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Intranasally Administered IGF-1 Inhibits Spreading Depression *In Vivo*

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Abstract

Spreading depression (SD) is a wave of cellular depolarization that travels slowly through susceptible gray matter brain areas. SD is the most likely cause of migraine aura and perhaps migraine pain, and is a well-accepted animal model of migraine. Identification of therapeutics that can prevent SD may have clinical relevance toward migraine treatment. Here we show that insulin-like growth factor-1 (IGF-1) significantly inhibited neocortical SD *in vivo* after intranasal delivery to rats. A single dose of IGF-1 inhibited SD within an hour, and continued to protect for at least seven days thereafter. A two-week course of IGF-1, administered every third day, further decreased SD susceptibility and showed no aberrant effects on glial activation, nasal mucosa, or serum markers of toxicity. SD begets SD *in vitro* by mechanisms that involve microglial activation. We add to this relationship by showing that recurrent SD *in vivo* increased susceptibility to subsequent SD, and that intervention with IGF-1 significantly interrupted this pathology. These findings support nasal administration of IGF-1 as a novel intervention capable of mitigating SD susceptibility, and as a result, potentially migraine.

Graphical Abstract

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Declaration of Conflicting Interests

Drs. Yelena Y. Grinberg and Richard P. Kraig are co-inventors (along with others) listed on a patent [US patent # 9,399,053 (pending in EU and Australia) "Treatments for migraine and related disorders"] that was issued 07/26/2016 after completion of this work, which is contained in the Ph.D. thesis of Y.Y.G. Dr. Kraig is a one of two co-founders of a start-up company, Seurat Therapeutics, Inc.TM that was formed in the fall of 2016 to commercialize IGF-1 as a novel therapeutic for migraine.

Authors' Contributions

Study concept and design; data acquisition and analysis; data interpretation; and writing of the manuscript were done by YYG and RPK. LZ was especially involved with all aspects of animal care / nasal administration work and assisted in writing of the manuscript. All authors approved the final version of the manuscript.

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Keywords

migraine; spreading depression; microglia; inflammation; neuroinflammation; insulin-like growth factor-1; neurotherapeutics

1. Introduction

Spreading depression (SD) is a transient cellular depolarization that travels through susceptible gray matter areas of brain at millimeters per minute (Ayata and Lauritzen, 2015; Bures et al., 1974; Somjen, 2001). It is the likely cause of migraine aura and perhaps migraine pain (Bolay et al., 2002; Karatas et al., 2013; Moskowitz et al., 1993; Nosedá and Burstein, 2013). SD is also a commonly used preclinical animal model to study migraine and assess the efficacy of potential therapeutics before translation to human clinical trials (Ayata et al., 2006; Charles and Baca, 2013; Costa et al., 2013; Eikermann-Haerter and Ayata, 2010; Eikermann-Haerter et al., 2012; Erdener and Dalkara, 2014; Nozari et al., 2010; Schytz et al., 2016).

Migraine is an immense healthcare burden with a prevalence of 18% among women and 6.5% among men in the United States (Lipton et al., 2001) and approximately 11% of the population worldwide (Stovner et al., 2007), a subset of whom experience high frequency and chronic migraine. In the United States migraine results in annual medical and lost work time costs of \$30 billion (Goldberg, 2005; Hu et al., 1999). However, existing therapeutics for high frequency and chronic migraine offer only fractional protection (Mack, 2011). Therefore, therapeutics that inhibit SD may be beneficial against migraine.

Increased physical and intellectual activity --often termed “environmental enrichment” in animal studies (Kraig et al., 2010; van Praag et al., 2000; Will et al., 2004), protects from SD (Pusic et al., 2014). Increased physical activity may also reduce susceptibility to migraine (Darabaneanu et al., 2011; Irby et al., 2016). Insulin-like growth factor-1 (IGF-1) rises with environmental enrichment (Chang et al., 2011; Ciucci et al., 2007) and protects in an animal model of stroke (Lin et al., 2009; Liu et al., 2001), suggesting it may also protect against SD. Indeed, IGF-1 is significantly protective against SD *in vitro* in hippocampal slice cultures (Grinberg et al., 2012; Grinberg et al., 2013). This effect involves reduction of brain

inflammation, including the pro-inflammatory cytokine tumor necrosis factor alpha (TNF α) and oxidative stress, which are factors that promote SD (Grinberg et al., 2012).

