SOMATOSTATIN RECEPTORS ON NEURONAL CILIA: EVIDENCE FOR NEUROPROTECTION

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Primary cilia are essential in brain development, as mediators of sonic hedgehog signaling. However, their role in mature neurons remains elusive. One means to elucidate their function may be to investigate the function of the somatostatin type 3 receptor (SstR3), which is concentrated on the primary cilia of neurons. The inhibitory and anticonvulsant properties of somatostatin suggest that ciliary SstR3 might protect neurons against excitotoxicity, as seen in epileptic seizures. C57BL/6 wild type (wt) and SstR3 knockout mice were administered vehicle or epileptogenic agents kainic acid (KA) or pentylenetetrazole. Seizure behaviors were rated on seizure severity scales. KA-induced seizure behaviors were more severe in SstR3 mutants than in wt. Correspondingly, the mutants showed greater reactive gliosis, as indicated by increased numbers of GFAP immunoreactive (GFAP(+)) astrocyte processes. In addition, seizure severity was associated with a greater percentage of neural stem cells having an ACIII(+) cilium. Following injections of pentylenetetrazole, SstR3 mutants reached maximum seizure levels faster than wt. These results support the hypothesis that ciliary SstR3 are neuroprotective in mature neurons, and may provide a new avenue for the treatment of seizures.
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CHAPTER 1

INTRODUCTION

Primary cilia are antenna-shaped eukaryotic organelles, occurring one per cell, and are common in most vertebrate cell types (Wheatley et al., 1996; Pazour and Witman, 2003). Their membrane is continuous with the cell’s plasma membrane, and they have a microtubule core called an axoneme. The axoneme consists of nine outer microtubule doublets and extends from the basal body, which is the mother centriole that migrates to a position near the cell surface, where it is anchored and serves as a template for ciliary assembly (Fig. 1A). Primary cilia lack the central microtubule pair, typical of motile cilia (Satir and Christensen, 2007; Marshall, 2008; Satir and Christensen, 2008). Intraflagellar transport (IFT) is required for the formation, maintenance, and function of the cilia by transporting proteins from the cell body to the cilia via molecular motors which travel along the microtubule doublets (Rosenbaum and Witman, 2002; Scholey, 2003; Scholey and Anderson, 2006; Blacque et al., 2008; Pedersen and Rosenbaum, 2008; Santos and Reiter, 2008; Gerdes et al., 2009; Silverman and Leroux, 2009).

Once believed to be vestigial organs, primary cilia are now regarded as essential for extracellular signal transduction. Primary cilia dysfunction and defects result in an array of disorders and diseases known as ciliopathies, with symptoms such as renal cysts, retinal degeneration, liver fibrosis, anosmia, ataxia, cardiac defects, and situs inversus (Davenport and Yoder, 2005; Hildebrandt and Otto, 2005). They provide important cellular sensory and signaling functions by mediating effects of a variety of physiological and developmental signals involved in chemotransduction, phototransduction, mechanotransduction, and development of the embryonic brain and other organs and tissues (Pazour et al., 2002; Pazour and Witman,
In the olfactory system, chemosensory cilia are found on olfactory sensory neurons. Signal transduction cascades are stimulated when an odorant binds to its respective receptor on the ciliary membrane, leading to activation of a G-protein mediated cascade that results in influx of Ca\(^{2+}\) and Na\(^{+}\), efflux of Cl\(^{-}\), and ultimately resulting in depolarization of the cell (Kleene, 2008; Su et al., 2009). In the visual system, photoreceptors are modified primary cilia (Horst et al., 1990), and IFT is essential for photoreceptor maintenance and function (Marszalek et al., 1990).
Primary cilia play a major role as a mechanosensory organelle as demonstrated in the kidney. Proper function of the kidney is dependent upon primary cilia serving as flow sensors, leading to hyperpolarization and a rise in intracellular Ca$^{2+}$ (Praetorius and Spring, 2001; Nauli et al., 2003; Pazour and Witman, 2003). In brain development, the necessity for primary cilia is well accepted. In vertebrates, primary cilia are regarded as essential for brain development, as they are required on neural stem cells for mitogenic signaling by sonic hedgehog (Shh), a protein well known for its regulation of brain development (Huangfu and Anderson, 2005; Palma et al., 2005; Chizhikov et al., 2007; Eggenschwiler and Anderson, 2007; Breunig et al., 2008; Han et al., 2008; Spassky et al., 2008).

However, the functions of primary cilia in mature post mitotic neurons are thus far not well understood. Recent findings have provided some insight into their function on terminally differentiated neurons. Neuronal primary cilia are widely distributed throughout the brain, and it has been shown that certain G-protein coupled receptors are concentrated in the primary cilia through localization sequences (Berbari et al., 2008a). Investigating the signaling proteins within the cilium is a key strategy to elucidating its function. Second messenger signaling proteins, including adenylyl cyclase III (ACIII), concentrate within the primary cilium, and are used as molecular markers in the immunohistochemical identification of primary cilia (Bishop et al., 2007). The findings that the serotonin type-6 receptor, melanin-concentrating hormone type 1 receptor, and somatostatin type 3 receptor (SstR3) localize to neuronal primary cilia in several brain regions suggest that cilia may function as a chemosensor of the extracellular milieu, mediating signal transduction (Hamon et al., 1999; Handel et al., 1999; Brailov et al., 2000; Wolfrum and Schmitt, 2000; Pazour et al., 2002).
Somatostatin

The inhibitory neuropeptide somatostatin (Sst) modulates neuronal excitability and neurotransmission. It has been found that Sst and Sst agonists modulate both Ca\(^{2+}\) and K\(^{+}\) channels in a variety of cell lines (reviewed by Csaba and Dournaud, 2001). Sst can cause a reduction of intracellular Ca\(^{2+}\) via activation of K\(^{+}\) channels resulting in cell hyperpolarization or by directly inhibiting high voltage-dependent Ca\(^{2+}\) channels (Boehm and Betz, 1997; Akopian et al., 2000; Csaba and Dournaud, 2001; Olias et al., 2004; Mergler et al., 2008; Segond von Banchet, 2008; Smith, 2009).

