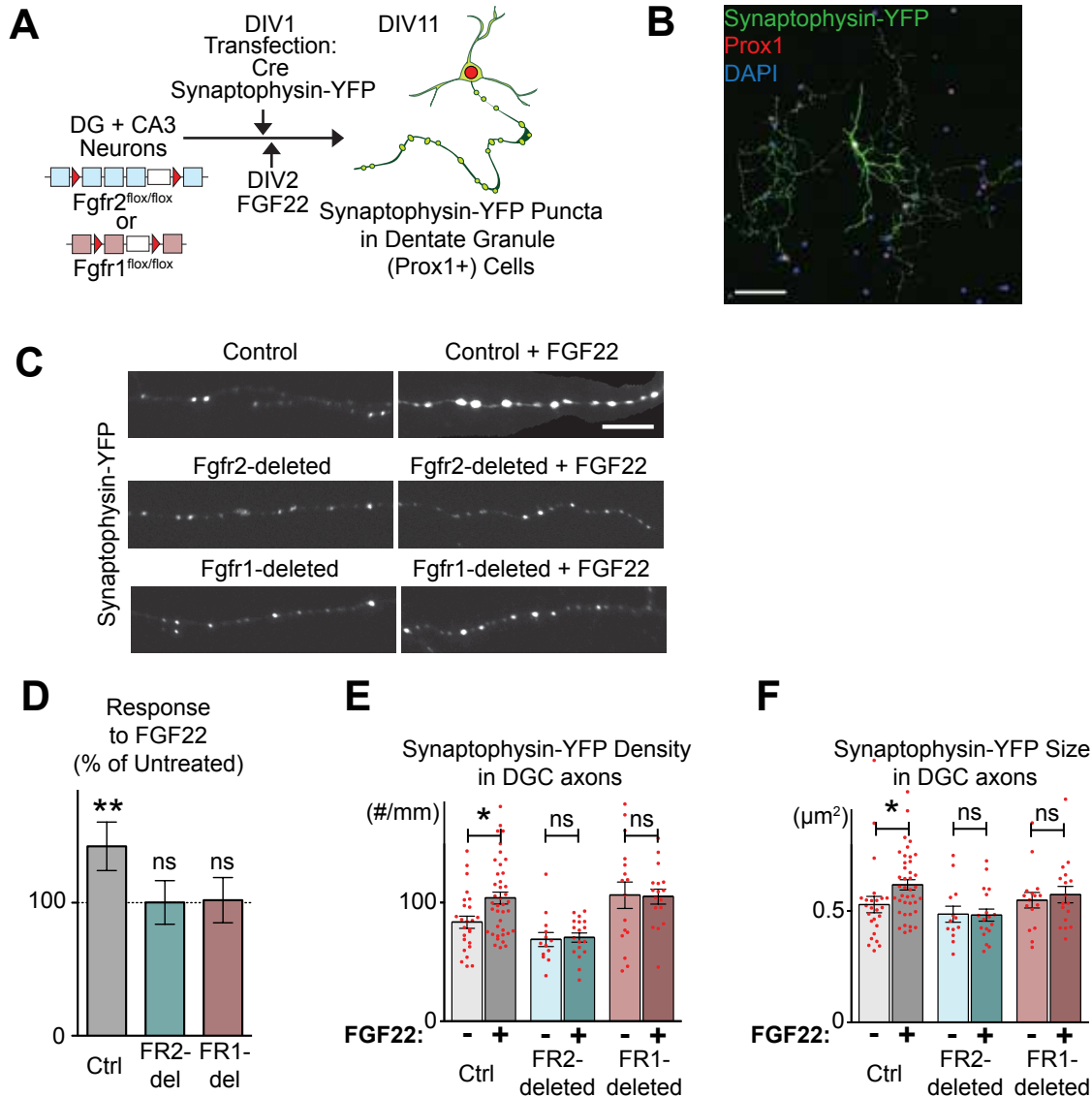
Figure 3.4. Selective deletion of Fgfr2 or Fgfr1 in dentate granule cells eliminates response to FGF22.

(A) Experimental scheme. DG and CA3 neurons were cultured from Fgfr2flox/flox or Fgfr1flox/flox pups. At DIV1, neurons were sparsely transfected with Cre to induce Fgfr deletion (Fgfr2-deleted, or Fgfr1-deleted) and with Synaptophysin-YFP to label synaptic vesicles. Control transfections consisted of Synaptophysin-YFP and empty vector without Cre. Neurons were treated with 3 nM FGF22 on DIV2. At DIV11, neurons were stained for Prox1 to identify DGCs. Synaptophysin-YFP puncta in axons from discretely transfected DGCs were analyzed.
(B) Example of Synaptophysin-YFP-transfected DGC.
(C-F) Fgfr2-deleted and Fgfr1-deleted DGCs do not respond to FGF22. (C) Representative images of Synaptophysin-YFP in DGC axons. (D) Quantification of FGF22 responsiveness, calculated as total Synaptophysin-YFP signal per mm of axon in FGF22-treated DGCs divided by that of untreated DGCs. (E) Synaptophysin-YFP density and (F) size within DGC axons. (n=[neurons, experiments] 25, 7 Control; 40, 7 Control+FGF22; 13, 3 Fgfr2-deleted; 18, 3 Fgfr2-deleted+FGF22; 16, 4 Fgfr1-deleted; 17, 4 Fgfr1-deleted+FGF22) Scale bars in (B) 100 µm, (C), 10 µm.
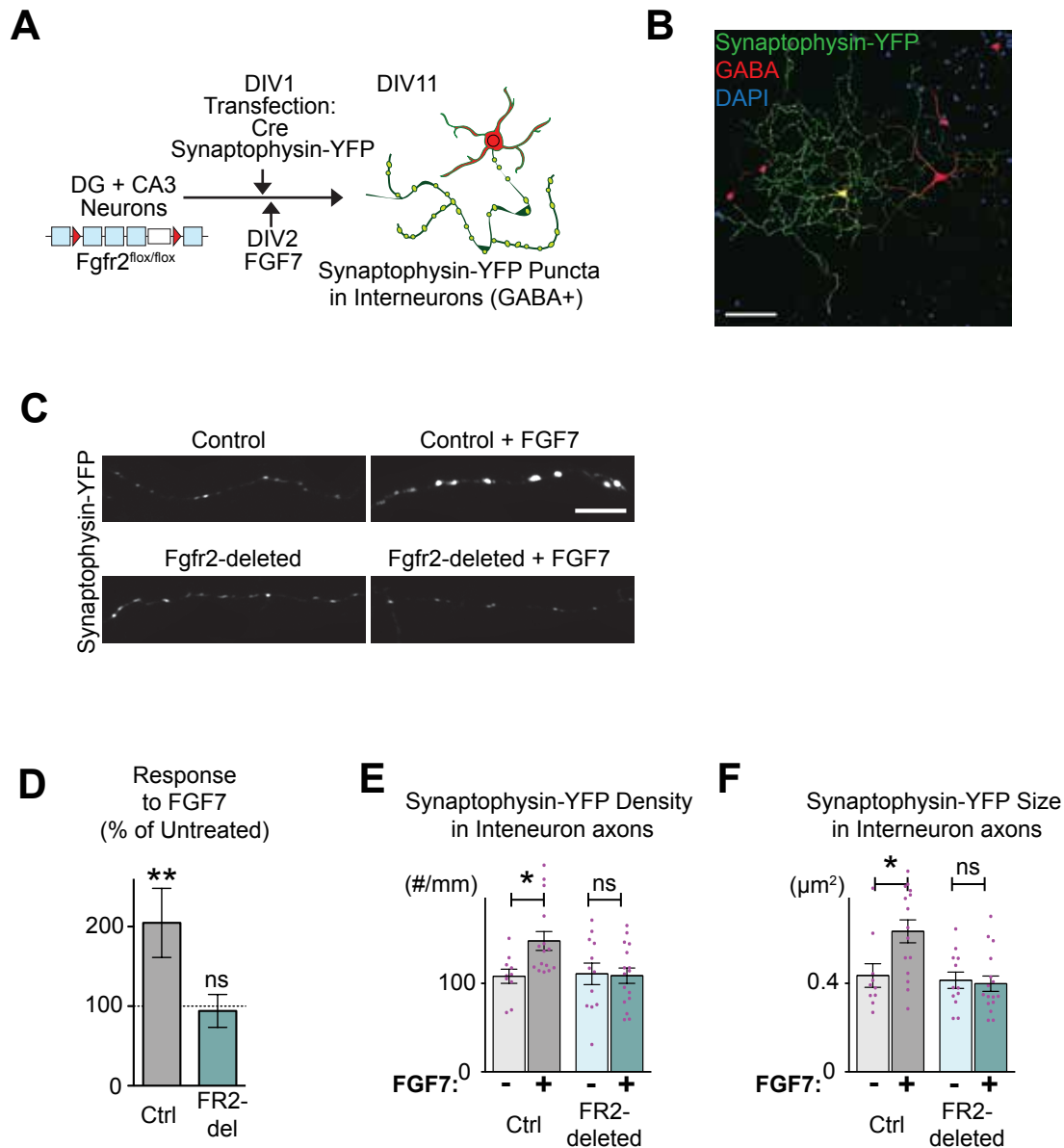
Figure 3.5. Selective deletion of Fgfr2 in interneurons eliminates response to FGF7.
(A) Experimental scheme. DG and CA3 neurons were cultured from Fgfr2flox/flox pups. At DIV1, neurons were sparsely transfected with Cre and Synaptophysin-YFP. 3 nM FGF7 was applied on DIV2. At DIV11, neurons were stained for GABA to identify interneurons. Synaptophysin-YFP puncta in axons from discretely transfected interneurons were analyzed. (B) Example of Synaptophysin-YFP-transfected interneuron.
(C-F) Fgfr2-deleted interneurons do not respond to FGF7. (C) Representative images of Synaptophysin-YFP in interneuron axons. (D) Quantification of FGF7 responsiveness, calculated as total Synaptophysin-YFP signal per mm of axon in FGF7-treated interneurons divided by untreated interneurons. (E) Synaptophysin-YFP density and (F) size within interneuron axons. (n=[neurons, experiments] 10, 3 Control; 15, 3 Control+FGF7; 12, 3 Fgfr2-deleted; 16, 3 Fgfr2-deleted+FGF22). Scale bars in (B) 100 μm, (C), 10 μm.

Lemmon and Schlessinger, 2010). Phosphorylation sites within the intracellular domain also provide docking sites for adaptor proteins, such as FRS2, PI3K and PLCγ, which activate downstream signaling pathways, such as the MAPK/ERK and AKT by FRS2, AKT by PI3K, or IP3/DAG/Ca^{2+} by PLCγ (Mohammadi et al., 1992; Mohammadi et al., 1996; Burgar et al., 2002; Hart et al., 2001; Songyang et al., 1993; Hadari et al., 1998; Ong et al., 2001). In order to study signaling pathways specifically downstream of FGFR2b, we created FGFR2b mutant constructs to perturb specific downstream signaling (Figure 3.6): a kinase dead (KD) mutant and mutants lacking the FRS2, PI3K, or PLCγ binding site. I performed two sets of experiments: in the first, I overexpressed the mutant FGFR2b constructs in WT neurons (Figure 3.7), and in the second I performed a rescue experiment in FGFR2-deleted neurons (Figure 3.8). FGFR2b constructs were co-transfected with synaptophysin-YFP into DGC-enriched WT neuronal cultures at DIV1, then treated with FGF22 at DIV2 and fixed at DIV11 (Figure 3.7 A). DGCs transfected with empty vector (control) responded to FGF22 (Figure 3.7 B, C) with an increase in the density (Figure 3.7 D) and size (Figure 3.7 E) of synaptophysin puncta. DGCs overexpressing the FGFR2b-KD, FGFR2b-FRS2, or FGFR2b-PI3K mutants did not respond to FGF22 in terms of the density or size of synaptophysin-YFP, while DGCs overexpressing FGFR2b-PLCγ remained fully responsive to FGF22 (Figure 3.7 B-D). Next, I examined whether the mutated FGFR2b constructs can restore FGF22-induced Synaptophysin-YFP response in Fgfr2-deleted DGCs. We co-transfected Fgfr2b constructs with Cre and Synaptophysin-YFP into DG+CA3 Fgfr2$^{flox/flox}$ neuronal cultures at DIV1, treated with FGF22 at DIV2, and assessed Synaptophysin-YFP clustering at DIV11 (Figure 3.8 A). As in Figure 3.4, DGCs transfected with non-Cre plasmid (control) responded to FGF22, while Fgfr2-deleted DGCs did not (Figure 3.8 B, C). Expression of FGFR2b-WT in Fgfr2-deleted DGCs rescued FGF22-responsiveness, but expression of FGFR2b-KD, FGFR2b-FRS2-deficient, or FGFR2b-PI3K-deficient mutants did not. Interestingly, FGFR2b-PLCγ-deficient rescued FGF22-responsiveness (Figure 3.8 C), although only the rescue of Synaptophysin-YFP density (Figure 3.8 D) and not size (Figure 3.8 E) was statistically significant. These data indicate that kinase activity of FGFR2b is required to respond to FGF22, and that FGFR2b requires signaling downstream of FRS2 and PI3K to induce excitatory presynaptic differentiation in response to FGF22.
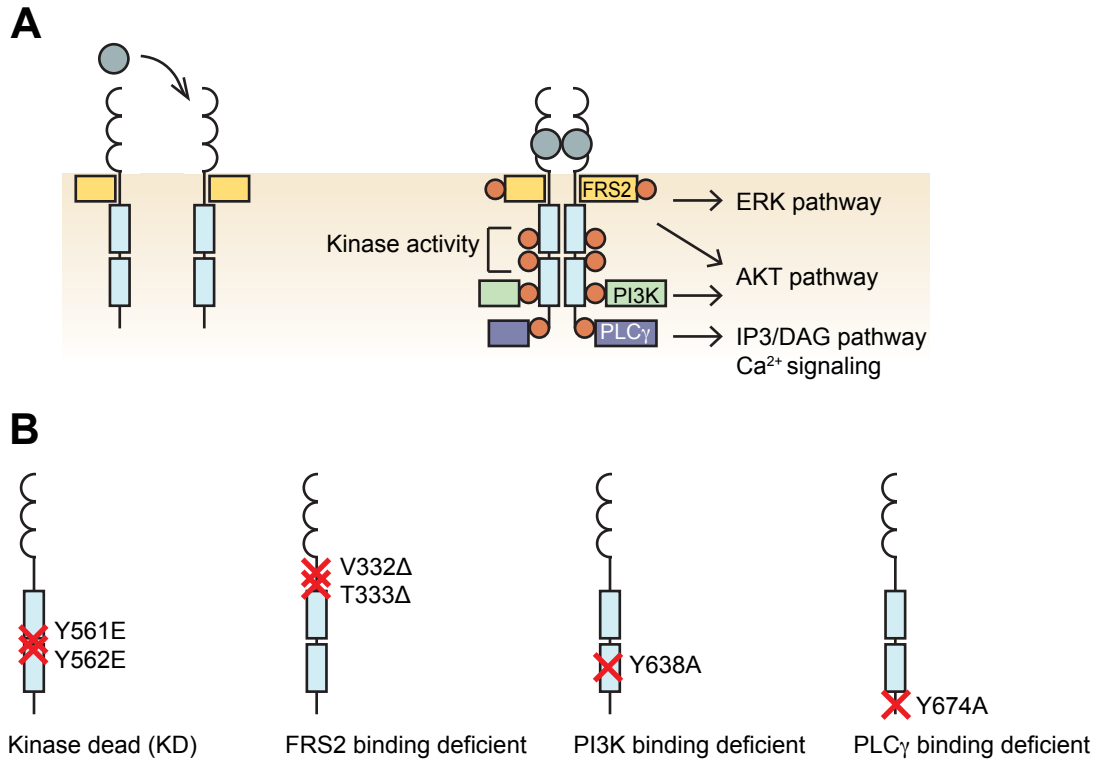
Figure 3.6. Schematic of FGFR2b mutants.
(A) FGF activates FGFR2b upon binding, causing dimerization and cross-autophosphorylation of the FGFR2b subunits. Phosphorylation sites provide docking sites for adaptor proteins, which are then phosphorylated and activated, ultimately leading to signaling through ERK, AKT, and PLCγ signaling pathways.
(B) Schematic of mutations: FGFR2b-KD: Y561E and Y562E mutations to inactivate kinase activity. FGFR2b-FRS2-deficient: V332 and T333 deletion to remove FRS2 binding site. FGFR2b-PI3K-deficient: Y638A mutation to remove PI3K binding site. FGFR2b-PLCγ-deficient: Y674A mutation to remove PLCγ binding site.