The goal of this study was to determine whether the protective effect of IGF-1 against SD can be translated to the *in vivo* condition, and whether IGF-1 produces any adverse effects. Intranasal administration of IGF-1 bypasses the blood-brain barrier for fast and non-invasive delivery to brain (Lin et al., 2009; Liu et al., 2001). Accordingly, we chose this route of administration for studies reported here. Experiments were performed in rats because of the wealth of data generated *in vivo* and *in vitro* indicating the utility of this species for modeling SD, and as a result, migraine. In addition, rats are a well-accepted animal model of the human condition. Our results showed that nasal administration of IGF-1 was highly effective in preventing SD *in vivo* and was not associated with adverse systemic effects. These results were previously presented in preliminary form (Grinberg and Kraig, 2013).

2. Results

2.1 A single dose of IGF-1 increased the threshold for SD

All animals displayed normal feeding, weight gain (Table 1), grooming, ambulatory activity, and drinking throughout experiments.

Intranasal administration allows for delivery of compounds, including IGF-1, into the brain within tens of minutes (Liu et al., 2001; Ross et al., 2004; Thorne et al., 1995). Since we show that IGF-1 begins to affect SD susceptibility within tens of minutes *in vitro* (Grinberg et al., 2012), we examined acute effects of intranasally administered IGF-1 on SD susceptibility *in vivo*. Perhaps not surprisingly, given the rapid entry of IGF-1 into brain, we found that a single dose of IGF-1 administered one hour before determining SD threshold, significantly increased threshold to SD 23-fold when compared to age-matched vehicle-treated sham controls (Fig. 2A, $n = 5$ animals/group). The one-hour timeframe was the average time between intranasal administration and evaluation of SD threshold due to the length of surgery and subsequent establishment of homeostasis in the electrical recording setup prior to KCl injection.

Single-dose treatment with IGF-1 also significantly increased SD threshold when evaluated days later (Fig. 2A). A single dose of IGF-1 administered one day prior to evaluating SD threshold increased threshold to SD 18-fold when compared to vehicle-treated sham controls ($n = 6$ and 5 animals/group, respectively). In another group of animals, SD threshold was evaluated three days after a single IGF-1 dose, and the threshold for SD was still significantly increased when compared to vehicle-treated sham controls ($n = 5$ animals/group). Three days after a single dose of IGF-1, there was still an 8-fold increase in SD threshold. Lastly, SD threshold was evaluated seven days following a single dose of IGF-1. A week after a single dose, IGF-1 continued to significantly protect against SD. In this paradigm, SD threshold was 4-fold increased when compared to vehicle-treated sham controls ($n = 6$ and 7 animals/group, respectively).

2.2 Multiple IGF-1 treatments resulted in greater protection from SD

Since our *in vitro* data suggested that recurrent exposure to IGF-1 results in increased protection (Grinberg et al., 2012), we hypothesized that this may also be true *in vivo*. IGF-1 or vehicle sham was administered every third day, with threshold evaluated a day after the last dose. Three doses of IGF-1 over one week significantly increased threshold to SD (Fig. 2B). Administration of IGF-1 every third day, i.e., dosing on day 0, 3 and 6, and recording on day 7 (as shown in Fig. 1B) resulted in a 37-fold increase in SD threshold when compared to vehicle-treated shams ($n = 5$ and 6 animals/group, respectively). We next observed that five doses of IGF-1 administered over two weeks also significantly increased threshold to SD. This administration schedule resulted in a 33-fold increase in the volume of 0.5 M KCl necessary to induce SD ($n = 7$ and 8 animals/group, respectively). As above, each set of experimental animals was compared to age-matched and treatment schedule-matched vehicle shams so as to control for effects of repeated anesthesia and animal handling. However, no changes were observed in any of the sham-treated groups, suggesting that our paradigm of up to five rounds of anesthesia do not appear to affect SD susceptibility at the times evaluated (Fig. 2).