All five classes of G\(_{i/o}\)-coupled somatostatin receptors (SstR1 – SstR5) are functionally coupled to the inhibition of adenylyl cyclase. The hippocampal region of the brain is thought to express only SstR1 – SstR4 (Csaba and Dournaud, 2001; Aourz et al., 2011). Pharmacological and physiological properties of the individual SstRs have been well studied. Although localization of the receptor subtypes has also been studied, the significance of this localization has yet to be established. SstR3 is the only SstR known to be localized on primary cilia. It is concentrated almost exclusively in the primary cilia on neurons, and is present on neuronal cilia in a majority of brain regions (Fig. 1B) (Handel et al., 1999; Schulz et al., 2000). The distribution and development of SstR3 has been described in rodents. In the hippocampus of both mice and rats, SstR3 is expressed on neuronal cilia in the CA fields and the granule cell layer of the dentate gyrus (Handel et al., 1999; Fuchs and Schwark, 2004; Berbari et al., 2008b; Stanic et al., 2009; Einstein et al., 2010). A recent study using SstR3 knockout mice has indicated a role for
this receptor in object recognition memory (Einstein et al., 2010). However, the effects of SstR3 on ciliary and cellular function are still unknown.

\[\text{Fig. 1B. The SstR3 localizes to the primary cilia. The SstRs are 7-transmembrane receptors that bind the inhibitory neuropeptide SST.}\]

Some of the SstRs have been implicated as major players in the anticonvulsant action in the hippocampus in several rodent models for limbic seizures (Vezzani and Hoyer, 1999; Qiu et al., 2008; Tallent and Qiu, 2008; Cammalleri et al., 2009). The activation of the SstRs following induced seizures results in the suppression of seizure activity, which has been demonstrated by the administration of Sst and Sst agonists to the hippocampus and the use of SstR knockout
mice (Moneta et al., 2002; Qiu et al., 2008; Aourz et al., 2011). The intrahippocampal administration of the SstR2 agonist octreotide reduced seizures in rats (Moneta et al., 2002). In a mouse model for limbic seizures, anti-convulsant effects were seen after SstR4 activation via an SstR4 selective agonist, and SstR4 knockout mice were more susceptible to seizures (Qiu et al., 2008). Aourz et al. (2011), reported that intrahippocampal administration of selective SstR2, SstR3, or SstR4 agonists resulted in anticonvulsant actions in the pilocarpine rat seizure model induced by the epileptogenic agent pilocarpine, implicating these receptors in the regulation of hippocampal excitability. Thus far, there have been limited reports of the inhibitory effects of SstR3. The use of SstR3 knockout mice has provided a means to elucidate effects of ciliary somatostatin signaling. Einstein et al. (2010) observed no gross abnormalities between wild type (wt) and SstR3-/- mice in cilia structure or incidence, suggesting normal ciliary function apart from a lack of SstR3 signaling in SstR3-/- mice. Further, no differences between the strains were observed in motivation to explore, as both wt and SstR3-/- mice approached novel objects with similar behaviors. Further understanding of the function of a receptor concentrated almost exclusively on cilia may yield clues to the functions of primary cilia in adult neurons.

Excitotoxicity and Kainic Acid

Excitotoxic cell death, also called glutamate excitotoxicity, occurs in response to excessive amounts of glutamate, which is highly toxic to neurons (Choi, 1987; Frandsen et al., 1989), astrocytes (David et al., 1996; Chen et al., 2000), and oligodendrocytes (Oka et al., 1993; Yoshioka et al., 1996; Alberdi et al., 2002; Matute et al., 2002; Alberdi et al., 2005). Excitotoxicity in cells is triggered by the excessive influx of Ca^{2+} into the cell in response to
overstimulation of the glutamate receptors on the neuron cell membrane, both N-methyl-D-aspartate (NMDA) and non-NMDA subtypes. As a result, the endoplasmic reticulum (ER) is stressed and the ER membrane disintegrates, causing the generation of reactive oxygen species and mitochondrial dysfunction, leading to apoptosis and necrosis in neurons (Reynolds and Hastings, 1995; Schinder et al., 1996; Nicholls, 2004) and other cell types (Matute et al., 2002; Matute et al., 2006).