Figure 3.7. *(Following page)* **FRS2 and PI3K signaling is required downstream of FGFR2b for presynaptic response to FGF22 on a WT background in DGCs.** (A) Schematic of experiment. CA3 and DGCs were cultured from WT P0 pups. At DIV1, the neurons were sparsely transfected with Fgfr2b mutant constructs and synaptophysin-YFP. Control transfections consisted of synaptophysin-YFP and empty vector without Fgfr2b. Neurons were treated with FGF22 at DIV2. At DIV11, neurons were fixed and stained for Prox1 to identify DGCs. (B-E) DGCs transfected with Fgfr2b-KD, Fgfr2b-FRS2-deficient, and Fgfr2b-PI3K-deficient constructs do not respond to FGF22. (B) Representative images of synaptophysin-YFP in axons from DGCs. (C) Total measured binary signal per axon length displays total FGF22-responsiveness in control and Fgfr2b-PLCγ groups. (D) Quantification of synaptophysin-YFP puncta density in axons. (E) Quantification of average size of synaptophysin-YFP puncta. (n = Control: 23 neurons, Control + FGF22: 23 neurons, over 5 experiments; Fgfr2b-KD: 23 neurons, Fgfr2b-KD + FGF22: 27 neurons, over 4 experiments; Fgfr2b-FRS2-deficient: 15 neurons, Fgfr2b-FRS2-deficient + FGF22: 16 neurons, over 3 experiments; Fgfr2b-PI3K-deficient: 10 neurons, Fgfr2b-PI3K-deficient + FGF22: 9 neurons, over 3 experiments; Fgfr2b-PLCγ-deficient: 11 neurons, Fgfr2b-PLCγ-deficient + FGF22: 14 neurons, over 3 experiments). *$p<0.05$, and "ns" not significant ($p>0.05$) using Student's t test to compare FGF22-treated with untreated neurons, within each transfection group. Data represents mean ± s.e.m. In scatter plots, each dot represents average value from an individual DGC analyzed. Scale bar in (B), 10 µm.

A DG+CA3 Neurons → DIV1 Transfection: Fgfr2b Construct Synaptophysin-YFP → DIV2 FGF22 → DIV11

Synaptophysin-YFP Puncta in Dentate Granule (Prox1+) Cells

B Synaptophysin-YFP

No FGF | +FGF22

Control

Fgfr2b-KD

FgfrR2b-FRS2-deficient

Fgfr2b-PI3K-deficient

Fgfr2b-PLCγ-deficient

C Total response to FGF22 (Normalized to Ctrl-No FGF)

FGF22: − + − + − + − + − +
Ctrl KD FRS2 PI3K PLCγ

D Synaptophysin-YFP Density in DGC axons (#/mm)

FGF22: − + − + − + − + − +
Ctrl KD FRS2 PI3K PLCγ

E Synaptophysin-YFP Size in DGC axons (μm²)

FGF22: − + − + − + − + − +
Ctrl KD FRS2 PI3K PLCγ

Figure 3.8. *(Following page)* **FRS2 and PI3K signaling is required downstream of FGFR2b for presynaptic response to FGF22.**

(A) Experimental scheme. DG and CA3 neurons were cultured from Fgfr2flox/flox pups. At DIV1, neurons were sparsely transfected with Cre, Fgfr2b constructs, and Synaptophysin-YFP. Empty vector was used as a control. Neurons were treated with FGF22 at DIV2 and stained at DIV11 for Prox1 to identify DGCs. Synaptophysin-YFP puncta in axons from discretely transfected DGCs were analyzed.

(B-E) Expression of FGFR2b-WT or FGFR2b-PLCγ-deficient, but not FGFR2b-KD, FGFR2b-FRS2-deficient, or FGFR2b-PI3K-deficient restores FGF22 responsiveness in Fgfr2-deleted DGCs. (B) Representative images of Synaptophysin-YFP in DGC axons.

(C) Synaptophysin-YFP response to FGF22, quantified as in Fig. 9D. (D) Synaptophysin-YFP density and (E) size within DGC axons. (n=[neurons, experiments] 7, 3 Control; 7, 3 Control+FGF22; 6, 3 FGFR2-deleted; 10, 3 FGFR2-deleted+FGF22; 13, 3 FGFR2b-WT; 18, 3 FGFR2b-WT+FGF22; 8, 3 FGFR2b-KD; 7, 3 FGFR2b-KD+FGF22; 9, 3 FGFR2b-FRS2-deficient; 13, 3 FGFR2b-FRS2-deficient+FGF22; 12, 3 FGFR2b-PI3K-deficient; 13, 3 FGFR2b-PI3K-deficient+FGF22; 13, 3 FGFR2b-PLCγ-deficient; 13, 3 FGFR2b-PLCγ-deficient+FGF22). Scale bar, 10 μm.

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A

DIV1
Transfection:
Cre
Fgfr2b Construct
Synaptophysin-YFP

DIV11

DG + CA3
Neurons

Fgfr2^flox/flox

DIV2
FGF22

Synaptophysin-YFP Puncta
in Dentate Granule (Prox1+) Cells

B

No FGF   +FGF22

Control

Fgfr2-deleted

WT-FGFR2b rescue

FGFR2b-KD rescue

FGFR2b-FRS2-deficient rescue

FGFR2b-PI3K-deficient rescue

FGFR2b-PLCγ-deficient rescue

Synaptophysin-YFP

C

Total response to FGF22
(Normalized to Ctrl-No FGF)

** * **

FGF22: - +   - +   - +   - +   - +   - +   - +
        Ctrl   Cre   Cre   Cre   Cre   Cre   Cre
        +   +   +   +   +   +   +
        Ctrl   Ctrl   WT   KD   FRS2   PI3K   PLCγ
                                    -def   -def   -def

D

Synaptophysin-YFP Density in DGC axons

(#/mm)

*   ns   ns   ns   ns   ns   **

FGF22: - +   - +   - +   - +   - +   - +   - +
        Ctrl   Cre   Cre   Cre   Cre   Cre   Cre
        +   +   +   +   +   +   +
        Ctrl   Ctrl   WT   KD   FRS2   PI3K   PLCγ
                                    -def   -def   -def

E

Synaptophysin-YFP Size in DGC axons

(µm²)

*   ns   ns   ns   ns   ns   ns

FGF22: - +   - +   - +   - +   - +   - +   - +
        Ctrl   Cre   Cre   Cre   Cre   Cre   Cre
        +   +   +   +   +   +   +
        Ctrl   Ctrl   WT   KD   FRS2   PI3K   PLCγ
                                    -def   -def   -def

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<u>Discussion</u>.

While synaptogenic molecules such as FGFs, Wnts, neurotrophins, Eph/ephrins, neurexins/neuroligins, and LRRTMs have been identified (Dai and Peng, 1995; Umemori et al., 2004; Terauchi et al., 2010; de Wit et al., 2011; Dickins and Salinas, 2013; Park and Poo, 2013; Siddiqui and Craig, 2011; Xu and Henkemeyer, 2012), the underlying mechanisms through which they organize presynaptic differentiation are still being investigated. In this chapter, I demonstrated, using both *in vivo* and *in vitro* evidence, that FGFRs function within presynaptic neurons to respond to target-derived FGF22 or FGF7 (DGCs or interneurons, respectively), and that FGFR2b utilizes FRS2 and PI3K signaling to promote synaptic vesicle accumulation. Together, I provide novel insights into mechanisms of excitatory and inhibitory presynaptic differentiation in the mammalian hippocampus that are critical to proper brain function.

I found that FGFR2b requires kinase activity as well as binding of FRS2 and PI3K for synaptic vesicle recruitment in response to FGF22 (Figures 3.7 and 3.8). All three are upstream of AKT signaling, and because the effect of mutating any of these three pathways had an equal effect in our experiments, it is reasonable to speculate that AKT signaling is involved in excitatory presynaptic differentiation downstream of FGFR2b. However, phosphorylation of FRS2 by FGFRs activates not only the PI3K/AKT pathway (through Grb2/Gab1 binding; Ong et al., 2001), but also the MAPK/ERK pathway (through Grb2/Shp2 binding; Hadari et al., 1998). Indeed, deletion of FGFR1 and/or FGFR2 leads to decreases in phospho-MAPK/ERK and phospho-AKT levels (Emmenegger et al., 2013, Zhao et al., 2008, Cai et al., 2013, Hoch and Soriano, 2006, Loilome et al., 2007, Kondo et al., 2007), and overexpression of FGFR2b or FGFR1 increases both phospho-AKT and phospho-ERK levels (Cha et al., 2008, Freeman et al., 2003, Acevedo et al., 2007). It is known that crosstalk can occur between the PI3K/AKT and MAPK/ERK pathways (Moelling et al., 2002), so possibly the MAPK/ERK pathway is being triggered congruently. Indeed, a previous study linked FGF2-induced synaptogenic activity to the MAPK/ERK pathway: application of MAPK/ERK specific inhibitors blocked FGF2's synaptogenic effect (Li et al., 2002). Finally, recent evidence suggests a role for WAVE regulatory complex (WRC) in the regulation of actin cytoskeletal dynamics downstream of synaptic cell adhesion molecules (Chia et al., 2014; Chen et al., 2014). Interestingly, FRS2

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contains a putative WRC binding consensus sequence (Chen et al., 2014), and PI3K can contribute to actin cytoskeletal dynamics (Cain and Ridley, 2009). An interesting possibility is that FGFR2b-FRS2/PI3K signaling may be controlling the local actin environment at the presynaptic terminal for presynaptic differentiation at the site of activation. Finally, two interesting questions remain: Does FGFR1b act through the same or distinct signaling pathways to control excitatory presynaptic differentiation? And do distinct signaling pathways contribute to FGFR-dependent excitatory and inhibitory presynaptic differentiation? In chapter 5, I will explore these and other questions that stem from my new insights into FGF-induced excitatory and inhibitory presynaptic differentiation.

CHAPTER IV

FGF-induced Gene Expression and Cell-Wide Changes in Neurons

<u>Introduction</u>

In the previous two chapters, I presented data describing the mechanisms of localized FGF22 and FGF7 signaling in inducing presynaptic differentiation in the CA3 region of the hippocampal circuit. The hippocampal circuit is grossly organized as a feed-forward loop (Figure 1.1): entorhinal cortex sends projections into the hippocampal circuit that synapse mainly on the dentate gyrus, dentate gyrus cells send projections to CA3 and are one major source of excitatory input into CA3, although CA3 pyramidal neurons also connect with each other and receive input from the entorhinal cortex; then, simplistically, CA3 projects to the CA1 region and CA1 projects to subiculum (Witter, 2007). FGF22 and FGF7 are expressed in CA3 at P8 and function during early stages of synapse development, although FGF22 is also expressed in the infrapyramidal blade of the dentate gyrus at this age (Terauchi et al., 2010). FGF22 requires FGFR2b and FGFR1b in dentate granule cells to induce excitatory presynaptic differentiation; FGF7 utilizes FGFR2b in interneurons to induce inhibitory presynaptic differentiation (Dabrowski et al., 2015). Furthermore, I showed that FGFR2b, at least in the context of activation by FGF22, requires kinase activity and signaling that is downstream of FRS2 and PI3K to recruit synaptic vesicles, suggesting that synaptogenic FGFs engage signaling pathways downstream of FGFRs (Dabrowski et al., 2015). FGFR signaling is known to control important shifts in gene expression during development, that underlie cell differentiation (Dailey et al.,

2005; Olivera-Martinez et al., 2014). This means that synaptogenic FGF signaling is uniquely poised to have specific trophic roles in hippocampal neural circuit development: specifically, synaptogenic FGFs may trigger changes in gene expression that could have broader effects on hippocampal circuitry, including through control of the neurogenic niche in the dentate gyrus and induction of dendritic development in dentate granule cells and interneurons. In this chapter, I will start by framing the question of "coordinated synapse development" (see Figure 1.1 D and Figure 4.1) using neurotrophic signaling in the peripheral nervous system as an example of a synaptogenic signal that has cell-wide effects and then briefly describe the reciprocal relationship of gene expression and synapse development. Ultimately, I will present some preliminary data and my experimental strategy for exploring a more cell-wide effect of FGF signaling guiding neuronal integration into the hippocampal neural circuit.