Repeated nasal administration of vehicle solution (sham) did not alter SD threshold. Both anesthesia (Kudo et al., 2008) [see also (Pusic et al., 2014)] and stress (Shyti et al., 2015) have previously been shown to affect SD. In order to control for effects of repeated handling and anesthesia on SD threshold we compared each dose/time of IGF-1 administration to a matched vehicle sham (Fig. 1). We anticipated that the repeated handling of animals in combination with light isoflurane administration used for vehicle administration might alter threshold to SD. However, one-way ANOVA revealed no significant ($p = 0.789$; group sizes noted above) difference in SD threshold between acute, 1, 3, 7, or 13 day sham vehicle recipient animals. The volume threshold to SD in nL of 0.5 M KCl, ranged from an average of 1.18 to 7.17 nL.

2.3 Nasal IGF-1 treatment did not show adverse systemic effects

To assess the effects of nasally administered IGF-1 on glial reactivity, we measured mRNA expression levels of genes associated with astrocytic activation (GFAP; glial fibrillary acidic protein) and with pro-inflammatory microglial activation (TNF α and iNOS; inducible nitric oxide synthase) in animals treated with IGF-1 versus vehicle alone (five doses administered over two weeks). We found no significant ($p = 0.723$, 0.847 and 0.667 respectively; $n = 4$ vehicle; $n = 5$ IGF-1) difference in expression levels of any of these genes (Fig. 3A). Similarly, recurrent nasal delivery of IGF-1 did not reveal semi-quantitative immunostaining evidence of astrocytic ($p = 0.736$, $n = 3$ animals/group) or microglial ($p = 0.898$, $n = 3$ animals/group) activation (Fig. 3B). Taken together, these data suggest that recurrent nasal administration of IGF-1 does not evoke adverse sequelae in the brain.

We also examined non-SD-related impact of recurrent delivery of nasal IGF-1. Animals receiving the greatest total dose of IGF-1 were weighed prior to the start of initial nasal administration and immediately prior to SD induction two weeks later. Five doses of IGF-1 administered over two weeks did not affect animal weight (Table 1; $p = 0.704$; $n = 5$ animals/group for vehicle sham and IGF-1 treated). Serum metabolic parameters were also

unaffected by nasal IGF-1 and no difference between treated, sham and naïve animals was seen (Table 1; $n = 3$ animals/group). One exception to this was serum globulin, which increased ($p = 0.034$) with IGF-1 treatment. However, globulin, like all the other serum markers remained within the normal range in naïve control, vehicle shams and IGF-1 treated animals. We also examined the impact of recurrent nasal IGF-1 exposure on hematoxylin/eosin staining of nasal mucosa (Fig. 4). No adverse effects were seen between naïve, sham, and IGF-1 treated animals ($n = 3$ animals/group) as measured by external examiners and consistent with the national Toxicology Services at the U.S. Department of Health and Human Services (Miller and Cesta, 2015). Specifically, no evidence of nasal mucosal cell necrosis, hyperplasia or evidence of inflammatory cell infiltration was seen, common sequelae to toxic exposure of the nasal epithelium (Harkema et al., 2006). Finally, we saw no clinical evidence of seizures or electrophysiological evidence of epileptiform activity (e.g., see Gajda et al., 2005) when DC records were expanded in amplitude and time for one-minute epochs in recurrent (two week) IGF-1 or vehicle-treated animals prior to SD threshold determinations.