As a potent agonist for the kainate subtype of ionotropic glutamate receptor, kainic acid (KA) is often used, via intravenous, intraperitoneal, intranasal, or intracerebral administration, to induce seizures to study status epilepticus (Olney et al., 1974; Ben-Ari, 1985; Yang et al., 1997; Mulle et al., 1998; Carriedo et al., 2000; Chuang et al., 2004; Rodriguez-Moreno and Sihra, 2004; Wang et al., 2005; Yang et al., 2005; Benkovic et al., 2006; Lee et al., 2008; Chihara et al., 2009; Morales-Garcia et al., 2009; Zheng et al., 2011). Kainic acid administration provides a good model of temporal lobe epilepsy (TLE). In part due to its high kainate receptor density (Darstein et al., 2003), the hippocampus is particularly vulnerable to KA-induced neurotoxicity, especially the hilar, CA1, and CA3 regions (Cantallops and Routtenberg, 2000; Bausch and McNamara, 2004; McLin and Steward, 2006; Kasugai et al., 2007; Zheng et al., 2011). However, different modes of administration have resulted in different levels of susceptibility to KA-induced excitotoxicity, especially in the C57BL/6 mouse strain, which is relatively resistant to seizure-induced behaviors and cell death (Schauwecker and Steward, 1997; Chen et al., 2002; Schauwecker, 2003; McLin and Steward, 2006; Kasugai et al., 2007; Zheng et al., 2011). The intranasal administration of kainic acid has been said to be a successful model in overcoming the resistance exhibited by C57BL/6 mice to systemic administrations of KA (Chen et al., 2002).
In response to KA administration and other modes of inducing status epilepticus, Shh-dependent neural progenitor proliferation is increased (Banerjee et al., 2005; Balu and Lucki, 2009). The transmembrane receptor Patched1 localizes to the primary cilium and binds Shh and regulates Shh-signaling at the primary cilium (Rohatgi et al., 2007). The use of cyclopamine, a blocker of Shh-signaling, reduces hippocampal progenitor cell proliferation in normal and seizure-induced mice (Banerjee et al., 2005), strongly implicating the involvement of primary cilia in status epilepticus-induced Shh-dependent neural progenitor proliferation. Additionally, three days after status epilepticus, there is an increase in proliferation of Type I neural stem cells in the dentate gyrus (Huttmann et al., 2003), which share some characteristics with radial glia (Brunne et al., 2010), and contribute to the increase in seizure-induced hippocampal neurogenesis (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998; Nakagawa et al., 2000; Blumcke et al., 2001; Deisseroth et al., 2004; Jessberger et al., 2005). Astrocytes have been shown to be relatively resistant to injury, as opposed to neurons and oligodendrocytes, in response to ischemia induced glutamate-mediated death (Petito et al., 1998) and excitotoxic conditions (Matute et al., 2006). Astrocytes also undergo reactive gliosis in response to status epilepticus, in which there is extensive neuron depolarization resulting from excessive amounts of glutamate, as characterized by an enhanced expression of the cytoskeletal proteins glial fibrillary acidic protein (GFAP) and vimentin and hypertrophy of the cell body and processes (Sofroniew and Vinters, 2010). Reactive gliosis is considered a hallmark of neurotoxicity (Torre et al., 1993; Wilhelmsson et al., 2004; Chen et al., 2005; Pekny and Nilsson, 2005; Wetherington et al., 2008), which is known to accompany epilepsy.
**Astrocytes**

Astrocytes, a major class of glial cells, are regulators of synaptic function throughout the brain (Pascual et al., 2005; Volterra and Meldolesi, 2005; Haydon and Carmignoto, 2006). They have functional neurotransmitter receptors, including glutamate receptors, and upon stimulation astrocytes undergo changes in intracellular Ca\(^{2+}\) concentrations (Hosli and Hosli, 1987; Porter and McCarthy, 1997). Oscillations in the concentration of cytoplasmic Ca\(^{2+}\) in astrocytes occur in response to continuous neuronal stimulation (Dani et al., 1992; van den Pol et al., 1992; Nedergaard, 1994; Dani and Smith, 1995; Yagodin et al., 1995). Astrocytes can take up glutamate at synapses and release it in a Ca\(^{2+}\)-dependent manner, such that waves in astrocytic Ca\(^{2+}\) can trigger astrocytic glutamate release (Simard and Nedergaard, 2004; Tian et al., 2005). Astrocytes are believed to produce growth factors to protect neurons from death and to promote proliferation and differentiation of precursor cells (Sandhu et al., 2009).

Activated astrocytes, such as seen in excitotoxic conditions, have long been thought to protect cells in the central nervous system. They take up potentially excitotoxic excess glutamate (Rothstein et al., 1996), protect against oxidative stress (Swanson et al., 2004), produce modulators such as pro- and anti-inflammatory cytokines, chemokines, and neurotrophic factors that are involved in neuron-glial communication (Dakubo et al., 2008; Braun et al., 2009; Sandhu et al., 2009), among many other protective and beneficial actions (reviewed by Sofroniew and Vinters, 2010). These findings demonstrate neuron-glial communication and suggest that astrocytic regulatory responses are influenced by neuronal activity.

*Astrocytes regulate neurotransmitter recycling by sequestering, inactivating, recycling,*
and restricting diffusion of neurotransmitters such as glutamate, GABA, and catecholamines (Bak et al., 2006; de Melo Reis et al., 2008; Sykova and Nicholson, 2008). Astrocytic neurotransmitter recycling takes place by a process called the glutamate-glutamine cycle, and is critical in maintaining ongoing synaptic function. When astrocytes take up glutamate, the enzyme glutamine synthetase (GL1) converts glutamate to glutamine, which can then be taken up by neurons and converted back to glutamate by the mitochondrial enzyme glutaminase (Chaudhry et al., 2002; Bak et al., 2006; de Melo Reis et al., 2008). However, this cycle has been shown to be compromised in disease states that affect the central nervous system, including epilepsy (Robinson, 2001; Sepkuty et al., 2002; Eid et al., 2004; Seifert et al., 2006; Tilleux and Hermans, 2007; Eid et al., 2008). Reactive astrocytes down-regulate glutamine synthetase, resulting in reduced conversion of glutamate to glutamine and available glutamine for neuron reuptake, suggesting that reactive gliosis may be involved in increased seizure susceptibility underlying TLE (Wilhelmsson et al., 2004; Ortinski et al., 2010).