In the peripheral nervous system, target-derived neurotrophic factors guide numerous aspects of integrating the recipient presynaptic neuron into the circuit. Target-derived signals can communicate the amount of target that needs to be innervated, the identity of the target, and the state of maturation of the target. In the periphery, target-derived cues are important for protection from developmental apoptosis, axon targeting, presynaptic development, neurotransmitter identity, cell body size, dendrite growth and postsynaptic maturation (Chun and Patterson, 1977; Voyvodic, 1989; Landis, 1990; Sharma et al., 2010; Harrington and Ginty, 2013). The fact that target-derived cues can both promote presynaptic maturation at the site of signaling, but also have distant function in inducing an appropriate level of postsynaptic maturation on the dendrites of the same cell provides a mechanism for how synapse development in one region can affect synapse development in a distant region, spanning distal tips of the cell. Not all of these changes are induced through gene expression: Target-derived NGF promotes postsynaptic development in the dendrites of the recipient neuron through a mechanism involving trans-neuronal trafficking of the entire NGF-TrkA signaling endosome from the axon terminal into the dendrites (Sharma et al., 2010). However, in other instances, gene expression induced by target-derived cues appears to be crucial for target-derived effects on dendrites. Motor neurons respond to a target-derived cue, GDNF, which induces Pea3 transcription factor activation and changes the dendritic morphology of the motor neurons in the spinal cord; furthermore this change in dendritic morphology is accompanied by a circuitry rearrangement, with a shift in the presynaptic partners (Vrieseling and Arber, 2006). Target derived BMP signaling controls the correct sensory
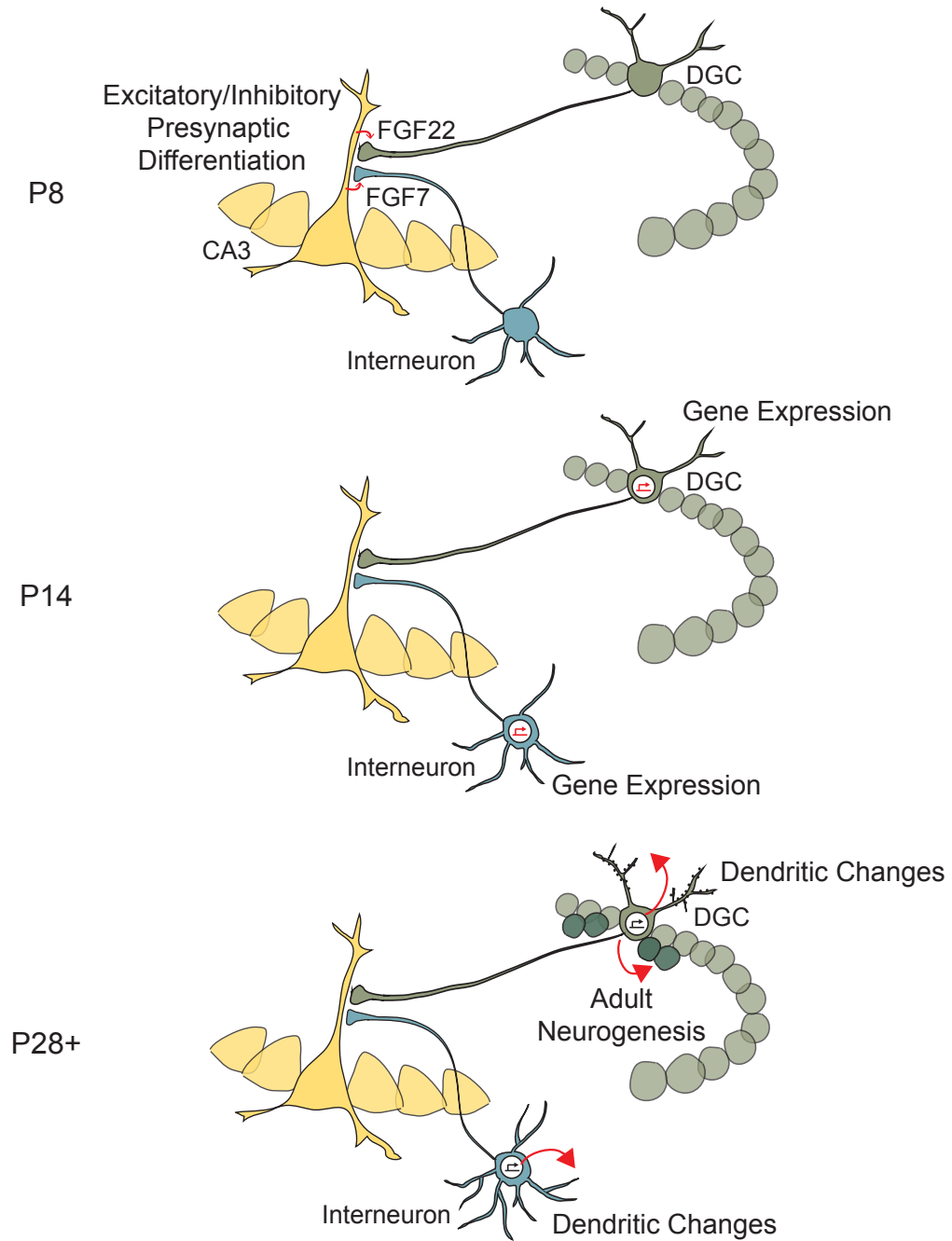
Figure 4.1. Model for target-derived role of synaptogenic FGFs in promoting neuronal integration into the hippocampal neural circuit.
At P8, CA3-derived FGF22 induces excitatory presynaptic differentiation and FGF7 inhibitory presynaptic differentiation in CA3. Synaptogenic FGFs may drive changes in gene expression in DGCs and interneurons at P14. By P28, FGF-induced gene expression can lead to changes throughout the cell, including dendritic changes in both DGCs and interneurons, and adult neurogenesis in the dentate gyrus.

innervation of the face by the trigeminal nerve through gene expression by regulating the transcription factor Onecut, which is required for correct targeting of sensory neuron projections (Hodge et al., 2007). Thus, when contacting the periphery, neurons repeatedly use target-derived growth factors to induce gene expression programs that are important for aspects of appropriate neural circuit formation. A similar function has yet to be shown between neurons of the central nervous system.

Gene expression and synapse development have a reciprocal relationship. In the hippocampus, there are drastic shifts in gene expression over the course of development from embryonic stages to the mature circuit, and many of these genes are involved in synapse development or function (Mody et al., 2001). Recently, a chromatin regulator, nucleosome remodeling and deacetylation (NuRD) complex was shown to control the expression of multiple genes involved in presynaptic differentiation in the cerebellum, and loss of NuRD led to impaired synaptogenesis in the cerebellum (Yamada et al., 2014). The molecular cues that promote drastic shifts in the trancriptome are still incompletely understood, but the shifts in the transcriptome reflect the changing requirements of the neuronal circuit. Synapse development can induce changes in gene expression, both through intracellular signaling pathways and due to synaptic activity itself. At the neuromuscular junction, neuregulin, which promotes synapse formation, induces gene expression of acetylcholine receptors in the postsynaptic muscle through ErbB2 and ErbB3 signaling (Jo et al., 1995). In neuronal cultures, EphB, a synaptogenic molecule, phosphorylates the NMDA glutamate receptor subunit GluN2B and costimulation with ephrinB2 and glutamate leads to gene expression of BDNF and CPG15, both of which have been in turn implied in synapse development (Takasu et al., 2002). Furthermore, synaptic activity triggers intracellular calcium waves in the postsynaptic neuron that induce gene expression through activation of CREB (Armstrong and Montminy, 1993; Bito et al., 1996; Hardingham et al., 2001; Flavell and Greenberg, 2008). Developmental transcription programs drive genes that are required for synapse development, while synapse development in turn triggers gene expression.

Another aspect of dentate gyrus development that target-derived FGF22 from the CA3 could be regulating is adult neurogenesis. A striking feature of the hippocampal circuit is that the subgranular zone in the dentate gyrus is one of a few areas where neurogenesis is maintained in the adult brain (Duan et al., 2008; Suh et al., 2009). Adult neural stem cells have the capacity to

differentiate into astrocytes or into immature DGCs; their maintenance requires careful regulation of the neurogenic niche and of the undifferentiated state of the neural stem cells through a combination of extrinsic and intrinsic signals (Duan et al., 2008; Suh et al., 2009). Furthermore, the neurogenic niche is highly regulated by synaptic inputs (Suh et al., 2009). Decreased neurogenesis correlates with depression (Sahay and Hen, 2007), and certain insults, such as epileptic seizures, aberrantly increase rates of adult neurogenesis, leading to abnormal circuit connectivity (Parent et al., 1997). Thus understanding how adult neurogenesis is regulated within the developing circuit has been of extreme interest (Jessberger and Gage, 2014). Adult (five month old) FGF22-KO mice have a decreased number of proliferating cells and immature neurons in the dentate gyrus compared to their WT counterparts (Lee and Umemori, 2013), suggesting a failure of maintaining the neurogenic niche with loss of FGF22.

FGF signaling regulates gene expression at multiple stages of development and in multiple organs (Dailey et al., 2005, Guillemot and Zimmer, 2011). FGF signaling causes phosphorylation of transcription factors, synthesis of new transcription factors, chromatin remodeling, and repressor recruitment (Dailey et al., 2005). FGF-induced gene expression is required to specify neural tissue (Streit et al., 2000), through activation of ERK (but not AKT) signaling (Stavridis et al., 2007), leading to upregulation of Sox genes, which are transcription factors required for neuronal cell identity (Pevny and Placzek, 2005). FGF signaling directly induces changes in chromatin regulation through controlling expression of the histone deacetylase HDAC1; expression of HDAC1 can lead to chromatin remodeling that may direct the switch of gene expression towards neural induction (Olivera-Martinez et al., 2014). FGF8 induces cerebellar fate in rhombomere 1 and an induction of a clear midbrain-hindbrain border during early neural development through multilevel control of gene expression, including upregulation of FGF18 and FGF17 signaling and induction of negative feedback (Liu et al., 2003). FGF signaling drives early neuronal development through controlling gene expression. Thus, later, synaptogenic FGF signaling could also regulate gene expression in dentate granule cells (in response to FGF22) and in interneurons (in response to FGF7).

What gene-expression driven cell-wide aspects of DGC or interneuron development could FGF22 and FGF7 be coordinating? Given that both FGF22 and FGF7 induce presynaptic differentiation, it is likely that synaptogenic FGF signaling induces genes important for presynaptic maintenance. However, following the example of target-derived neurotrophic effects

in the peripheral nervous system, FGF22 and FGF7 could also promote dendrite growth and branching, postsynaptic differentiation (including maturation of dendritic spines), and in the case of FGF22, secretion of factors that promote maintenance of the neurogenic niche. Indeed, some evidence has surfaced for long-lasting effects of loss of FGF22 and FGF7 on neural circuit formation: Firstly, FGF22-KO and FGF7-KO mice have altered seizure susceptibility as adult mice, suggesting that early changes in excitatory/inhibitory balance have a long-lasting impact (Terauchi et al., 2010). Furthermore, FGF7-KO mice display a rearrangement of the mossy fiber circuit from DGCs and an increase in the rate of adult neurogenesis by 2 months of age in the absence of seizures, that nonetheless histologically resembles a brain after experiencing seizures (Lee et al., 2012). Finally, FGF22-KO mice have decreased rates of adult neurogenesis (Lee and Umemori, 2013). Here, I will describe changes in DGC dendritic spines in young adult FGF22-KO mice, and describe our strategy for identifying FGF22-induced gene expression in DGCs and FGF7-induced gene expression in interneurons.

Methods

Mice. FGF22-KO and FGF7-KO mice were described previously (Terauchi et al., 2010), Thy1-GFP-M mice are available at Jackson (Feng et al., 2000), VGAT-Venus mice have been previously described (Wang et al., 2009). All mice were on a C57BL/6 background. All animal care and use was in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committees at Boston Children's Hospital and University of Michigan.

FGF22 gene expression. To assess what genes are induced in DGCs by FGF22 (Figure 4.2), gene expression in DGCs was compared between FGF22-KO mice and WT mice at P14, the time of transition between early and more mature patterns of activity (Leinekugel et al., 2002). DGCs were isolated by microdissecting out the cell layer of the dentate gyrus. RNA was extracted, and ran on a microarray by a previous graduate student, Aaron Reifler. I later confirmed expression levels of selected genes by microdissecting the dentate granule cell layer from two WT and four FGF22-KO mice at P14, isolating RNA and performing quantitative real-time PCR (QPCR) using Sybr Green QPCR kit (Biorad) and a Biorad QPCR machine.

FGF7 gene expression. To assess what genes are induced in interneurons by FGF7 (Figure 4.2), gene expression was compared in CA3 interneurons between FGF7-KO and WT mice at P14, the time of transition between early and more mature patterns of activity (Leinekugel et al., 2002). The FGF7-KO and WT mice were crossed with VGAT-Venus mice, in which Venus-GFP is expressed in interneurons. To isolate interneurons, CA3 was dissected from P14 mice and cells were dissociated using a papain-based protocol (Brewer and Torricelli, 2007). Briefly, CA3 was dissected out of brains in a mammalian ringers solution (1.38 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM HEPES) supplemented with 5% trehalose (MR-TH), which increases neuron survival (Saxena et al., 2012). The CA3 was then finely chopped into 7-10 pieces and placed into 1 ml HABG-TH (5% trehalose, 2% B27 supplement, 0.5 mM L-glutamine in Hibernate-A medium) until ready for dissociation. The CA3 fragments were next transferred into a papain solution (34 units papain/ml, 0.05 mM AP5, 0.5 mM Glutamine, 0.005% DNaseI in MR-TH) and incubated at 30°C for 30 minutes with occasional gentle swirling. The remainder of the procedure was carried out on ice. CA3 cells were spun down at 200xg, resuspended in 1.2 ml HABG-TH, spun down and resuspended twice more, and then gently triturated 15 times using a fire-polished glass Pasteur pipette. Cells were allowed to settle for one minute, then passed through a cell strainer. Remaining fragments were resuspended in 1.2 ml HABG-TH and triturated 15 times and passed through the cell strainer. Dissociated cells were spun for 3 minutes, resuspended in 2 ml HABG-TH, spun down and finally resuspended in FACS buffer (10 mM Glucose, 10 mM HEPES, 0.1% Fraction V BSA in L15 medium). FACS sorting was performed at the Boston Children's Hospital Intellectual and Developmental Disabilities Research Center Flow Cytometry Core Facility, led by Dick Bennett and performed by Noreen Francis on a Becton Dickinson FACSAria machine. Cells that were negative for DAPI (live cells) and positivity for GFP (interneurons) were collected into 2 ml of HABG-TH. Cells were then spun for 5 minutes at 4°C to pellet the cells and total RNA was isolated using a Qiagen RNeasy kit. Total RNA was submitted to the Biopolymers Facility at Harvard Medical School for Next Gen sequencing, with amplification using a Nugen SPIA kit, cDNA library preparation; DNA-seq is being performed.