2.4 Multiple SDs increase susceptibility to subsequent SD, an effect inhibited by IGF-1

We previously showed that induction of recurrent SD increases susceptibility to subsequent SD in hippocampal slice cultures (Grinberg et al., 2013). To determine whether this observation translates to the *in vivo* condition, we evaluated SD threshold followed by induction of a total of six SDs (or sham stimuli using saline instead of 0.5 M KCl) on day 0, then reevaluated SD threshold the following day (Fig. 5). SD threshold was measured on the second day in a different area of the cortex to avoid tissue injury effects. Since SD can spread across the entire cortex of a lissencephalic animal, but may decay in amplitude with distance (Bures et al., 1974), we risked missing extra SDs that only propagated a short distance. We rarely noted SDs with small (e.g., less than 20 mV and always 5 mV or more in amplitude) DC potential amplitudes and consistently saw an SD-like DC potential change after KCl injections used to elicit recurrent SD. We found that induction of six SDs resulted in significantly lower (> 2 -fold) potassium threshold to SD the following day. However, induction of six SDs immediately followed by nasal IGF-1 administration resulted in significantly increased (5-fold) SD threshold compared to counterparts that experienced SD alone ($n = 5$ animals/group). Specific 0.5 M KCl volumes to elicit SD threshold were 6.0 ± 0.8 , 2.2 ± 0.2 , 14.8 ± 1.4 nL in animals pre-exposed to sham injections, to 6 SDs, and to 6 SDs followed by nasal IGF-1, respectively.

3. Discussion

We evaluated the efficacy and safety of IGF-1 administered nasally to rats to protect from SD. We found that a single dose of IGF-1 increased threshold to SD (i.e., made brain resistant to SD) acutely and a single dose continued to protect against SD out to seven days, the longest time point evaluated in this study. Multiple doses of IGF-1 provided additive effects without adverse CNS or systemic consequences that we could detect here. Furthermore, we show for the first time *in vivo*, that recurrent SD increases susceptibility to subsequent SD, an effect that is also inhibited by nasal treatment with IGF-1. This work

extends our previous *in vitro* findings that show IGF-1 protects against SD in hippocampal slice cultures.

In our previous work we measured electrical threshold to SD using hippocampal slice cultures, a well-accepted and physiological *in vitro* model of brain tissue (Grinberg et al., 2012; Grinberg et al., 2013; Kunkler and Kraig, 1998; Kunkler and Kraig, 2004; Kunkler et al., 2005; Pusic et al., 2011; Pusic et al., 2014). A bipolar stimulating electrode can be reproducibly placed at the dentate gyrus, and therefore consistently elicit a local neural tissue current pulse to sensitivity determine SD threshold in hippocampal brain slice cultures. This is not the case for pial surface electrical stimulation *in vivo* where stimulating electrodes can be expected to produce more widespread, and therefore variable, current density in either a monopolar or bipolar configuration (Chehrizi and Collins, 1981; Nathan et al., 1993). Also, pial surface electrical stimulating electrodes, unlike those placed on the slice culture dentate gyrus, cannot be reproducibly positioned over underlying neural circuits to result in consistent electrical activation of the brain. As a result, we chose potassium stimulation here to evaluate SD threshold. However, we did not place potassium on the cortical surface and measure subsequent SD frequency over a defined period (Caggiano and Kraig, 1996; Moskowitz et al., 1993). Instead, we reasoned that accurately measurable micro-injections of KCl would be a sensitive and reproducible means to establish differences in SD threshold between treated and sham controls. We found that to be the case here, as in previous studies, given the consistently small standard error of means measured and small sample sizes needed to attain statistical significance with Power levels always >0.80 and most often ~1.00 (Pusic and Kraig, 2015; Pusic et al., 2015; Pusic et al., 2014). Accordingly, we believe the KCl micro-injection strategy to elicit SD in whole animals described in this work is a useful refinement of methods to elicit SD that can reduce the number of experimental animals that would otherwise be used (Pusic and Kraig, 2015; Pusic et al., 2015; Pusic et al., 2014).