Previously, it was hypothesized that cilia might be neuroprotective, by conferring a hyperpolarizing bias on the membrane potential of neurons (Fuchs and Schwark, 2004). If ciliary SstR3 is neuroprotective it would protect neurons against various stressors. We tested this possibility by determining whether the genetic deletion of SstR3 renders mice more susceptible to excitotoxic conditions that accompany epileptic seizures. Seizures were induced via intranasal administration of KA, and to test whether resulting effects were specific to either KA or the intranasal route of administration, another epileptogenic agent, pentylenetetrazole (PTZ) was administered via intraperitoneal (i.p.) injection. As KA is a potent glutamate agonist, and is used to model partial seizures with hippocampal involvement from early stages
PTZ acts as a γ-aminobutyric acid (GABA) antagonist. GABA is the primary inhibitory neurotransmitter, which binds the GABA receptor, and mediates inhibitory synaptic transmission (Olsen, 1982). PTZ is used as a model to induce generalized cortical seizures, general tonic-clonic seizures (PitKäen et al., 2006), with late hippocampal involvement (Brevard et al., 2006). Brains were examined three days after treatment as the optimum time point for reactive gliosis.
CHAPTER 2

MATERIALS AND METHODS

Subjects

Adult SstR3 knockout (SstR3-/-) mice were compared with age- and gender-matched wt mice of the same background (C57BL/6). Subjects were born in the mouse colony at the University of North Texas. Wildtype breeding pairs were obtained from Jackson Laboratories (Bar Harbor, ME). SstR3-/- breeder mice, described elsewhere (Zeyda, 1999; Zeyda and Hochgeschwender, 2008), were a kind gift from Dr. Melanie Tallent (Drexel University, Philadelphia, PA). Animal care and surgical procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of the University of North Texas.

Seizure Induction

A. Kainic acid

A solution of kainic acid (KA; 10 mg/1.3 mL dH₂O (Chen et al., 2004)) or dH₂O was administered at a dose of 21 mg/kg body weight intranasally under light isoflurane gas anesthesia (n = 16). Mice were held down on their backs by hand, and half the volume of KA or vehicle was administered continuously for 5 minutes to each naris, with a 2 minute break in between. After treatment, mice were immediately returned to their cages, and placed on their backs to retain residual KA and to observe the time required for the mouse to turn over.

B. Pentylentetrazole

A solution of pentylentetrazole (PTZ; 7.5 mg/1 mL 0.9% saline in dH₂O; (Qiu et al., 2008)) or vehicle was administered at a dose of 50 mg/kg body weight by i.p. injection (without
anesthesia) \((n = 16)\). After injection, mice were immediately returned to their cages.

**Seizure Behaviors**

Mice were observed for 4 hours after KA administration and periodically for 30 minutes at a time over the next 24 hours. Seizures were rated on the seizure severity scale used by Hesp et al. (2007) originally described by Racine et al. (1972), where 0 = no symptoms and 5 = rearing and falling.

PTZ-administered mice were observed over a 20-min period after PTZ injection and periodically for 20 minutes at a time over the next 24 hours. Seizures were rated on a PTZ seizure intensity scale, where 1 = sudden behavioral arrest and 6 = tonic-clonic seizures and/or wild jumping (Luttjohann et al., 2009). The times from injection to maximum seizure severity, and onset of seizure to maximum seizure severity were recorded. All observations were recorded by at least 2 observers blind to strain and treatment of the mice.

**Perfusion and Brain Tissue Processing**

Three days after administration of the epileptogenic agent, mice were sacrificed by injection of an overdose of 15 g/kg body weight of 20% urethane in dH\(_2\)O. The mice were perfused transcardially with 0.9% saline in dH\(_2\)O, followed by 4% paraformaldehyde (PFA) in 0.4 M phosphate buffer. Brains were removed and post-fixed in 4% PFA in 0.4 M phosphate buffer, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and frozen at -80° C 2-methylbutane, which does not freeze or interact with the tissue and has good heat conduction. Brains from KA-treated mice were sliced using a sliding microtome to 30 µm thick sections. Sections were stored at -20° C in cryoprotectant until used.
Immunohistochemistry

Brain sections were rinsed well in tris-buffered saline (TBS), mounted on gelatin-subbed slides, and allowed to air-dry for at least 3 hours. To increase the efficiency of protein-antibody binding, an antigen retrieval method was used, which unmask the epitopes of the antigens by breaking the carboxyl bonds made between PFA and the proteins during fixation. Sections were pre-incubated for 30 min in TBS containing 3% normal serum and 0.03% Triton X-100, and were incubated overnight in primary antibodies diluted in the pre-incubation solution. Primary antibodies included rabbit anti-ACIIl for cilia (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-GFAP for astrocytes (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-GFAP for astrocytes (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-SRY (sex-determining region Y)-box 2 (Sox2) for stem cells (1:250, Santa Cruz Biotechnology, Santa Cruz, CA). Following incubation in primary antibodies, sections were rinsed with TBS and incubated for 1.5-2 hours in secondary antibodies diluted 1:500 in TBS with 3% normal serum. Secondary antibodies included goat anti-rabbit IgG Alexa Fluor® 568, goat anti-mouse IgG Alexa Fluor® 488, donkey anti-rabbit IgG Alexa Fluor® 568, and donkey anti-goat IgG Alexa Fluor® 488 (Invitrogen, Carlsbad, CA). Nuclei were stained for DNA with 4’,6-diamidino-2-phenylindole (DAPI 1:1000; Invitrogen, Carlsbad, CA). Sections were coverslipped with Vectashield (Vector Labs, Burlingame, CA), sealed with nail polish, and stored at 4°C.