Imaging. Thy1-GFP-M (FGF22+/- or FG22-/-) and VGAT-Venus (WT or FGF7-/-) coronal sections of 50 μm – 100 μm were made on a vibratome. Sections were stained for GFP (1:5000,

A WT versus FGF22-KO:

B WT versus FGF7-KO:

Fluorescently-labeled Interneurons

Dissection of DG cell layer

Dissection of CA3
Fluorsescence-activated cell sorting

Microarray

RNA-seq
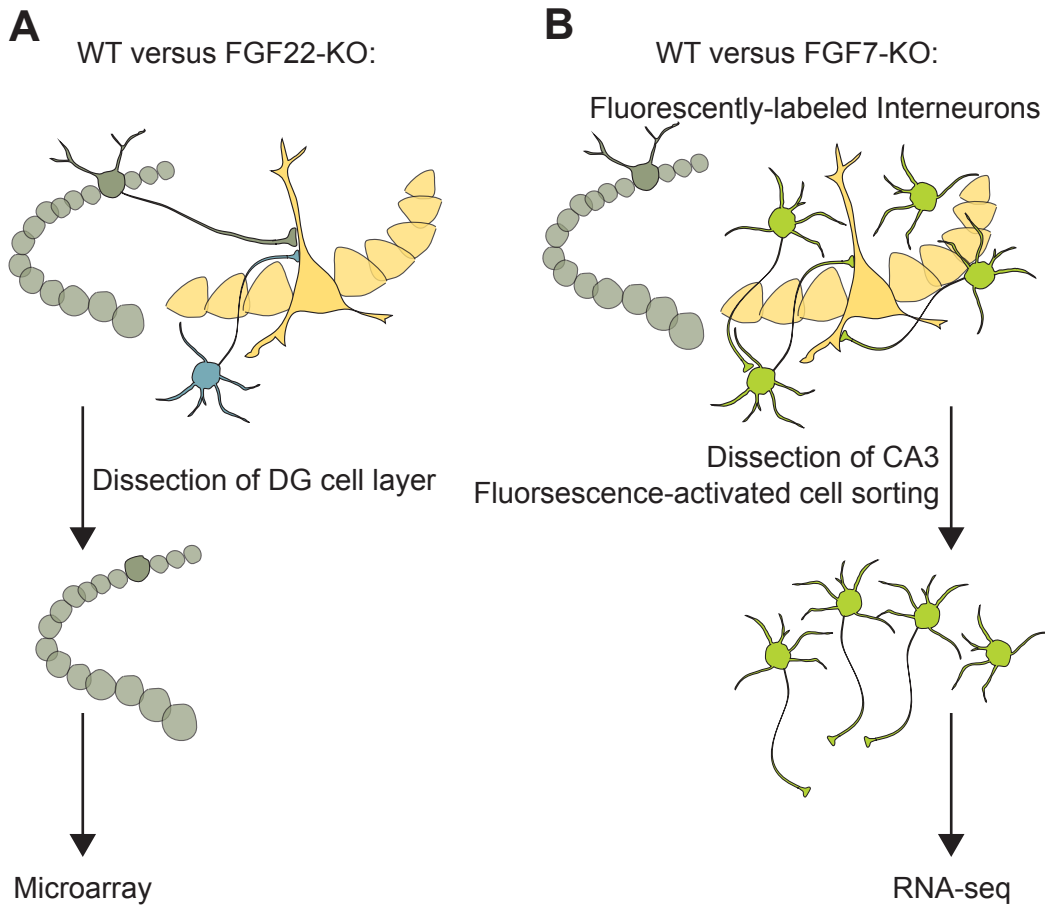
Figure 4.2. Strategy to measure FGF-induced genes.
A) To detect FGF22-induced genes in dentate granule cells, the dentate gyrus cell layer
was dissected from WT and FGF22-KO mice. RNA was isolated and prepared for
microarray to compare gene expression.
B) To detect FGF7-induced genes in interneurons, mice were crossed with a strain of
mice with fluorsescently-labeled interneurons. CA3 was dissected, and dissociated cells
were fluorescence activated cell sorted (FACS) to isolate interneurons. RNA was isolated
and prepared for RNA-seq to compare gene expression.

Aves). Images were acquired on a Zeiss LSM 700 Confocal microscope using 25x, 40x, and 63x objectives.

Results and Discussion

Loss of FGF22 leads to increased dendritic spine density in DGCs *in vivo*

FGF22 induces presynaptic differentiation in area CA3, where the primary excitatory input comes from DGCs, and I found that FGFR2 and FGFR1 function presynaptically in DGCs to respond to FGF22 (Dabrowski et al., 2015). To investigate whether DGCs from FGF22KO mice have defects in their dendritic morphology or postsynaptic development, indicative of a failure to properly integrate into the hippocampal circuit, I investigated DGC development in the FGF22KO and WT mice crossed with GFP-M mice, in which neurons sparsely and at random express GFP under the Thy1 promoter (Feng et al., 2000). I found FGF22-KO mice have an increased density of dendritic spines in the suprapyramidal blade, compared to WT mice (Figure 4.3). From my own observations across multiple FGF22-KO, FGF22-het and/or WT animals, I had the following preliminary observations: the increase in dendritic spine density is more pronounced in the suprapyramidal blade than in the infrapyramidal blade. Dendritic spine morphology is different: with longer spines that have smaller spine heads; the proportion of "mushroom-shaped" spines is lower in FGF22-KO mice than in controls. Although expression of GFP in neurons in the Thy1-GFP-M animals is sparse, it is too dense in the dentate gyrus to provide adequate resolution of dendrites from individual neurons; however, differences in dendritic morphology that I noted were: dendritic shafts are thinner in FGF22-KO than in FGF22-het or WT animals; the dendrites have more uniform thickness compared to FGF22-het or WT, which display a range of dendrite thickness. Finally, I also observed occasional ectopic dentate granule cells in the inner molecular layer of FGF22-KO dentate gyrus, but not FGF22-het or WT. Regarding the difference in dendritic spine density between suprapyramidal and infrapyramidal blades of the dentate gyrus, it is known that the two blades have slightly different anatomy and functional connectivity (Schmidt et al., 2012). Dendritic spine density tends to be higher in the suprapyramidal blade (Claiborne et al., 1990), the suprapyramidal blade receives more input from the lateral entorhinal cortex while the infrapyramidal blade receives more input

A

DG

CA3

B

FGF22-het

Animal 1   Animal 2

FGF22-KO

Animal 1   Animal 2
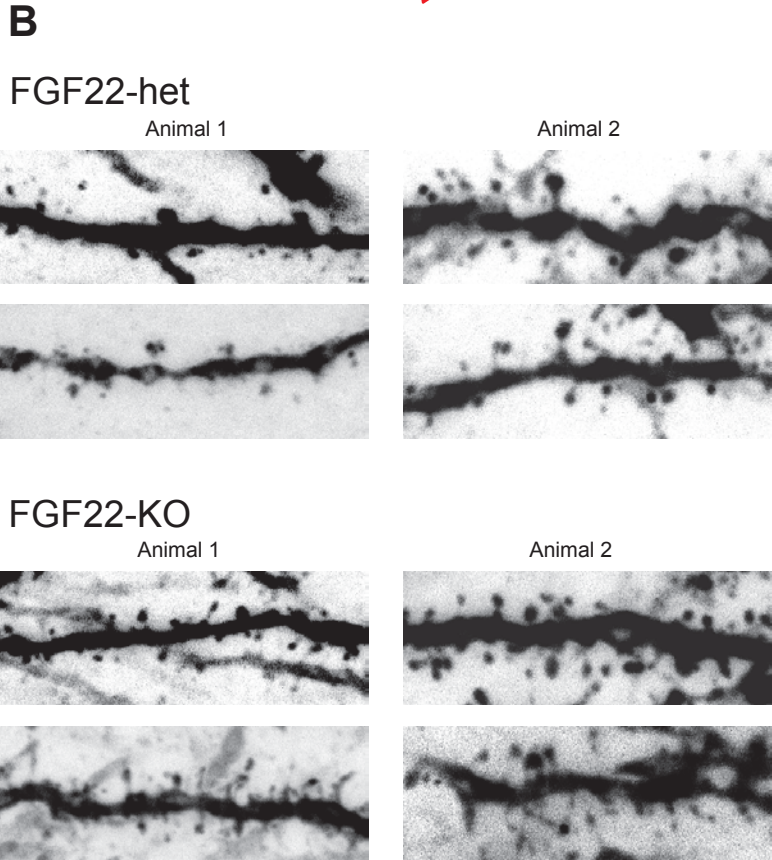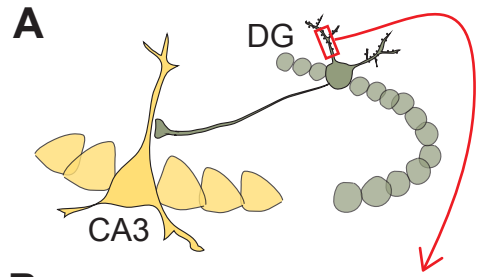
C Dendritic Spine Density
(Relative to Control)

100%

0
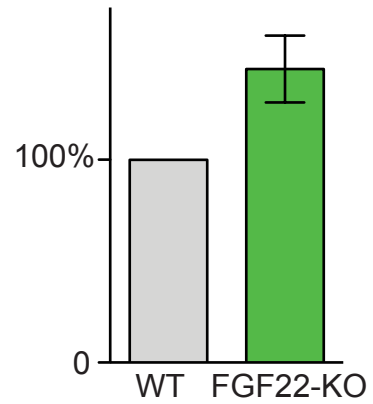
WT   FGF22-KO

Figure 4.3. Density of dendritic spines increases on FGF22-KO DGCs.
A) Partial schematic of hippocampal circuit to show molecular layer in suprapyramidal blade of dentate gyrus, where dendritic spine change is observed.
B) Example images of dendritic spines in FGF22-het and FGF22-KO (from two separate animals, each).
C) Quantification of FGF22-KO dendritic spines relative to WT.

from the medial entorhinal cortex (Squire, 1992; Tamamaki, 1997), and finally the suprapyramidal blade may have a greater role in pattern separation than the infrapyramidal blade (Schmidt et al. 2012). Thus, FGF22 may have a role in postsynaptic development of DGCs in the dentate gyrus affecting the connectivity between the entorhinal cortex and dentate gyrus. The change in synapse number could be due to an increase in functional excitatory synapses, or an increase in immature, non-functioning excitatory synapses with or without a concurrent decrease in functional excitatory synapses: thus synaptic function in dentate granule cell of FGF22-KO animals merits closer investigation.

FGF22 induces gene expression in DGCs, including genes implicated in neurogenesis and dendritic spine formation

FGF22 is expressed by CA3 pyramidal neurons and subsets of dentate granule cells in the infrapyramidal blade of the dentate gyrus at P8 (Terauchi et al., 2010). By P21, expression levels of FGF22 are markedly decreased throughout the dentate gyrus (Toth et al., 2013), suggesting that changes observed in dendritic spines of DGCs are a result of earlier FGF22 expression. One model is that early, target-derived, synaptogenic FGF22 induces changes in gene expression that lead to altered dendritic spine growth. In order to identify the genes controlled by FGF22 that could be promoting adult neurogenesis (Lee and Umemori, 2013) and dendritic spine density, we compared gene expression in FGF22KO and WT DGCs at P14, a time at which early presynaptic differentiation in response to FGF22 is ending (see Dabrowski et al., 2015), but at which FGF22 could already have induced genes important for network development in the DGCs. From the seventy candidate genes identified by microarray, I selected genes that have a significant change in gene expression on microarray (an absolute DiffScore of more than ±13, which corresponds to a p-value of 0.05) and that have been linked to neurogenesis or postsynaptic development (Figure 4.4). Many of the genes that I do not describe here have not been directly linked to dendritic spine development or neurogenesis yet, but could still play an uncharacterized role. Of note, some of the genes (BMP4, IGF2, COL1A1, COL4a1, neuronatin, Gabra2) that I selected to describe below were also identified for their patterns of expression levels over hippocampal development, from embryonic to adult (online supplemental gene expression data for Mody et al., 2001), underscoring their importance in guiding developmental shifts in the hippocampal

A

Gene	DiffScore	Protein	Expression in KO Relative to WT
Bmp4	-21.096	Bone morphogenetic protein 4 (Bmp4)	
Igf2	-23.166	Insulin-like growth factor 2 (Igf2)	
Erf	-18.133	Ets2 repressor factor (Erf)	
Zfp365 (DBZ)	-29.716	Zinc finger protein 365 (Zfp365) / DISC1-Binding Zinc finger protein (DBZ)	
Col1a1	-33.444	Procollagen, type I, alpha 1 (Col1a1)	
Col4a1	-21.645	Procollagen, type IV, alpha 1 (Col4a1)	
Col6a1	-52.859	Procollagen, type VI, alpha 1 (Col6a1)	
Lrrtm3	-33.386	Leucine rich repeat transmembrane neuronal 3 (Lrrtm3)	
Slc9a3r1 (Nhe3)	-18.583	Solute carrier family 9, isoform 3 regulator 1 (Slc9a3r1) / Sodum-hydrogen exchanger 3 (Nhe3)	
Mylk	-18.278	Myosin, light chain kinase (Mylk)	
Nnat	14.191	Neuronatin	
Adra2a	-25.554	Adrenergic receptor, alpha 2a (Adra2a)	
Gabra2	15.156	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 2 (Gabra2)	
Cd200	16.997	Cd200 antigen (Cd200)	

0   100%   200%

B

Expression in KO Relative to WT

Figure 4.4. FGF22-induced Gene expression in Dentate Granule Cells.
A) Selected genes from microarray of gene expression in dentate granule cells of FGF22-KO relative to WT at P14. Genes then confirmed by QPCR highlighted in green. Genes ordered by potential role in DGCs (as described in main text.) B) Expression of selected genes by QPCR in dentate granule cells of FGF22-KO relative to WT at P14.

network. I will describe below the rationale for how the following genes may mediate FGF22-induced changes in the hippocampal circuit, but ultimately these candidates will need to be verified experimentally for their biological function.

Among the candidate genes that control the neurogenic niche, I have selected two secreted proteins (BMP4 and IGF2) and two cytosolic proteins (ERF and DBZ) to discuss here, all of which were downregulated in FGF22KO DGCs. Furthermore, I confirmed that BMP4 and IGF2 were downregulated in FGF22KO DGCs relative to WT DGCs using QPCR (Figure 4.4).