Our observed effects of IGF-1 on SD threshold exceed the mitigation of SD observed using common current prophylactic migraine treatments in a similar rodent preparation (Ayata et al., 2006). For example, Ayata and coworkers found that both topiramate and valproate required a minimum of four weeks of treatment to show an effect, and that reduction in SD susceptibility to potassium was only approximately two-fold (Ayata et al., 2006)—whereas IGF-1 offered fourfold protection a week after a single dose. One study shows immediate protection from a single high intravenous dose of topiramate immediately prior to evaluation of SD susceptibility (Akerman and Goadsby, 2005). Other means of administration (e.g., intraperitoneal injections and oral gavage) required weeks to affect SD elicited by non-injurious methods (Ayata et al., 2006; Unekawa et al., 2012). A similar time frame for the efficacy of topiramate is observed in patients with episodic migraine (Silberstein et al., 2007; Silberstein, 2017).

The delivery route of IGF-1 used here may help explain its marked efficacy against SD. IGF-1 enters brain following intranasal delivery, bypassing the BBB, and reduces stroke injury in rats (Liu et al., 2001; Thorne et al., 2004). Other cytokines [i.e., interleukin-11 (Pusic et al., 2014) and interferon gamma (Pusic and Kraig, 2015)] of similar molecular size also effectively enter brain after nasal delivery and significantly inhibit SD. Thorne et al.

(2004) used radiolabeled IGF-1 to show that within 30 minutes of intranasal administration, radioactivity and IGF-1 signaling activity can be found throughout brain –a speed of delivery which the authors suggested can occur via a convective extracellular transport route. Indeed, it has now been shown by a number of groups that intranasal delivery allows compounds of a limited size to enter brain via the perivascular spaces (Lochhead et al., 2015), also termed the glymphatic route. Because we used the same administration protocol, molecule, and vehicle here, we hypothesize that IGF-1 was similarly able to enter brain, and thus affect SD threshold. Our results here provide further support for the utility of therapeutics delivery to brain via the nasal route as suggested by others (Dhuria et al., 2010; Djupesland et al., 2014; Hallschmid et al., 2008; Jiang et al., 2015b; Patel and Patel, 2017).

There are two additional considerations relevant to intranasal administration of IGF-1 *after* SD –a paradigm used for data shown in Figure 5. First, Schain et al. (2017) recently showed that SD induces transient closing of the paravascular spaces, impairing glymphatic flow. This transient closing of paravascular spaces would potentially impair IGF-1 access to areas of brain affected by the SD we induced immediately prior to intranasal administration (Fig. 5). Second, SD activates collagenase MMP-9, including within the matrix of cortical blood vessels, and this contributes to transiently increased blood brain barrier permeability hours later (Gursoy-Ozdemir et al., 2004). Brain blood vessel MMP-9 activity has been shown to facilitate localized IGF-1 transport into brain parenchyma (Nishijima et al., 2010). Together, these findings suggest that MMP-9 activity may be upregulated in the hours post-SD, a time window when serum IGF-1 should peak given our administration schedule (Fig. 5), and perhaps facilitate IGF-1 transport into SD-affected areas of brain. We can predict the concentrations of IGF-1 found in brain vs. blood following intranasal administration (Thorne et al., 2004), however, it is difficult to predict what local IGF-1 concentrations would result from SD-induced MMP-9 activation. We hypothesize that enhanced IGF-1 exposure to SD-affected areas of brain would result in enhanced protection from subsequent SD.

Recurrent administration of IGF-1 was not harmful. Intranasal delivery of IGF-1 does result in eventual increases in blood IGF-1 levels (Thorne et al., 2004). Perhaps the modest serum increase in globulin seen in IGF-1 treated animals reflects such systemic spread, given that we used a human form of IGF-1 in rats, which as a result could trigger immune reactions (Table 1). However, the globulin elevation seen with IGF-1 remained within normal limits. Importantly, parenteral administration of IGF-1 can result in hypoglycemia (Lewitt et al., 1991). Furthermore, recurrent nasal IGF-1 had no impact on kidney or liver function as well as nasal mucosa or brain glia histology.