Microscopy, Quantification and Data Analysis

Sections were examined with epifluorescence microscopy (Nikon Optiphot). The incidence of astrocytes in the hilus was examined by counting the number of GFAP(+) cells having the typical astrocytic stellar morphology. Astrocytes were counted in one field of view
under a total magnification of 200X, in the hilus of both sides of the brain for each animal. A small population of the quantified GFAP(+) cells was randomly selected within each field of view and the number of processes extending from each cell was quantified. The averages for each animal were calculated. Confocal image stacks were taken to evaluate the intensity differences of GFAP expression between animal strains and conditions. Images were taken using a Zeiss 200M microscope and spinning disk confocal scanner (McBain Systems, Simi Valley, CA). Slices in the image stack were taken at 640X magnification at 1 µm intervals. Compiled images were adjusted for brightness and contrast as needed, using ImageJ.

The numerical density of neural stem cells in the hilus was examined by counting the total number of Sox2(+) cells in one field of view with a total magnification of 200X, in the hilus of both sides of the brain for each animal. A population of cells was randomly selected within each field of view and the number of Sox2(+) cells having an ACIII(+) cilium was counted to quantify the incidence of ciliated neural stem cells. Cells were counted only if they were not intersected by any edge of the image. Groups were compared using independent samples t-tests and Kruskal-Wallis non-parametric independent samples tests where appropriate, and the correlation between seizure severity and ciliated Sox2(+) cells was analyzed using Spearman’s rho non-parametric correlation, where parametric statistics were not applicable. The level for statistical significance was set at $p < 0.05$.  

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CHAPTER 3

RESULTS

After intranasal administration of KA, there was a statistically significant difference in the seizure behavior between wt and SstR3-/-. The seizure behaviors observed in the SstR3-/ knockout mice were more severe, with a median seizure rating of 4.0 as compared with the wt median seizure rating of 2.0. As the behavioral seizure ratings were made using an ordinal scale, a non-parametric Kruskall-Wallis test was used and revealed a statistical significance. The SstR3-/- had significantly more severe seizures than wt mice ($\chi^2(1) = 5.671, *p < 0.05$, Fig. 2).

![Fig. 2](image)

**Fig. 2.** KA-induced seizure severity in SstR3-/- knockout and wt mice. The SstR3-/- showed heightened seizure-susceptibility compared with the wt mice. Seizure-severity was rated based on Racine’s seizure severity scale as outlined by Hesp et al. Seizure behavior in SstR3-/- was double that seen in wt mice. (4.0 vs. 2.0, median, Kruskal-Wallis, $\chi^2(1) = 5.671, *p < 0.05$) ($n = 4$ mice per group)
To determine whether the brains were also differentially affected, brains were examined for signs of reactive gliosis, which is a hallmark of epilepsy. Three days after administration of intranasal KA or vehicle, the KA-treated SstR3-/− mice showed an increase over vehicle control in reactive gliosis (Fig. 3), as reflected by the level of GFAP expression and the number of GFAP(+) labeled processes per cell (t(6) = 4.49, p < 0.001, d = 7.95, Fig. 4). In parallel with the greater seizure intensity observed in the SstR3-/− mice, there was a strain difference in reactive gliosis, with KA administration leading to more severe reactive gliosis in the SstR3-/− knockout than in the wt mice. The number of processes on each GFAP(+) cell in the SstR3-/− was significantly greater than that observed in wt (t(6) = 4.49, p < 0.01, d = 3.53, Fig. 4).

Based on the increase in Shh-dependent proliferation that occurs in neural progenitors in status epilepticus (Banerjee et al., 2005; Balu and Lucki, 2009), together with the requirement for cilia in Shh signaling (Corbit et al., 2005; Huangfu and Anderson, 2005), we asked whether there were increases in the number of progenitors or the percentage of ciliated progenitors. We counted the number of hilar Sox2(+) progenitor cells per mm² and the percentage of Sox2(+) progenitor cells having a primary cilium. The numerical density of Sox2(+) cells in the hilus of KA-administered mice of both SstR3-/− and wt strains increased as compared with vehicle control mice. In addition, the percentage of Sox2(+) cells having a cilium (Fig. 5) increased with seizure severity, based on data from all groups of mice taken together (r_s = 0.893, p < 0.05, Fig. 6).
Fig. 3. Seizure-induced reactive gliosis in wt and SstR3-/- mice. KA-induced seizures and seizure intensity were associated with reactive astrocytes, as evidenced by the increase in staining intensity of GFAP(+) cells and number of processes per GFAP(+) cell, 3 days post administration of KA or vehicle. (n = 4 mice per group)
Fig. 4. Seizure-induced reactive gliosis in wt and SstR3-/− mice. Seizure intensity was directly correlated with reactive astrocytes, as evidenced by the increase in the number of processes on each GFAP(+) cell, 3 days post administration of KA or vehicle. Reactive gliosis was significantly greater in the KA SstR3-/− as compared with the KA wt. (**p < 0.01; ***p < 0.001; mean ± SEM) (n = 4 mice per group)
Fig. 5. ACIII(+) primary cilia on Sox2(+) cells in the hilus of the dentate gyrus. Sox2(+) cells with ACIII(+) primary cilia (arrows) in the hilus of a WT control mouse. SGZ, subgranular zone of the dentate gyrus; DGL, dentate gyrus-granular layer.
To test whether the heightened seizure susceptibility is specific to either KA or the intranasal route of administration, a similar experiment was done with intraperitoneal injections of PTZ. While all PTZ mice reached the highest levels of seizure intensity (5-6), SstR3-/- knockout mice showed more rapid seizure progression. SstR3-/- reached their peak seizure levels in less than half the time as wt, either when measured from PTZ injection (10.2 ± 1.76 vs. 5.1 ± 0.82 min, p < 0.05) or from onset of seizure behaviors (3.5 ± 1.5 vs. 7.9 ± 0.6 min, p < 0.05, Fig. 7).