Proteins secreted in a paracrine manner have important roles in controlling stem cell niches through regulating the proliferation and differentiation of the stem cells (Scadden, 2006; Duan et al., 2008). BMP4 and IGF2 may be secreted from mature DGCs to regulate neurogenesis in the subgranular zone. Regulation of intrinsic signaling within neural stem cells is also important for maintenance of the neurogenic niche (Duan et al., 2008). A decrease in the expression of ERF and DBZ may reflect a decrease in their expression in mature DGCs, where they could have a role in inducing extracellular and secreted proteins important for maintenance of the neurogenic niche, or they could represent a decrease in the expression of intrinsic factors within the neurogenic niche due to decreased pro-neurogenic signaling from mature DGCs.

BMP4 is a secreted growth factor that is highly expressed throughout the adult brain, and in the dentate gyrus it is highly expressed both in the granule cell layer and the molecular layer (Mikawa et al., 2006). BMP4 has a complex role in neurogenesis that is both age and concentration dependent. BMP4 inhibits neurogenesis in vitro and promotes differentiation towards a neuronal fate (Kalyani et al., 1998; Li et al., 1998; Shou et al., 1999; Howard et al., 2000; Rios et al., 2004), but at lower concentrations it promotes neurogenesis, specifically by promoting survival of newly generated cells (Shou et al., 2000; Mabie et al., 1999). In adult animals, constitutive overexpression of BMP4 led to decreased neurogenesis; in fact, BMP4 was downregulated in response to activity, which increases neurogenesis (Gobeske et al., 2009). BMP4 inhibits neural differentiation by inhibiting the activity of a neurogenic transcription factor in neural stem cells, thus maintaining their undifferentiated state (Kamiya et al., 2011). BMP4 overexpression in dentate gyrus *in vivo* slowed down the rate at which newly born DGCs matured (Bond et al., 2014); BMP4 inhibition increased neurogenesis at the cost of losing quiescent cells (Bonaguidi et al., 2005; Bond et al, 2014). This suggests that FGF22 may induce

BMP4 in the dentate gyrus to maintain neural stem cells in an undifferentiated state during adulthood.

In contrast, FGF22 may induce IGF2 to promote neurogenesis. IGF2 is a secreted growth factor that has numerous roles in brain development (Fernandez and Torres-Aleman, 2012). IGF2 in the cerebrospinal fluid promotes embryonic neurogenesis throughout the cerebral cortex – IGF2-KO animals have markedly decreased cortical size at birth (Lehtinen et al., 2011). IGF2 expressed by neural stem cells in the dentate gyrus promotes the proliferation of neural stem cells (Bracko et al., 2012). Thus, FGF22 may be important for regulating a balance between maintenance and differentiation of neural stem cells. ERF is a transcriptional repressor that inhibits neuronal precursor proliferation and promotes neural differentiation in the developing neural plate of Xenopus (Janesick et al., 2013). Decreased ERF expression in FGF22-KOs may lead to a depletion of the subgranular zone neurogenic niche before adulthood. DBZ was identified for its interaction with Disrupted-in-Schizophrenia 1 (DISC1) (Hattori et al., 2007). DISC1 promotes adult neurogenesis in response to GABA-ergic signaling in the subgranular zone (Kim et al., 2012). DISC1 is also important in slowing the rate of maturation of newborn dentate granule cells – loss of DISC1 leads to smaller soma size, abnormal dendrites, and mislocalization of newborn dentate granule cells into the molecular layer of the dentate gyrus (Duan et al., 2007). Thus, dysregulation of DBZ in FGF22-KOs may lead to dysregulation of DISC1 and a failure of immature neurons to respond appropriately to neurogenic factors, leading to defects in adult neurogenesis.

FGF22-induced genes in the dentate gyrus that control neurogenesis could reflect a highly complex switch from neurogenesis during early development to adult neurogenesis, in which the balance must be maintained between stem cell maintenance, proliferation, and neuronal differentiation. The defect in neurogenesis in FGF22-KO mice could be due to decreased proliferation of neural precursors and/or altered programs of neural differentiation in the dentate gyrus that have led to maintenance of fewer precursors. Since FGF22 is expressed in the infrapyramidal blade of the dentate gyrus at P8, (although expression is nearly gone by P21) (Terauchi et al., 2010; Toth et al., 2013), FGF22 expressed directly within the dentate gyrus could be inducing genetic programs required for the maintenance of the neurogenic niche. Indeed, there appears to be more adult neurogenesis in the infrapyramidal blade compared to the suprapyramidal blade (Snyder et al., 2009). Another interesting model is that target-derived

FGF22 from the CA3 induces expression of genes involved in maintenance of the neurogenic niche in the dentate gyrus (model outlined in Figure 4.1). Further experiments (described in Chapter 5) will be required to distinguish between the role of CA3-derived versus DG-derived FGF22, although it is possible that both are involved.

FGF22-KO mice have an increased density in dendritic spines in the molecular layer of the suprapyramidal blade in the dentate gyrus at P28 (Figure 4.3), three weeks after high expression of FGF22 in CA3 at P8 (Terauchi et al., 2010). At P8, FGF22 is also expressed in the infrapyramidal blade, but not the suprapyramidal blade where the defects were observed. I propose a model in which target-derived FGF22 from the CA3 controls changes in gene expression that then induce changes in dendritic spines of DGCs (model in Figure 4.1), although the contribution of CA3-derived versus DG-derived FGF22 still needs to be clarified (see Chapter 5 for more detail). From the microarray data, I have selected several candidate genes that may play a role in dendritic spine density: Col1a1, Col4a1, Col6a1, Lrrtm3, Nhe3, Mylk, Neuronatin, DBZ (mentioned above), Adra2a, Gabra2, and CD200. All of the genes are downregulated in FGF22KO DGCs, except for Neuronatin, Gabra2, and CD200, which are upregulated in FGF22KO. I have confirmed the changes in expression for IGF2, CD200, and LRRTM3 (Figure 4.4) using QPCR. I will describe here how these genes could be inducing a change in dendritic spine density.

Dendritic spines are the protrusions from dendrites and the postsynaptic locations of excitatory synapses. The spines are highly dynamic during development that stabilize with maturation; their morphology and number is carefully regulated by a host of molecular cues, including secreted factors, extracellular matrix proteins, cell adhesion proteins, regulators of cytoskeleton, metabolic regulators, and neurotransmitter proteins (Bhatt et al., 2009). An increase in dendritic spines in FGF22-KO may represent either an increase in mature, stable spines on the dendrites (with a concurrent increase in excitatory drive), or an increase in the number of unstable, highly motile dendritic spines (with a possible decrease in excitatory drive). Thus, loss of FGF22 could lead to loss of a negative signal for pruning away synapses, or loss of a stabilizing signal to increase mature stable synapses and decrease unstable synapses.

In terms of stabilizing cues, there are a number of FGF22-induced genes that could be important for stabilization of dendritic spines, and the loss of which could lead to an increase in unstable spines. First, careful regulation of the extracellular matrix, including composition of

collagens in the extracellular space, is important for synapse stabilization (Fox et al., 2007; Kurshan et al., 2014). Thus, FGF22-induced collagens (Col1a1, Col4a1, and Col6a1) could play important roles in stabilizing dendritic spines; their loss could lead to destabilization and an increase in immature spines. Likewise, cell adhesion molecules are important for maturation and stabilization of the postsynaptic density, including members of the leucine-rich transmembrane family (de Wit and Ghosh, 2014). Clustering of LRRTMs induces postsynaptic differentiation (Linhoff et al., 2009), thus FGF22-induced LRRTM3 may be important for stabilization of dendritic spines in DGCs. Nhe3 binds Shank2 and to the actin cytoskeleton (Han et al., 2006;), and Shank2 is a postsynaptic density protein important for postsynaptic stabilization (Boeckers et al., 2002), thus FGF22-induced regulation of Nhe3 levels in postsynaptic densities may be important for dendritic stabilization. DBZ, which binds to DISC1 (Hattori et al., 2007), may also be involved in regulating the postsynaptic density through its interaction with DISC1, which is important for regulating dendritic spines (Carlisle et al., 2011; Duan et al., 2007; Hayashi-Takagi et al., 2009). Regulation of the actin cytoskeleton is important for dendritic spine development (Bhatt et al., 2009); Mylk regulates myosin light chain, and both Mylk and myosin light chain regulate actin cytoskeleton in dendritic spines (Zhang et al., 2005; Amparan et al., 2005, Lei et al., 2001, Naisbitt et al., 2000). FGF22-induced Mylk may be important for regulating cytoskeletal dynamics to control appropriate synaptic density. Additionally, dendritic spines require regulation of local protein translation (Martin and Zukin, 2006). Neuronatin is suppressed by FGF22 (upregulated in FGF22KO DGCs compared to WT): it is a proteolipid that regulates calcium levels and induces neural fate in embryonic stem cells, and appears to control local translation in dendrites of cultured hippocampal neurons (Joseph, 2014), suggesting that upregulation of neuronatin in FGF22-KO DGCs may misregulate postsynaptic protein translation, leading to defects in dendritic spine morphology. Thus, FGF22 may regulate the density of dendritic spine on numerous levels, from extracellular matrix to control of the actin cytoskeleton and local protein synthesis in spines.

Dendritic spine density in DGCs in response to FGF22 may be a result of altered regulation of postsynaptic function in FGF22-KO DGCs. The adrenergic receptor, Adra2a, which is induced by FGF22, regulates postsynaptic function at dendritic spines in the prefrontal cortex (Wang et al., 2007). Blocking Adra2a in cultured neurons leads to increased density of spines and increased length of dendritic spines (Hu et al., 2008). On the other hand, the GABA-A

receptor subunit, Gabra2, is suppressed by FGF22 signaling and has also been implicated in dendritic spine development – loss of Gabra2 in adult born neurons in the olfactory bulb leads to decreased dendritic spine density (Pallotto et al., 2012). Thus, the increased spines in FGF22KO DGCs could be due to decreased Adra2a or increased Gabra2, through modulation of postsynaptic function and maturation.

Finally, FGF22-KO DGCs may have increased dendritic spine density through an altered interaction with microglia. Microglia have an important role in eliminating dendritic spines through phagocytosis (Salter and Beggs, 2014; Ueno and Yamashita, 2014). CD200 is a protective signal against microglial phagocytosis (Elward and Gasque, 2003). CD200 is upregulated in FGF22-KO DGCs, thus may lead to an overexpression of a protective signal on dendritic spines and a failure to eliminate them in a microglia-dependent manner, which may lead to an increase in the density of mature dendritic spines in FGF22-KO DGCs.

Synaptogenic FGFs may be acting as a target-derived cue (as in the model in Figure 4.1) to promote the integration of neurons into the neural network. Indeed, FGF22 induces changes in gene expression in DGCs (Figure 4.4), and DGCs have increased dendritic spine density (Figure 4.3). At the time of writing this chapter, the results of the RNA-seq screen for genes induced by FGF7 in interneurons had not yet come in. We will be interested in comparing the genes induced by FGF7 in interneurons with the genes induced by FGF22 in DGCs, to understand which genes may be required in both DGCs and interneurons to promote the integration of neurons into the circuit. Furthermore, I have examined interneurons in the CA3 of WT and FGF7-KO mice that were crossed with the Vgat-Venus mice to fluorescently label interneurons. My preliminary observations are that CA3 interneurons in the stratum oriens, which form synapses onto CA3 pyramidal neurons in the stratum radiatum and stratum lucidum (Freund and Buzsaki), have smaller cell body size, decreased number of primary dendrites, and decreased dendritic branching. Because these observations are preliminary, they are being followed up by other members in the lab. However, if synaptogenic FGFs prove to act as target-derived factors for dendritic and postsynaptic development, and regulation of the neurogenic niche, this will present a novel mechanism of coordinated synapse development in the central nervous system (Figure 1.1D).

CHAPTER V

Conclusions and Future Directions

I have described here my work on the role of FGFRs in excitatory and inhibitory synapse differentiation. First in Chapter 2, using *in vivo* and *in vitro* data, I demonstrated that distinct, overlapping sets of FGFRs are responsible for excitatory versus inhibitory presynaptic differentiation in response to FGF22 or FGF7 in the CA3 of the hippocampus: FGFR2b and FGFR1b are required for excitatory presynaptic differentiation in response to FGF22, and FGFR2b alone is required for inhibitory presynaptic differentiation in response to FGF7 (Dabrowski et al., 2015). Next in Chapter 3, using an *in vitro* assay, I demonstrated that the receptors are functioning in the presynaptic cell to induce synaptic vesicle accumulation within the axon: FGFR2 and FGFR1 in dentate granule cells respond to FGF22, and FGFR2 in interneurons responds to FGF7. Using an *in vitro* assay, I demonstrated that FGFR2b requires kinase activity, and binding of the adaptor proteins FRS2 and PI3K to induce presynaptic differentiation in DGCs in response to FGF22, likely through a mechanism involving AKT signaling (Dabrowski et al., 2015). Finally, in Chapter 4, I presented preliminary data on the roles of FGF22 in incorporating DGCs into the hippocampal circuit by modulating dendritic development, possibly through control of genes induced by FGF22 in dentate granule cells. Together these data suggest a model in which FGFR2b and FGFR1b, through their differential use at excitatory versus inhibitory axon terminals, provide redundant specificity to FGF signaling in excitatory versus inhibitory presynaptic differentiation.

These studies open up many future directions, which can be categorized into six broad questions: 1) What is the relationship between FGFR2b and FGFR1b signaling in excitatory

neurons and are there differences in FGFR2b function in excitatory versus inhibitory neurons? 2) Do FGFR2b and FGFR1b recruit different proteins to the excitatory and inhibitory presynaptic bouton? 3) Do FGFRs expressed in other cell types have a role in FGF22-induced excitatory presynaptic differentiation or FGF7-induced inhibitory presynaptic differentiation? 4) Do genes induced by FGF22 and FGF7 in dentate granule cells and interneurons, respectively, promote DGC and interneuron integration into the hippocampal circuit? 5) What are the functional consequences of loss of FGFR2b or FGFR1b in the hippocampal circuit? And ultimately, 6) What is the role of FGF signaling in epilepsy and neurological disease? I will outline here plans for future experiments to address these questions and further our understanding of FGF signaling in the development of healthy brain circuits.