Nasal delivery of IGF-1 to brain did not adversely impact brain electrophysiological function. Recent evidence shows that IGF-1 directly injected into brain can promote seizure activity in an acute animal model of seizures (Jiang et al., 2015a), although IGF-1 has also been shown to be protective in animal models of epilepsy (Miltiadous et al., 2010). Notably, we did not observe any evidence of seizure activity. Instead, we found that IGF-1 inhibited SD, an electrophysiological phenomenon that requires a sufficient volume of gray matter brain to be synchronously hyperactive for induction (Bures et al., 1974; Somjen, 2001).

Demonstrating the mechanisms by which nasal delivery of IGF-1 protects against SD is beyond the scope of this study. However, prior *in vitro* studies conducted in hippocampal brain slice cultures suggest how IGF-1-based protection against SD may proceed *in vivo*. We have shown that IGF-1 significantly decreases SD triggered *in vitro* in hippocampal slice cultures, and that this effect involves decreased levels of TNF α and decreased production of reactive oxygen species from microglia; factors which otherwise promote SD (Grinberg et al., 2012, 2013).

Though the role of microglial activation in initiation of a single SD has been questioned (Shibata and Suzuki, 2017), our lab has shown that microglia are essential for induction of single SDs via synaptic activation (Pusic et al., 2014). Furthermore, there is evidence that the increased neural circuit excitatory drive necessary to elicit a single SD involves increased TNF α and reactive oxygen species that in turn play a role in SD initiation. First, physiological neuronal activity increases reactive oxygen species production on a time course consistent with the point of increased neuronal excitability onset (Bito et al, 1996; Hongpaisan et al., 2004). Second, microglia rapidly release TNF α upon stimulation from soluble agents released by neuronal activity (Hide et al., 2000). Third, excitatory neuronal *N*-methyl-D-aspartate receptor function (necessary for SD initiation) increases within two minutes of exposure to physiological levels of TNF α (Wheeler et al., 2009). Minutes after application of TNF α to hippocampal neurons in culture or in acute hippocampal brain slices, surface expression and function of neuronal α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors is increased while surface membrane expression and function of gamma-aminobutyric acid receptors is decreased (Pribrag and Stellwagen, 2014), changes which could promote SD by increasing excitability (Bures et al., 1974; Somjen, 2001).

Since SD itself generates TNF α and oxidative stress, we have proposed that recurrent epochs of SD might enhance SD susceptibility and have found that IGF-1 curtails this enhanced susceptibility by opposing these mechanisms (Grinberg et al., 2012, 2013). Sukhotinsky and coworkers (2011) show that a single SD per day repeated for two weeks, but not one week, reduces SD susceptibility in mice. However, the number of SDs that occurs with acute, high frequency, or chronic migraine is not clear. Importantly, it is well-recognized that even lethal stimuli when brought below threshold can be protective. This effect is known as “physiological conditioning hormesis” (Calabrese et al., 2007). We commonly use recurrent SD (e.g., six SDs elicited over an hour) not as a model of migraine aura *per se* but instead, as a model of the hyperexcitability phenotype seen in the brain of migraineurs as well as a non-injurious means to trigger oxidative stress consistent with that seen in SD and migraine (Kraig et al. 2010; Grinberg et al., 2012; 2013). We used recurrent SD here (Fig. 5) to confirm and extend our *in vitro* results (Grinberg et al., 2012; 2013) to the *in vivo* condition that SD begets SD, an effect that is inhibited by IGF-1.

In conclusion, aberrant CNS hyperexcitability and oxidative stress are recognized to occur in migraine patients and are increasingly recognized as a phenomena worthy of further study to unravel the underlying pathophysiology of migraine and apply this information to the development of improved therapies -- for further information see recent reviews (Aurora and Brin, 2017; Borkum, 2016; Goadsby et al., 2017). It is notable that Viggiano and coworkers

(2005) show that trigeminal pain transmission requires reactive oxygen species production. In a series of experiments using multiple animal preparations, Shatillo and colleagues show that SD triggers oxidative stress, not only in the cortical area involved in SD, but also in the ipsilateral trigeminal nucleus (Shatillo et al., 2013). They show that oxidative stress (from exposure to H₂O₂) causes increased calcitonin gene-related peptide (CGRP) release (Shatillo et al., 2013). CGRP antagonists are a novel migraine target now in clinical trials which reduce migraine pain (Edvinsson, 2015) and also can retard SD (Tozzi et al., 2012). Perhaps IGF-1 works upstream of CGRP to reduce the hyperexcitability and oxidative stress involved not only in SD, but perhaps more importantly in nociceptive activity in the trigeminal system associated with SD and by extension, migraine.