Fig. 6. Seizures were linked to an increase in the percent of Sox2(+) cells with an ACIII(+) cilium. Three days after KA administration, the percent of Sox2(+) cells with an ACIII(+) cilium increased with seizure severity. ($r_s = .893, p < 0.05; n = 6$)
Fig. 7. SstR3-/- knockout mice progressed more rapidly than wt in seizure activity after PTZ administration. PTZ induced faster seizure progression in SstR3-/- than wt, whether measured by (A) injection time to peak seizure severity (10.2 ± 1.76 vs. 5.1 ± 0.82 min, *p < 0.05) or (B) onset of seizure behaviors to peak seizure severity (3.5 ± 1.5 vs. 7.9 ± 0.06 min, *p < 0.05). All PTZ mice reached the highest levels of seizure intensity (5-6). Seizure behavior was rated based on a PTZ seizure severity scale (Luttjohann et al, 2009). (*p < 0.05; mean ± SEM) (n = 4 mice per group)
Currently, functions for primary cilia are still being investigated. Evidence is growing to support the idea that adult neuronal primary cilia are organelles with extracellular signaling capabilities (Fuchs and Schwark, 2004; Berbari et al., 2009; Green and Mykytyn, 2010), yet a definitive function remains elusive. The function of SstR3 is also unclear, although recently, Einstein et al. (2010) found that SstR3-/- mice show a deficit in object recognition memory. In addition, other than the lack of SstR3 signaling, SstR3-/- show reasonably normal brain function and behavior (Zeyda and Hochgeschwender, 2008).

The results of the present study suggest a neuroprotective function for neuronal SstR3. Seizure-induced excitotoxicity and cell death have previously been linked (Choi, 1992; Fujikawa et al., 2000; Chen et al., 2010; Kovac et al., 2012). The behavioral differences exhibited by the wt vs. the SstR3-/- suggests that a bigger reaction occurred in the brain of the SstR3 knockout mice. The increased seizure behavior in the mutant mice suggests more neuron firing and greater neuron vulnerability in the mutant mice. Moreover, the nearly exclusive concentration of the SstR3 on primary cilia supports the importance of primary cilia in post mitotic neurons. The significant increase in seizure severity seen in the SstR3-/- as compared with the wt is evidence for the importance of the SstR3 in seizure susceptibility. This finding suggests that the ciliary SstR3 is important in detecting Sst in the extracellular space. Indeed, Sst is released in excitotoxic states, as seen in status epilepticus (Hashimoto and Obata, 1991; Manfridi et al., 1991; Tallent and Siggins, 1999; Vezzani and Hoyer, 1999; Tallent and Qiu, 2008). The results suggest the potential value of SstR3 agonists for the treatment of seizures. Although it remains
to be determined whether human neuronal cilia also have SstR3, the possibility that various receptors on neuronal cilia may regulate responses to excitotoxicity opens a potential new avenue for the treatment of seizures, injury, and neurodegenerative diseases in which excitotoxicity is critical.

The literature on epilepsy research suggests substantial variation in the sensitivity to excitotoxic effects of KA depending on a number of factors including species, age, and strain. In rats, systemic injections of KA produced widespread neuronal death within the hilus, CA1, and CA3 hippocampal regions (Morales-Garcia et al., 2009). Within mouse strains there is variation in sensitivity, with the C57BL/6 strain being the most resistant in terms of behavior and cell death (Chen et al., 2002; Schauwecker, 2003; Kasugai et al., 2007; Zheng et al., 2011). Nevertheless, in some studies, C57BL/6 mice have shown significant neuronal damage in the CA1 and CA3 regions, with lesser damage in the polymorphic layer of the dentate gyrus (Benkovic et al., 2004, 2006). In the present study, the seizure severity in SstR3-/- mice strongly implicates the SstR3 as anticonvulsive. In seizure-sensitive gerbils, Kang et al. (2003) reported a down regulation in Sstr3. The present results contrast with previous findings that after subcutaneous KA injections, SstR3-/- knockout mice failed to show significant differences from wt in seizure severity, although SstR3-/- mice showed reduced latency to seizure stages 1 and 2 (Qiu et al., 2008). The inconsistency between the current and previous results could be attributed to the route of administration, since C57BL/6 mice are resistant to subcutaneous administration of KA (Schauwecker and Steward, 1997), but showed vulnerability to the intranasal administration of KA (Chen et al., 2002).

In the present study, evidence of astrocyte activation in response to seizure induction
was also observed in the SstR3-/- and wt. Chen et al. (2005) reported that KA administered intranasally led to increases in GFAP expression in C57BL/6 mice. Increased GFAP activity has also been detected in young wt rats when treated with KA (Ravizza et al., 2005). Another study examining C57BL/6 wt versus interleukin (IL)-18 knockout mice reported similar findings in regard to condition and between strain, where reactive gliosis was increased in KA treated animals, and the IL-18 knockout expressed more reactive gliosis than the KA wt (Zhang et al., 2007). The difference in expression of reactive gliosis between the wt and SstR3-/- KA-treated mice seen in the present study may be a secondary effect of seizure severity rather than strain difference per se.