1. FGFR2b and FGFR1b – dissecting differences

FGFR2b and FGFR1b are required in DGCs for FGF22 to induce excitatory presynaptic differentiation; FGFR2b, but not FGFR1b, is required for FGF7 to induce inhibitory presynaptic differentiation (Dabrowski et al., 2015). The overlap in FGFR2b requirement for excitatory and inhibitory presynaptic differentiation, but uniqueness of FGFR1b requirement for excitatory differentiation, leads to several questions: since FGFR2b is acting in different cell types, why is FGFR1b required for excitatory presynaptic differentiation, and not just the differential expression of FGFR2b in excitatory or inhibitory axon terminals? Does FGFR1b have a unique role in signaling excitatory presynaptic differentiation or does it somehow suppress inhibitory presynaptic differentiation? Do FGFR2b and FGFR1b have similar functions in recruitment of excitatory synaptic vesicles? Are both receptors required to activate gene expression in response to FGF22? Here, I will outline some experiments to address these questions (Figure 5.1).

I have demonstrated that signaling downstream of FRS2 and PI3K is required for FGFR2b to respond to FGF22, but what is the response of FGFR1b to FGF22 or FGFR2b to FGF7? To address this, *in vitro*, cell autonomous rescue experiments transfecting mutant FGFR1b constructs into dentate granule cells and mutant FGFR2b constructs into interneurons, together with synaptophysin-YFP, can be performed (Dabrowski et al., 2015). Additionally, to address whether the extracellular and intracellular domains of FGFR2b and FGFR1b are
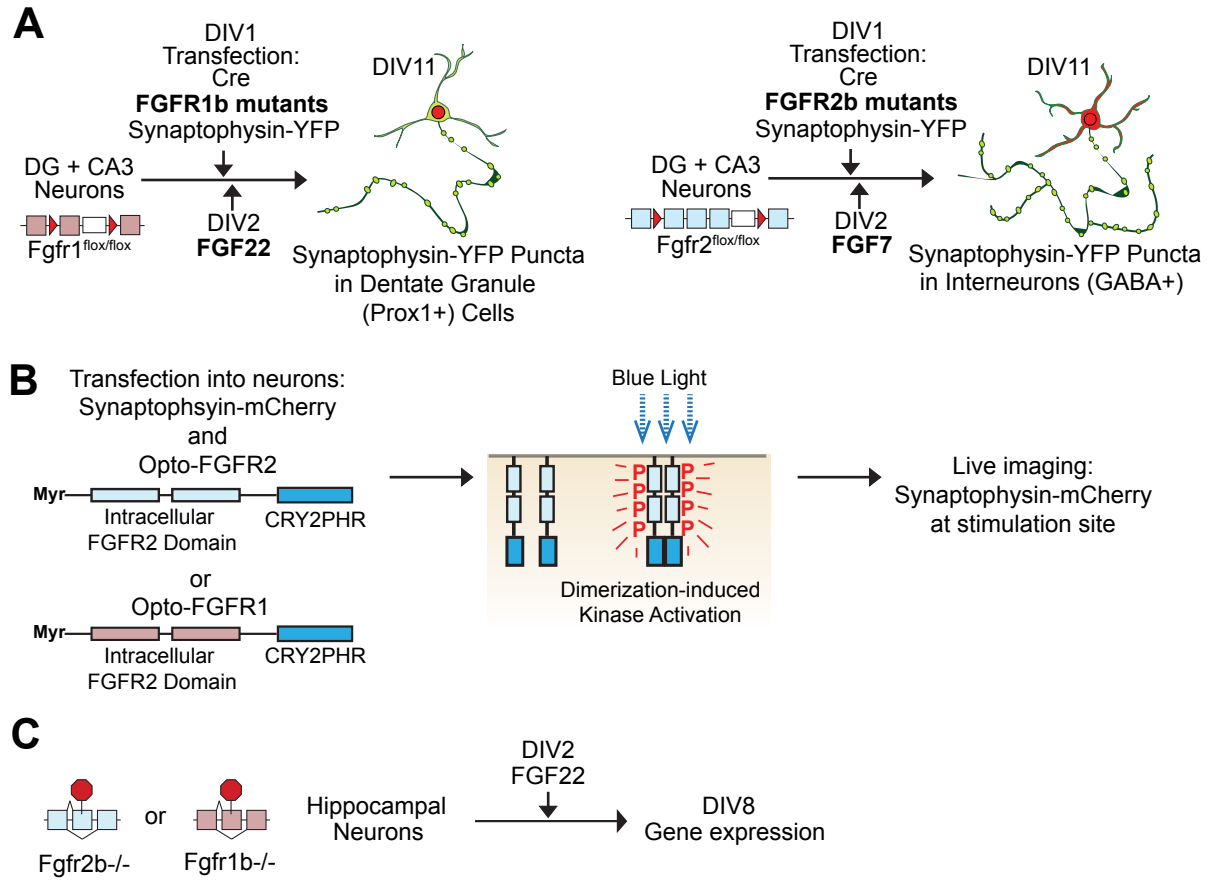
Figure 5.1. Schematic of experiments to dissect signaling differences between FGFR2b and FGFR1b.
A) Dissecting signaling pathways downstream of FGFR1b in response to FGF22 and downstream of FGFR2b in response to FGF7.
B) Schematic of Opto-FGFR experiment to track recruitment of synaptic vesicles to activated FGFRs (Opto-FGFR2 or Opto-FGFR1) in real time.
C) Dissecting the contribution of FGFR2b and FGFR1b to FGF22-induced gene expression.

interchangeable for rescue of FGF22 responsiveness, chimeric FGFR2b-1b mutants can be made, with the extracellular domain of one receptor fused to the intracellular domain of the other receptor. As a reminder of the experimental design, cell autonomous manipulation is achieved by sparse transfection of neuronal cultures with synaptophysin-YFP, a fluorescently-tagged marker of synaptic vesicles, along with Cre (to induce cell-autonomous receptor deletion in transfected conditional KO neurons) and/or FGFR mutant constructs. These experiments can answer several questions: Does FGFR2b activated by FGF7 also recruit FRS2 and PI3K for inhibitory presynaptic differentiation, similar to excitatory activated by FGF22? Does FGFR1b require FRS2 and PI3K in response to FGF22, similar to FGFR2b? Is the intracellular portion of FGFR2 fused with extracellular FGFR1b (or vice versa) functionally equivalent to WT FGFR2b? If there are differences between FGFR2b and FGFR1b response to FGF22, it is possible that it is due to different affinity of FGF22 for the receptor, or it could due to differences in the intracellular kinase activity between FGFR2 and FGFR1. In terms of signaling pathways required for presynaptic differentiation, FGFR2b in response to FGF22 or FGF7 and FGFR1b in response to FGF22 could all utilize the same signaling pathways. Alternatively, FGFR2b could use the same signaling pathways, but FGFR1b could use distinct pathways, such as the PLCγ pathway. Another alternative possibility is that excitatory and inhibitory synaptic vesicle accumulation requires different signaling pathways downstream of FGFR2b. The three pathways I test (ERK, AKT, and PLCγ) are the major pathways downstream of FGFRs, however there are additional pathways implicated in FGFR signaling transduction, such as the STAT pathway, and mechanisms of negative feedback, that could be unique between receptors (Ornitz and Ioth, 2015). Some downstream regulation is unique: for example, in the absence of ligand activation, the C-terminus of FGFR2 is bound by Grb2, displacing PLCγ (Timsah et al., 2014), and supporting the possibility of unique signaling pathways between FGFR2b and FGFR1b. Signaling downstream of the receptors merits further study.

Are synaptic vesicles immediately recruited to the site of FGFR activation on the scale of minutes to hours? For example, Wnt7a application to cultured hippocampal neurons induces excitatory synapses within an hour in a transcription-independent manner (Ciani et al., 2011). Immediate recruitment of synaptic vesicles to the site of FGFR activation suggests a direct mechanism of action. To monitor local, temporal dynamics of synaptic vesicle recruitment to the site of FGFR activation, one would want to locally manipulate FGFR activation and use live

imaging to monitor the recruitment of synaptic vesicles. One possibility would be to locally apply FGF22 or FGF7, and monitor the accumulation of synaptic vesicles at the site of application over the next twenty-four hours. However, because FGF22 and FGF7 may diffuse away from the site of application, a recently developed optogenetic technique for manipulation of the FGFR activation can be utilized (Opto-FGFR; Kim et al., 2014 and Grusch et al., 2014). Opto-FGFRs are constructs in which the intracellular domain of an FGFR was fused at the C terminus with the photolyase homology region of photosensory cryptochrome 2 from *Arabidopsis thaliana* (Kim et al., 2014) or the light-oxygen-voltage domain of aureochrome photoreceptors from stramenopiles (Grush et al., 2014). Endogenous cryptochromes and aureochrome photoreceptors dimerize when exposed to blue light, thus the Opto-FGFR construct dimerizes when blue light is shined upon it, inducing cross auto-phosphorylation and activation of FGFR signaling, as measured by ERK, AKT, and PLCγ phosphorylation, and cell growth and migration. The extracellular portion is excluded to avoid interference from endogenous ligands. To study the dynamics of synaptic vesicle recruitment to the site of FGFR activation, Opto-FGFR1 and Opto-FGFR2 constructs would be transfected into neurons along with synaptophysin-mCherry (using red fluorescence might minimize the possibility of activation of Opto-FGFR from the synaptophysin fluorescence during imaging). Laser activation using blue light will be followed by live imaging of the neurons and evaluation of the intensity and size of Syn-mCherry at the site of stimulation relative to unstimulated sites. These experiments will help address whether there are differences between the receptors in terms of their temporal dynamics of synapse vesicle recruitment. Furthermore, mutated constructs of the Opto-FGFRs can be made, using the intracellular domains of those shown in Figure 3.6, to test the contribution of individual signaling pathways to the local recruitment of synaptic vesicles in response to FGFR activation.

Using Opto-FGFR, another question that can be answered is if FGFR activation induces an increase in synaptic vesicle exocytosis at the site of synaptic vesicle accumulation. During spontaneous synaptic vesicle pausing in the absence of postsynaptic contact, synaptic vesicles at pause site frequently exocytose (Ahmari et al., 2000). However, as a synapse differentiates, the synaptic vesicles begin to form heterogenous pools, some of which are in the readily-releasable pool, while others are in recycling or reserve pools (Alabi and Tsien, 2012). From electron-microscope imaging *in vivo*, FGF22 and FGF7 appear to promote the recruitment of synaptic

vesicles into the readily releasable pool (Terauchi et al., 2010). Thus, to address the dynamics of acutely-accumulating synaptic vesicles in response to FGFR stimulation, Opto-FGFR can be expressed in neurons *in vitro* together with a synapto-pHluorin, such as VGLUT1-pHlorin for excitatory synapses (Balaji and Ryan, 2007), a synaptic marker in which a pH-sensitive GFP is in the vesicle lumen and fluoresces when synaptic vesicle exocytose and are alkalinized. By providing a potassium challenge that depolarizes the axon, the magnitude of exocytosis (measured as an increase in VGLUT1-pHluorin fluorescence) at the site of FGFR-activation can be assessed. An increase in exocytosed synaptic vesicles at the site of FGFR activation supports the idea of direct recruitment of synaptic vesicles into a readily-releasable pool.

The ultimate readout of FGFR signaling is gene expression. We have identified genes expressed in response to FGF22 in DGCs and are identifying genes induced in response to FGF7 in interneurons (see Chapter 4). Do both FGFR2b and FGFR1b contribute to gene expression? Do they control expression of unique genes, or are the two receptors redundant? To answer these questions, the ability of FGF22 to induce candidate gene expression in dentate granule cells in hippocampal neuron cultures from FGFR2b-KO, FGFR1b-KO, or WT mice can be tested. We interrogated changes in gene expression in FGF22-KO animals at P14 *in vivo*, about one week after peak expression of FGFs in the CA3, thus candidate genes could be assessed *in vitro* one week after FGF22 application. RNA could be isolated from the whole hippocampal culture and target genes could be measured by QPCR. To measure gene expression specifically in DGCs, fluorescence *in situ* hybridization on the cultured neurons could be performed to detect mRNA of the target genes in Prox1+ DGCs (Swanger et al., 2011). It is possible that only one of the two receptors induce gene expression in response to FGF22, or that the two receptors act through distinct pathways to induce gene expression and induce different genes. This could shed light on the differences in signaling induced by FGFR2b and FGFR1b activation.

Understanding the detailed dynamics of synaptogenic FGFR2b and FGFR1b has broader implications for FGF studies. There are eighteen secreted FGF ligands, but only seven FGFRs, and yet FGF signaling displays a remarkable degree of specificity in their function (Ornitz and Itoh, 2015; Guillemot and Zimmer, 2011; Dailey et al., 2005). FGF22 and FGF7 maintain their specific effect on excitatory and inhibitory presynaptic differentiation when bath applied *in vitro*, that is FGF22 induces a more robust increase in VGLUT1 puncta than VGAT puncta, while FGF7 induces a more robust increase in VGAT than in VGLUT1 puncta (unpublished results,

observed by myself and multiple lab members). In the absence of spatial restriction of FGF secretion, how does FGF7 activation of FGFR2b in interneurons have a more robust effect on inhibitory presynaptic differentiation than FGF22 activation of FGFR2b in interneurons does? Little is known about the differences in how different FGFs activate the same FGFR; or how the same FGFR responds to different ligands. Probing these questions would be of broader interest to the FGF scientific community and shed light on the specificity of the promiscuous nature of FGF signaling.