4. Materials and Methods

4.1. Animal surgery and spreading depression threshold determination

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Chicago and were conducted in accordance with the guidelines of the National Institutes of Health and Guide for the Care and Use of Laboratory Animals (2011), and were patterned after ARRIVE guidelines.

Adult (214–414 g) male Wistar rats (Charles River Laboratories, Wilmington, MA) were housed (two animals/cage before surgery, and one animal/cage after surgery) in static micro isolator cages with corn cob bedding and were provided with Enviro-Dri nesting material (Shepherd Specialty Papers, Watertown, TN) for enrichment. They were maintained on a 12-hour light-dark cycle with controlled temperature and humidity in our Central Animal Facility. Rats were fed 2918 irradiated Teklad diet (Envigo, Madison, WI) and provided acidified water *ad libitum*. Rats were observed daily after treatments. Rats were removed from the central animal facility via standard transport to the Kraig laboratory and returned to the central facility if destined to survive after recordings using procedures approved by the Animal Resource Center. This travel distance was only two floors nearly vertically away from one another.

This project involved the use of 109 rats as follows: 11 naïve animals, 50 vehicle sham controls, and 48 IGF-1 treated animals. Specific group sizes per experiment are listed in the Results section where the findings of each data set are described. Animals were randomly placed into each of these three groups and all analyses (e.g., amount of KCl injected) were only apparent after SD threshold was determined (Figs. 2 and 5). Computer-based digital imaging strategies were unbiased as they were applied equally to samples from sham controls and IGF-1 treated animals as described below (Figs. 3 and 4). All experiments with live animals were performed near the midpoint of their light cycle (i.e., 6 AM to 6 PM).

Animal preparation and recording procedures were completed using aseptic technique as previously described (Caggiano and Kraig, 1996; Kunkler and Kraig, 2003; Pusic and Kraig, 2015; Pusic et al., 2015; Pusic et al., 2014) (Fig. 1). These previously delineated procedures include rationale for the use of inhalational isoflurane as the anesthetic for rat SD studies as described in this work (Pusic et al., 2014). Briefly, rats were anesthetized via inhalational isoflurane (Butler Schein Animal Health, Dublin, Ohio), delivered with a nasal mask (5%

induction, 3% during surgery, and 2–3.5% in oxygen during electrophysiological recordings) with outflow gas exhausted via vacuum to prevent room contamination. Bupivacaine (0.25% solution; 0.05 mL; Hospira, Lake Forest, IL) was injected into the scalp to either side of what would become the left of center incision used for craniotomies. Eyes were coated with Artificial Tears™ (#17478-062-35; Akorn, Lake Forest, IL).

Adequacy and consistency of anesthesia level in spontaneously breathing animals was maintained as follows. Arterial oxygen was monitored throughout with a cutaneous oximeter (2500A VET; Nonin Medical, Plymouth, MN) placed on a hind paw and ranged from 95–100%. Animal internal temperature was monitored via rectal thermometer ($37 \pm 0.5^\circ\text{C}$), and maintained using either an infra-red heating lamp during surgeries or a recirculating water-based heating table during electrophysiological recordings. In addition, animals were continuously clinically monitored for uniform respiratory rate and depth of respiration as well as animal color and periodic absence of withdrawal to hind paw pinch [Guide for the Care and Use of Laboratory Animals (2011); Tremoleda et al., 2012]. We recognize that use of volatile anesthesia in spontaneously breathing animals will cause a rise in arterial carbon dioxide tension (Kraig et al., 1991; Moskowitz et al., 1993; Caggiano and Kraig, 1996), which can inhibit SD initiation. However, this would create a systematic confound across all animal groups that were uniformly anesthetized as described above.