It has been suggested that astrocytes play a dynamic role by integrating neuronal inputs and modulating synaptic activity. It is generally thought that astrocytes are supportive and protective of neurons, and that reactive gliosis as induced by seizure activity has beneficial effects (Sofroniew and Vinters, 2010). However, there is a growing view that once expressed, reactive gliosis contributes to disease progression (Vesce et al., 2007) and inhibits neuroregeneration (Wilhelmsson et al., 2004; Sofroniew and Vinters, 2010). In the present study, three days after treatment, wt mice showed less reactive gliosis in response to KA treatment than did the SstR3-/- mice. Although a time course sampling was not conducted, reactive gliosis is known to occur for an extended period of time (Dihne et al., 2001). Examination of one time point may provide a representative idea of resulting reactive gliosis. The concept of reactive astrocytes contributing to disease progression would suggest that the lack of seizure intensity observed in the wt mice could be associated with the limited increased expression of reactive gliosis, and not solely due to the neuroprotective effects of the SstR3 and
the neuronal primary cilia. The expression of SstR3 on astrocytes has yet to be reported. It can then be suggested that neuronal ciliary SstR3 may additionally protect against negative effects of reactive gliosis, secondary to their protective effects against seizure activity.

KA-induced status epilepticus has previously been linked to neuronal cell death, especially in the CA3, CA1, and hilus regions of hippocampus (Benkovic et al., 2004, 2006; Morales-Garcia et al., 2009). There have been reports of excitotoxic loss of neurons in the hippocampus (Vezzani and Hoyer, 1999), of the CA1 region in KA-treated rats (Perez et al., 1995), and in the hippocampus of pilocarpine-treated rats (Kwak et al., 2008) and naturally seizure-sensitive Mongolian gerbils (Kang et al., 2003). Due to the limited amount of cell death that occurs in the seizure-induced cell death resistant C57BL/6 mice, cell death was not evaluated in the present study. Because behavioral seizure severity following KA administration has been shown to be related to the degree of GFAP(+) astrocyte activation as well as to the amount of neuron cell stress and death (Chen et al., 2002), it is likely that there was more cell stress and death in the mutant than the wt KA-seizure groups. Analysis of cell vulnerability in SstR3 mutants is currently in progress in our lab.

Seizures are associated with increased cell proliferation and neurogenesis within hours and up to 1 to 2 weeks after seizure induction, most notably in the hippocampal region (Bengzon et al., 1997; Parent et al., 1997; Nakagawa et al., 2000). Brief, single, and intermittent seizures induced by hippocampal kindling stimulation in rats leads to neural cell proliferation in the dentate gyrus (Bengzon et al., 1997). Pilocarpine induced status epilepticus and seizures induced via continuous perforant path stimulation in rats leads to an increase in granule cell neurogenesis (Parent et al., 1997). Nakagawa et al. (2000) reported increased genesis of
dentate granule cells after KA-induced seizures and electrical kindling, yet observed that repeated stimulation after establishing generalized seizures did not continue to result in an increase of dentate progenitor cell division.

Primary cilia have been implicated in proliferative responses to seizure. They are essential for mediating sonic hedgehog (Shh) induced proliferation (Corbit et al., 2005; Huangfu and Anderson, 2005) and have been identified on neural stem cells in the subgranular zone of the hippocampus (Breunig et al., 2008; Han et al., 2008) and the subventricular zone (Maslov et al., 2004). Shh transcripts are up-regulated in seizures, which are associated with Shh-dependent proliferation of both neuronal and astrocytic progenitors in the hippocampus (Banerjee et al., 2005; Balu and Lucki, 2009). The Shh signaling inhibitor, cyclopamine, blocks hippocampal progenitor proliferation both in normal mice and mice with induced seizures (Banerjee et al., 2005). Altogether, Shh’s mitogenic effects have been indicated as essential for adult neurogenesis and have implicated primary cilia as being essential in normal and seizure-induced neural cell proliferation.

In the present study, a seizure-related increase in the percent of Sox2(+) cells with ACIII(+) cilia was observed ($r_s = 0.893$, $p < 0.05$, Fig. 6). As reviewed by Suh et al. (2009), Sox2 is a transcription factor essential for maintenance of neural stem cells. Adenylyl cyclase has been implicated in regulation of primary cilium length, and inhibition of ACIII results in cilia elongation (Ou et al., 2009). While many neuronal primary cilia express both ACIII and SstR3, and some express ACIII alone, none have been detected that express SstR3 alone (Berbari et al., 2007). It has been demonstrated in Chinese hamster ovary cells that SstR3 is selectively coupled to ACIII via the $G_{i\alpha1}$ subunit (Law et al., 1994). SstR3-/- and wt mice that received KA
showed an increase in the percentage of Sox2(+) cells with ACIII(+) cilia. However, three days after seizure induction, there was a correlation between seizure severity and the percent of Sox2(+) cells with ACIII(+) cilia, where the SstR3−/− suffered more severe seizures and showed a corresponding increase in the percent of Sox2(+) cells with ACIII(+) cilia ($r_s = 0.893, p < 0.05$, Fig. 6). It may be that cilia respond dynamically to seizure conditions and ciliogenesis can be induced by seizures, or that ACIII is up-regulated in neural primary cilia as a result of seizure activity.