2. Proteins recruited by FGFRs in presynaptic boutons

FGF22 induces excitatory presynaptic differentiation and FGF7 induces inhibitory presynaptic differentiation in hippocampal CA3 (Terauchi et al., 2010). I showed that FGFR2b and FGFR1b are required in the presynaptic excitatory neurons (dentate granule cells) and FGFR2b is required in the presynaptic inhibitory neurons (interneurons) (Dabrowski et al., 2015). I also showed that FGFR2b and FGFR1b, when overexpressed, localize to presynaptic boutons in axons (Dabrowski et al., 2015). Finally, I found that FGFR2b requires kinase activity, and signaling downstream of FRS2 and PI3K to induce excitatory presynaptic differentiation in response to FGF22 (Dabrowski et al., 2015). This suggests that FGFRs form signaling complexes in the presynaptic bouton that recruit synaptic vesicles to the differentiating presynaptic terminal. Questions remain: What sorts of proteins complex with activated FGFRs to induce excitatory versus inhibitory presynaptic differentiation? Are the proteins recruited by FGFR2b and/or FGFR1b to excitatory versus inhibitory axon terminals different? My results suggest that FRS2 and PI3K will be in that complex, but there are two distinct FRS2 proteins, FRS2α and FRS2β, and multiple proteins bind to FRS2 when FGFRs are activated (Ong et al., 2000). I hypothesized at the end of Chapter 3 that FRS2 and PI3K could be activating either the AKT pathway, or alternatively, could be recruiting the WAVE receptor complex (WRC) (Chen et al., 2014) to induce changes in F-actin – directly detecting WRC at activated FGFRs would strongly implicate it in presynaptic differentiation. Likewise, there are differences in regulation of synaptic vesicles at excitatory and inhibitory presynaptic terminals (Augustin et al., 1999; Gitler et al., 2004), suggesting that there may be distinct mechanisms to recruit synaptic vesicles

in response to FGF22 and FGF7. Differences in the required pathways to recruit synaptic vesicles at excitatory and inhibitory terminals could explain why FGF22 and FGF7 utilize distinct sets of FGFRs for presynaptic differentiation (Dabrowski et al., 2015). This question is related to the previous question (see Figure 5.1), but now I focus on specific proteins recruited to FGFR2b or FGFR1b in response to synaptogenic FGFs, with the hope of uncovering novel protein interactions (Figure 5.2). I propose to use proteomics to A) probe the proteins immunoprecipitated with FGFR2 or FGFR1 from excitatory or inhibitory neurons in the presence or absence of FGF22 or FGF7, and B) probe the proteins present at excitatory and inhibitory synapses in the presence or absence of synaptogenic FGF signaling. I acknowledge that the experiments I propose below are technically challenging, because FGFRs are expressed in many cell types in the hippocampus but the effect I am looking for is very local, because available antibodies are not specific for –b verus –c splice forms of FGFR2 or FGFR1, and because proteomics requires a fairly high sample volume. However, the answers these experiments yield may be very interesting for gaining a deeper understanding of the differences between FGFR2b and FGFR1b signaling, and context-dependent differences in FGFR signaling.

To address what proteins assemble at activated FGFRs in excitatory versus inhibitory synaptic terminals, one could immunoprecipitate FGFR2 or FGFR1 from excitatory or inhibitory neurons and identify associated proteins. I propose to isolate excitatory or inhibitory neurons from the hippocampal CA3 from P8 animals, which is when FGF22 and FGF7 are highly expressed in CA3 (Terauchi et al., 2010), stimulate the isolated neurons with FGF22 or FGF7, and then immunoprecipitate FGFR2 or FGFR1. In order to isolate the subpopulations of neurons, one could use FACS sorting (as described in chapter 4); for excitatory neurons this would require a fluorescent reporter expressed in excitatory neurons, for example by combining Cre-induced YFP expression with a Cre expressed under the VGLUT1 promoter (Allen Institute for Brain Science, 2009); for inhibitory neurons, VGAT-Venus mice described in Chapter 4 could be used. After isolating the neuronal sub-population, excitatory neurons would be stimulated with FGF22 and FGFR2 and FGFR1 immunoprecipitated, and inhibitory neurons stimulated with FGF7 and FGFR2 immunoprecipitated. The samples would need to be manipulated in the presence of phosphatase inhibitors. The proteins could then be run out using two-dimensional protein electrophoresis to identify differences in spots, which could then be excised and identified using matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS); or, all of the
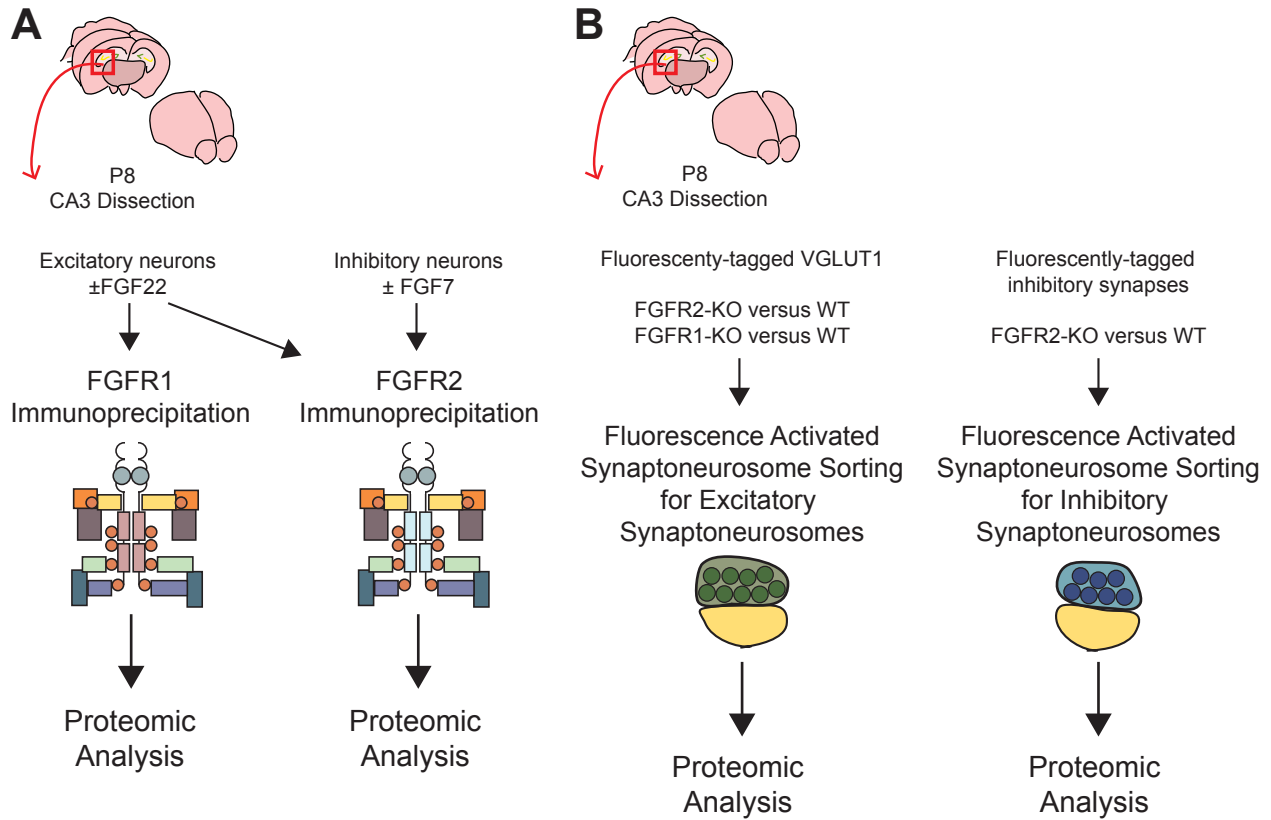
Figure 5.2. Schematic of experiments to probe for changes in FGFR-induces synaptic proteasome.
A) To test differences proteins recruited to FGFR2b and FGFR1b in response to FGF22 or FGF7 in excitatory or inhibitory neurons, neurons will be fluorescence activated cell sorted (FACS) to isolate an excitatory or inhibitory neuron population at P8. Excitatory neurons will be incubated with FGF22 then FGFR2 or FGFR1 will be immunoprecipitated and processed for proteomic analysis. Inhibitory neurons will be incubated with FGF7, then FGFR2 immunoprecipitated and processed for proteomic analysis.
B) To study the differences in the proteins recruited to the synapse by FGFR2b or FGFR1b, excitatory synaptoneurosomes will be isolated using fluorescence-activated synaptoneurosome sorting (FASS) to compare excitatory synapses by using proteomic analysis in the presence and absence of FGFRs, and inhibitory synaptoneurosomes will be isolated using FASS and compared with proteomic analysis to see the difference in the presence and absence of FGFR.

immunoprecipitated proteins could be eluted and analyzed using electrospray ionization mass spectrometry (ESI-MS) (Gundry et al., 2009). In both cases, special protocols to target phosphoproteins could be used to identify phosphorylated proteins (Mann et al., 2002). In addition to downstream cytosolic proteins, it is possible that immunoprecipitation of the complexes will reveal interactions with other transmembrane proteins, such as an interaction of FGFR1 with NCAM (Dityatev et al., 2004). Understanding what proteins are recruited to FGFRs in response to synaptogenic activation will help shed light on the activated downstream signaling pathways (and relates strongly to question 1 in this section – dissecting the differences between FGFR2b and FGFR1b), and it will also help identify novel presynaptic proteins that may be associated with the FGFR signaling complex to induce presynaptic differentiation.

It is possible that the presynaptic proteins affected by FGFR signaling will not be directly bound to the receptor, or may not remain bound to the receptor during immunoprecipitation. To gain a deeper biochemical understanding of synaptic vesicle recruitment, one would want to know how synaptogenic FGFR signaling affects the proteasome of excitatory or inhibitory presynaptic boutons. However the challenge is to isolate native excitatory and inhibitory synapses in order to narrow the experimental focus on the immediate vicinity of FGFR signaling. I propose to use fluorescence activated synaptoneurosome sorting (FASS) (Biesemann et al., 2014), a modification of synaptoneurosome isolation (Hollingsworth et al., 1985), that would allow enrichment of selected excitatory or inhibitory synapses. Using the FASS protocol, one could isolate excitatory synaptoneurosomes from the hippocampal CA3 of FGFR-KO or WT animals expressing a fluorescently-tagged VGLUT1 and then sort using flow cytometry to enrich for VGLUT1-positive synaptoneurosomes. Excitatory synaptoneurosomes from FGFR1-KO, FGFR2-KO and control animals (or FGFR2b-het, FGFR1b-KO, and WT animals) would be isolated, to understand the specific roles of FGFR2b and FGFR1b in differentiating the excitatory presynaptic terminal. Isolation of inhibitory synaptoneurosomes would require crossing FGFR2-KO (conditional FGFR2-KO or FGFR2b-het) mice with mice that express a fluorescently-tagged VGAT, however fluorescently-tagged VGAT mice are not currently available. One possibility could be to use mice expressing YFP-labeled Neuroligin 2 (Soto et al., 2011), which marks inhibitory postsynaptic densities (Varoqueaux et al., 2004). Another possibility is to sort synaptoneurosomes from the VGAT-Venus mice described in Chapter 4 (Wang et al., 2009), but this would lead to enrichment for all of the synapses, excitatory and

inhibitory, onto the fluorescently-labeled interneurons because of fluorescent labeling of the postsynaptic side, in addition to inhibitory synapses onto CA3 pyramidal neurons. Once synaptoneurosomes were isolated from FGFR-KO and WT mice, the proteins could be run out using two-dimensional protein electrophoresis to identify differences in proteins; or, all of the immunoprecipitated proteins could be run on high throughput mass spectrometry. One potential problem may be that using fluorescently-tagged VGLUT1 will yield synaptoneurosomes with weaker fluorescence in the FGF22-KO, since they inherently have less accumulation. However, synaptic vesicle accumulation is not entirely abolished in FGF22-KO mice, and with proper thresholding, synaptoneurosomes may still be obtained. Another major limitation of isolating excitatory, and especially inhibitory synaptoneurosomes to perform proteomics is to obtain enough starting material for protein sequencing. However, if enough material can be obtained, by pooling samples from multiple animals, the results could yield novel substrates of FGFR signaling, and help clarify potential differences between mechanisms for recruiting synaptic vesicles in excitatory and inhibitory axon terminals.

3. Probing possible roles of FGFRs in other cell types for presynaptic differentiation.

I showed that FGF22 requires FGFR2b and FGFR1b that function presynaptically in the DGC, and FGF7 requires FGFR2b that functions presynaptically in interneurons to induce presynaptic differentiation (Dabrowski et al., 2015). However, FGF22 and FGF7 are secreted molecules, which means they can act on any cell in the vicinity of secretion, for example they could have an autocrine effect on the CA3 pyramidal neuron or act on astrocytes. Although I demonstrated presynaptic FGFR requirement for presynaptic function, by demonstrating their requirement in dentate granule cells and interneurons, I did not specifically exclude a function of FGFR2b and/or FGFR1b postsynaptically or in astrocytes in responding to FGF22 and FGF7. It is possible that FGFR2b and/or FGFR1b have additional roles in synapse development in response to CA3-derived FGFs. Indeed, Wnt7a, which induces presynaptic differentiation, acts on Frizzled receptors that are localized both presynaptically and postsynaptically (Salinas, 2012).

To address whether FGFRs are required in other cell types *in vivo* to respond to FGF22 or FGF7, the best strategy would be to delete FGFRs in specific cell types by crossing the

conditional FGFR-KO mice with mice expressing cell-type specific, inducible Cre to delete the receptor in both a cell-type specific and temporally controlled manner, from P0 to P8, when presynaptic differentiation was assessed in the conditional FGFR-KO mice (Dabrowski et al., 2015). Tamoxifen inducible Cre under a GFAP promoter (Ganat et al., 2006; Guo et al., 2011) can be used to delete floxed-FGFR2 or floxed-FGFR1 in astrocytes *in vivo* at birth, followed by measurement of excitatory and inhibitory presynaptic differentiation at P8 by using VGLUT1 and VGAT, although a significant drawback of this strategy is that the Cre targets newborn neurons in the dentate gyrus as well as astrocytes, and dentate granule cells are important excitatory input onto CA3. Cre under the Grik4 promoter (Nakazawa et al., 2002) can delete floxed-FGFR2 or floxed-FGFR1 in CA3 neurons, postnatally, although a drawback may be weak expression in the first postnatal week. Deletion of FGFRs in astrocytes may affect presynaptic differentiation, since both FGFR2 and FGFR1 have been found to be important in the differentiation of astrocytes (Smith et al., 2006; Stevens et al., 2010a; 2010b; 2012), which may lead to presynaptic underdevelopment. Deletion of FGFR2 or FGFR1 in CA3 neurons may affect VGLUT1 accumulation, because CA3 neurons are synaptically interconnected (Witter, 2007), but should not affect VGAT accumulation. On the other hand, I predict definite decreases in VGLUT1 after deletion of FGFR2 or FGFR1 in dentate granule cells by using Cre driven by the Pomc promoter (McHugh et al., 2007), and decreases in VGAT accumulation after deletion of FGFR2 from interneurons. Numerous strains of mice expressing inducible Cre under interneuron-specific promoters have been developed (Taniguchi et al., 2011), allowing not only functional localization of FGFR2 function to presynaptic interneurons, but also identification of which specific subtype of interneuron FGF7 is acting on.

4. Cell-wide effects of FGF22-induced genes and FGF7-induced genes

In Chapter 4, I described an analysis of the changes in gene expression induced by FGF22 in DGCs and by FGF7 in interneurons. I also described preliminary results demonstrating that DGCs in FGF22KO mice have an increased density of dendritic spines in the suprapyramidal blade of the dentate gyrus. It is now important to demonstrate the mechanism through which these changes are occurring. My hypothesis is that FGF22 and FGF7 are acting as

target-derived cues from CA3 pyramidal neurons onto DGCs and interneurons, respectively, to promote aspects of neuronal and dendritic maturation through regulation of gene expression (Figure 4.1), analogous to the role of target-derived neurotrophins acting on sympathetic neurons in the peripheral nervous system (Harrington and Ginty, 2013). Thus, it will be important to interrogate the role of FGF22-and FGF7-induced genes in DGC and interneuron maturation.

The verification of the hypothesis that FGF22 is acting as a target-derived cue (see Figures 1.1 and 4.1) can be broken down into smaller, tractable experiments: 1) CA3-derived FGF22 is inducing candidate gene expression in DGC; 2) the candidate gene induces dendritic changes; and, if FGF22 is inducing dendritic changes: 3) the candidate gene is necessary for FGF22 to induce dendritic changes and 4) the candidate gene rescues dendritic changes in FGF22-KO.

To test whether CA3-derived FGF22 induces candidate gene expression *in vitro*, one can use two approaches: bath apply FGF22 to neuronal cultures, or apply FGF22 specifically to axons (mimicking a target-derived effect) by culturing DGCs in a microfluidic chamber, in which cell bodies are separated from axons (Taylor et al., 2005). Changes in gene expression (compared to untreated controls) can be detected by performing QPCR on the cultured neurons, or, alternatively by performing fluorescence-*in situ* hybridization on cultured neurons together with antibody staining for Prox1 to identify DGCs (Swanger et al., 2011). In a model of target-derived effect, expression of candidate genes should be induced by FGF22 application both by bath application and axonal application.

To test the role of FGF22 and FGF22-induced genes on dendritic spine density *in vitro*, increases in dendritic spine density in FGF22-KO neurons in DGCs would first have to be observed in culture. (Defects described in Chapter 4 were *in vivo*.) Bath application of FGF22 should "rescue" spine density and decrease density back to WT levels. The roles of individual genes could be assessed by using shRNA in culture – for the genes that are induced by FGF22, knockdown (for example with shRNA) in culture should lead to increased dendritic spine density (and for Adra2a, this has already been observed, e.g. Hu et al., 2008). If FGF22-KO DGC dendritic spine density is increased relative to control, overexpression of candidate genes should rescue the density to normal levels, and knockdown of candidate genes should prevent the decrease to WT levels by bath application of FGF22.

The *in vivo* approach requires sophisticated genetic manipulations to selectively manipulate FGF22 expression in CA3 and candidate gene expression in the dentate gyrus. FGF22 is highly expressed in CA3, and also in the infrapyramidal blade of the dentate gyrus at P8 (Terauchi et al., 2010). Thus demonstrating that increased dendritic spine density or decreased neurogenesis is caused by target-derived FGF22 from CA3 will reinforce the model. To induce FGF22-deletion selectively in CA3, mice expressing Cre under a Grik4 promoter (Nakazawa et al., 2002) can be crossed with conditional FGF22-KO mice. First, CA3-selective FGF22-KO mice should be investigated for increases in dendritic spine density in the dentate gyrus and decreased adult neurogenesis, to establish whether FGF22 is acting as a target-derived cue. Next, to test whether CA3-derived FGF22 induces candidate gene expression *in vivo*, gene expression should be assessed in DGCs from CA3-selective FGF22-KO mice. To test whether candidate gene expression induces dendritic changes *in vivo*, it should be knocked down at P14 (when genes were first identified; Chapter 4) in the dentate gyrus, for example by infecting dentate gyrus cells with lentivirus carrying shRNA, which should lead to increased dendritic spine density and/or decreased adult neurogenesis. Lentiviral overexpression of the candidate gene at P14 should have an opposite effect on dendritic spine density and/or adult neurogenesis, and should rescue the effect of CA3-selective FGF22-KO.

Because FGF22-KO mice also display changes in adult neurogenesis (Lee and Umemori, 2013), the change in dendritic spine phenotype may be due to a change in the population of DGCs, specifically an increased proportion of mature DGCs relative to immature DGCs. To test whether the change in density of dendritic spines is caused by a change in the proportion of mature and immature DGCs, neurons with the same "age" should be assessed. Retrovirus only infects dividing cells (Kron et al., 2010), and can be used to sparsely label neurons at P0 with GFP. DGCs in the suprapyramidal blade of the dentate gyrus born at the same time in FGF22-KO and WT mice should be compared.

Together, demonstrating effects of target-derived FGF22 on dentate gyrus neurogenesis and dendritic spine development may demonstrate a novel mechanism of neuronal circuit development.

Ultimately, the most important purpose of synapse formation is functional connectivity between neurons. I showed that FGFR2b-het mice and conditional FGFR2-KO mice have defects in both excitatory and inhibitory presynaptic differentiation, while FGFR1b-KO and conditional FGFR1-KO mice have defects only in excitatory, but not inhibitory presynaptic differentiation (Dabrowski et al., 2015). I did not show whether this led to functional changes within the circuit, which is a question worth asking on a broad level: Are electrophysiological defects demonstrable in FGFR-KO mice? Do FGFR-KO mice have altered connectivity in the hippocampus because of defective presynaptic differentiation? Do FGFR-KO mice have changes in hippocampal-dependent learning tasks caused by deficient presynaptic differentiation in CA3? Area CA3 is important for encoding spatial memory (Papp et al., 2007) and for pattern separation (Leutgeb and Leutgeb, 2007). Loss of excitatory drive from DG to CA3 and loss of inhibitory tone within CA3 may lead to defective information transfer through the circuit and defective pattern separation that is required for encoding memories or spatial information.

To answer whether FGFR-KO mice have functional synaptic defects, slice recordings from hippocampal slices of FGFR-KO mice and WT littermates could be used. First, to test whether there are excitatory or inhibitory synaptic defects, miniature excitatory postsynaptic currents (mEPSCs) or miniature inhibitory postsynaptic currents (mIPSCs) could be measured in CA3. mEPSC frequency is decreased in FGF22-KO mice, and mIPSCs frequency is decreased in FGF7-KO mice (Terauchi et al., 2010). By stimulating dentate granule cells and performing whole cell patch recordings in CA3 (e.g. Toth et al., 2000), paired-pulse facilitation at the mossy fiber synapse in CA3 could be measured to determine the readily-releasable pool of excitatory synaptic vesicles; and, with a high-frequency stimulation paradigm, long-term potentiation at the mossy fiber synapse of FGFR-KO and WT could be compared. Mechanisms of mossy fiber LTP remain controversial, but some researchers propose that it is induced by presynaptic changes (Nicoll and Malenka, 1995), so decreased excitatory presynaptic differentiation in FGFR2b-het and FGFR1b-KO mice could lead to changes in the induction of synaptic plasticity at the mossy fiber bouton. Understanding the relationship between FGFR-induced presynaptic differentiation

and its functional outcomes for plasticity will help shed light on what developmental mechanisms are required for forming a synapse capable of undergoing plasticity changes.

Next, the question of how loss of synaptogenic FGFR affects the hippocampal circuit connectivity comes up. Both FGFR2b and FGFR1b are required in DGCs to respond to FGF22 (Dabrowski et al., 2015). However, at later stages of hippocampal circuit development, FGF22-KO DGCs display dendritic defects, wherein they have increased dendritic spines (Chapter 4), which may affect the incorporation of DGCs into the hippocampal circuit and lead to decreased connectivity from the enthorhinal cortex (see Figure 1.1). Additionally, FGF22-KO display decreased adult neurogenesis (Lee et al., 2012). Do either of the FGFR-KO DGCs display defects in the dendritic spines or decreased neurogenesis due to loss of FGF22 signaling? To assess neurogenesis, the number of proliferating and immature DGCS in FGFR2b-het and FGFR1b-KO mice can be measured (see Lee and Umemori, 2013). Dendritic spines should be observed in FGFR2b or FGFR1b mice, for example by crossing them with the GFP-M strain of mice. A drawback is that FGFR signaling is promiscuous and responds to many ligands, not only FGF22, (e.g. Ornitz and Itoh, 2015), so the effects of FGFRs on circuit development may go beyond FGF22-dependent functions, and should be viewed as a function of the FGFR signaling itself.

Finally, connectivity in CA3 is important in a number of computational tasks, notably rapid one-trial contextual learning and pattern completion-based memory recall (Nakashiba et al., 2008). FGFR1b-KO mice have decreased excitatory presynaptic differentiation onto CA3, FGFR2b-het mice have, in addition to decreased excitatory differentiation, altered inhibitory presynaptic differentiation in CA3. GABAergic inhibition controls spike timing of pyramidal neurons (Mann and Paulsen, 2007). Furthermore, it is likely that, since FGF22-KO mice have decreased neurogenesis, either FGFR2b-het or FGFR1bKO mice have decreased adult neurogenesis, which is important for encoding pattern separation (Clelland et al., 2009; Nakashiba et al., 2012). FGFR1 is implicated in neurogenesis (Ma et al., 2009) and FGFR2 is implicated in differentiation, but not proliferation (Kang et al., 2009), but the isoform-specific contribution is unknown. It is likely that FGFR-KO mice will display defects in learning tasks that require hippocampal CA3, which include rapid encoding of information that can be tested, for example, on novel platform water maze tasks or contextual fear-conditioning (Kesner, 2007). Indeed, FGFR2-KO mice have impaired learning and memory (Stevens et al., 2010a, 2010b;

2012). I predict that FGFR2b-het and FGFR1b-KO mice will display deficits on specific behavioral tasks, reflective of their deficits in CA3 synapse formation.

6. FGF signaling in neurological diseases, with a focus on epilepsy.

Neurological diseases, such as epilepsy, have complicated etiologies. Temporal lobe epilepsy is caused by an interplay of a brain insult and genetic predisposition (Chang and Lowenstein, 2003; Noebels, 2015; Staley, 2015). Notably, brain trauma elicits a rearrangement of the hippocampal neural circuit that leads to progressive susceptibility to develop spontaneous seizures, a process known as epileptogenesis (Pitkanen and Lukasiuk, 2011). Current medication mainly targets seizures, but does not affect the underlying process of epileptogenesis (Chang and Lowenstein, 2003; Pitkanen and Lukasiuk, 2011). Thus, there is a need for targeted therapy to halt epileptogenesis.

FGF22-KO mice and FGF7-KO mice display altered susceptibility to seizures (Terauchi et al., 2010; Lee et al., 2012; Lee and Umemori, 2013). They do not display spontaneous seizures, but have altered responses to seizure induction, thus making them good models for understanding the mechanisms of variable seizure susceptibility in the population. Furthermore, FGF22 and FGF7 are re-expressed after kainate-induced seizures (unpublished data), suggesting that their re-expression may have a role in shaping the post-seizure circuit that is increasingly prone to seizures. FGF22-KO and FGF7-KO mice may be informative models for understanding the molecular events underlying epileptic network rearrangement and for finding treatments targeting epileptogenesis.

In order to better understand the role of FGFRs in epileptogenesis several experiments can be performed. Do FGFR2b-het and FGFR1b-KO mice have seizure phenotypes? Loss of FGFR2b affects both FGF22-induced excitatory synapses and FGF7-induced inhibitory synapses: thus, it could lead to heightened seizure sensitivity, resistance, or neither. FGFR1b-KO mice should be seizure-resistant, since they are predominantly responding to FGF22 to induce excitatory presynaptic differentiation. If FGFR1b-KO mice do not have a change in seizure

susceptibility, this suggests that FGR2b is responsible for FGF22-induced changes in seizure susceptibility. On the other hand if FGFR1b-KO mice are seizure resistant, targeting FGFR1b signaling or FGF22 interaction with FGFR1b signaling may prove to be an important, and specific, therapeutic target.

Another important question is whether FGF22 functioning early during development or FGF22 re-expressed after seizures is key to promoting seizure susceptibility. To address this, conditional FGF22-KO mice can be crossed with inducible Cre mice to delete FGF22 either at P0 (to target developmental presynaptic differentiation) or concurrently with seizure kindling (to target pathological synaptic remodeling). If FGF22-deletion at kindling does not alter seizure susceptibility, that suggests that FGF22 activity during synaptogenesis predisposes the circuit to seizures. However, if FGF22-deletion at kindling causes resistance to seizure induction, this suggests that FGF22 re-expressed in response to seizures drives epileptogenesis. This outcome would suggest that targeting FGF22 could have therapeutic benefit.

Finally, FGF22 induces the expression of a number of genes that have roles in neural circuit remodeling (Chapter 4). Do FGF22-induced genes have a role in epileptogenesis? FGF22 re-expressed during kindling may be required for epileptogenic hippocampal circuit changes; it may also induce gene expression that leads to the circuit remodeling. FGF22-KO mice do not develop full seizures, even with an extended kindling protocol (Terauchi et al., 2010; Lee and Umemori, 2013), which makes it unfeasible to obtain a post-seizure sample from FGF22-KO to compare to post-seizure WT mice. However, the genes induced by FGF22 during development may be re-expressed after seizures, so it may be worthwhile to understand the roles of the FGF22-induced genes both in neural circuit development (Chapter 4), and in epileptogenesis. If targeting FGF22 proves to be a technically difficult strategy, targeting one of the downstream genes induced by FGF22 may prove to have therapeutic benefit.

Conclusion

The circuitry of the brain is incredibly complex and the mechanisms governing the startling accuracy with which the brain develops are being uncovered. Specific neuronal circuits are being parsed out with the help of optogenetic manipulation of individual neuron excitability,

viral gene expression, and sophisticated genetic mouse models (Steinberg et al., 2015). The trajectory of individual neurons incorporating into the neuronal circuits is being traced with increasing accuracy (e.g. Ge et al., 2006). And increasingly sophisticated studies have yielded an increase in the number of identified synaptogenic molecules (de Wit and Ghosh, 2014; Williams and Umemori, 2014; Dabrowski and Umemori, 2011). It will be important to understand formation of synapses within the context of an ever-changing circuit, where everything is, in the end, connected. So far, synaptogenic molecules within the central nervous system have been characterized primarily for their local effects at inducing synapse formation. Understanding the signaling through which synapses develop, and the potential effect of synapse development on the integration of neurons within the circuit (see Figure 1.1), will help shed light on the emergence of highly specific, functioning neuronal circuits. In this context, my work on identifying the receptors and the mechanisms through which FGF22 and FGF7 induce specific presynaptic differentiation (Dabrowski et al., 2015), and my work towards understanding gene expression and cell wide changes induced by the synaptogenic FGFs will make important contributions towards a deeper understanding of brain development, in health and in disease.

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