The increase in the number of Sox2(+) cells with an ACIII(+) cilium in the hilus could represent up-regulation of Sox2 or ACIII in cells that were already in the hilus at the time seizures were induced. This possibility is compatible with previously demonstrated up-regulation of stem-cell markers including Sox2, GFAP, vimentin, and nestin. Phi et al. (2010), reported an increase in Sox2 expression in tissue from patients diagnosed with the embryonic supratentorial brain tumor primitive neuroectodermal tumor (PNET), in which there is increased neural stem cell proliferation and differentiation. Studies have shown an increase in GFAP and vimentin cytoskeletal protein expression post-seizure activity (Aronica et al., 2000; Pekny, 2001). Increased expression of nestin is seen in the dentate gyrus of patients suffering from TLE (Blumcke et al., 2001). However, it is possible that some Sox2(+) subgranular zone progenitors migrated into the hilus, as happens with dentate granule neurons in status epilepticus (Gong et al., 2007). The relationship between seizure activity and protein expression on primary cilia can be further examined using additional primary cilia markers including ADP-ribosylation factor-like protein 13b (Arl13b), a GTPase required for ciliary structure (Larkins et al., 2011).
To test whether the observed behavioral and cellular differences in the SstR3 knockout mice were specific to KA or to an intranasal route of administration, the effects of PTZ administered i.p. were also evaluated. The results suggested that the effects may be generalized to other excitotoxic stressors, as PTZ-induced seizure behaviors progressed more rapidly in the SstR3-/− mice. Qiu et al. (2008) reported a similar tendency in response to PTZ administration: a Chi-squared analysis of only the percentages reaching stage 3 and 4 seizures showed that a greater percentage of SstR3-/− mice than wt reached these stages, although the majority of the mice in their study reached only stage 1 or stage 2 seizures, for which there was no significant strain difference. In comparing four strains of mice (wt and knockouts of SstR2, SstR3, and SstR4) in regard to latency to various stages, they reported a significant effect of strain (ANOVA). Additional pair-wise comparisons suggested that the latencies to stages 1 and 2, but not 3 and 4, were reduced in SstR3-/− compared to wt. However, they did not find increased seizure susceptibility in the SstR3-/− mice as compared with the wt in response to KA-administration (subcutaneous).

The analysis of SstR3-/− knockout mice shows promise in the quest to elucidate the role of neuronal primary cilia in adults. In interpreting the phenotype of any unconditional gene knockout, it is important to consider whether there are developmental anomalies that could account for differences present in adults. For example, it may be that the responses to seizure activity expressed in the brain are not solely due to the absence of the receptor, but may be attributed to other changes that may have occurred in the brain throughout development. In the case of SstR3-/− mice, no irregularities in ciliary function or structure have been detected in
SstR3-/ mice (Einstein et al., 2010), and there were no evident abnormalities in the brain or behavior of the SstR3-/ null mutant (Zeyda and Hochgeschwender, 2008).
CHAPTER 5

CONCLUSION

The results presented in this study support the hypothesis that ciliary SstR3 has a neuroprotective, stabilizing function in mature neurons. Mice lacking SstR3 showed greater seizure susceptibility as measured both behaviorally and by analyses of reactive gliosis in the brain. The mechanisms by which SstR3 influences brain function in adult neurons have yet to be elucidated, but may include an SstR3-mediated hyperpolarizing bias on the neuronal membrane potential and second messenger effects that discourage apoptosis. This study also supports the idea that SstR3-/ mice are a useful model to use to further investigate the functions of primary cilia in the adult brain. Additional investigation may further characterize the cellular consequences of seizure susceptibility, including neurogenesis, cell death, and mitogenic activity.
REFERENCES


Berbari NF, Lewis JS, Bishop GA, Askwith CC, Mykytyn K (2008b) Bardet-Biedl syndrome proteins are required for the localization of G protein-coupled receptors to primary cilia.


Chen Z, Duan RS, Quezada HC, Mix E, Nennesmo I, Adem A, Winblad B, Zhu J (2005) Increased microglial activation and astrogliosis after intranasal administration of kainic acid in


precursor cell development in the mouse optic nerve by sonic hedgehog from retinal


Davenport JR, Yoder BK (2005) An incredible decade for the primary cilium: a look at a once-

David JC, Yamada KA, Bagwe MR, Goldberg MP (1996) AMPA receptor activation is rapidly toxic
to cortical astrocytes when desensitization is blocked. J Neurosci 16:200-209.

de Melo Reis RA, Ventura AL, Schitine CS, de Mello MC, de Mello FG (2008) Muller glia as an
active compartment modulating nervous activity in the vertebrate retina:


Dihne M, Block F, Korr H, Topper R (2001) Time course of glial proliferation and glial apoptosis

23:345-373.

Eid T, Thomas MJ, Spencer DD, Runden-Pran E, Lai JC, Malthankar GV, Kim JH, Danbolt NC,


Horst CJ, Johnson LV, Besharse JC (1990) Transmembrane assemblage of the photoreceptor
connecting cilium and motile cilium transition zone contain a common immunologic epitope. Cell Motil Cytoskeleton 17:329-344.


Maslov AY, Barone TA, Plunkett RJ, Pruitt SC (2004) Neural stem cell detection, characterization,


Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727-3738.


receptor messenger RNAs, binding sites and somatostatin release in kainic acid-treated rats. Neuroscience 65:1087-1097.


Schinder AF, Olson EC, Spitzer NC, Montal M (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. J Neurosci 16:6125-6133.


Smith PA (2009) N-type Ca(2+) -channels in murine pancreatic beta-cells are inhibited by an exclusive coupling with somatostatin receptor subtype 1. Endocrinology 150:741-748.


Zeyda T, Hochgeschwender U (2008) Null mutant mouse models of somatostatin and