Craniotomies were created on a table-top nose clamp with an anesthesia mask and ear bars setup to stabilize animals. After craniotomy surgery, anesthetized animals were quickly transferred for recordings to a rat stereotaxic unit (model #1530; David Kopf Instruments, Tujunga, CA) modified to fit into a Narishige medium-sized animal recording setup using Narishige micromanipulators (Canberra and M1; Narishige International, Amityville, NY) to position microelectrodes under Wild M-8 stereomicroscope visualization (Leica Micro System, Inc.; Buffalo Grove, IL). The exposed craniotomy sites were superfused with sterile normal saline (~ 2 mL/min) warmed to 37°C using an in-line heater and control unit (model SH-27B, TC-344C; Warner Instruments, Hamden, CT).

To elicit SD, 0.5 M sterile KCl was injected via a glass micropipette (#6010 glass; A-M, Corporation, Everett, WA), with a tip diameter of $12 - 15\ \mu\text{m}$. Injections were made at 2 mm caudal to bregma, 2 mm lateral to the midline, and $750\ \mu\text{m}$ below the pial surface using a nitrogen gas-driven PicoSpritzer-II electronic valve system (Parker Hannifin, Hollis, NH), whose injection periods were registered directly to permanent digital recordings of interstitial DC potential changes. Injections of KCl were doubled in duration (e.g., 0.2 sec at 20 PSI, then 0.4 sec at 20 PSI) every four-to-five minutes if the previous injection failed to elicit an SD. Upon successful SD induction, the injection electrode was pulled up and an injection of the same pressure and duration was reproduced into 3-In-ONE™ light machine oil (WD-40 Company, San Diego, CA) contained in a depression well microscope slide (#48333-002; VWR Scientific Products, Buffalo Grove, IL). The injection volume was calculated using the injection sphere diameter in oil measured with a calibrated eye-piece micrometer on a compound microscope. Injection volumes measured from injection into machine oil likely do not fully reflect injections made *in vivo* (Nicholson, 2001). However, any discrepancy would not be consequential as it represents a systemic error that would be equally applied to all experimental conditions.

SD threshold data is presented as nL volume of 0.5 M KCl needed to trigger neocortical SD. Light machine oil was specifically used since its density was such that droplets of 0.5 M KCl floated in the oil and did not sink to the bottom of the slide well. For recurrent SD (six SDs evoked every 9–10 minutes over an hour), 0.5 M KCl nL volume was increased 2–3-fold to reliably evoke SD (Pusic et al., 2015). Craniotomies for the latter animals were retreated with subcutaneous Bupivacaine and closed under aseptic technique using wound clips (Autoclip; Clay Adams, Parsippany, NJ) with animals housed in single cages until the next day for the second day SD threshold measurements.

For both sets of animals (acute measurement of SD threshold and SD threshold 24 hours after recurrent SD) recordings were performed using microelectrodes (#6030 glass; A-M, Corporation, Everett, WA) filled with 150 mM NaCl with tip diameters of 2–8 μm , placed 6 mm caudal to bregma, 4.4 mm to the left side of the sagittal suture at a depth of 750 μm . A reference electrode made from a Pasteur pipette filled with 150 mM NaCl in 3.5% agar was placed on the left temporalis muscle. For second day induction of SD, KCl microelectrodes were placed 3 mm anterior to bregma and 2 mm lateral to the midline, 750 μm below the pial surface. Records were amplified with an Axoprobe A-1 system (Axon Instruments, Burlington, CA) and digitized using a Digidata 1440A analog-digital conversion board (Axon Instruments) for storage of the electrophysiological and injection timing records.

4.2 RT-qPCR

In a select group of animals treated with IGF-1 versus vehicle alone (five doses administered over 13 days), animals were deeply anesthetized on the 14th day with 5% inhalational isoflurane in oxygen, quickly decapitated and brains removed. Brains were sectioned down the midline and frozen in isopentane on dry ice and stored at -80°C . This sequence of steps took less than an hour for each animal. Matching segments of frontal neocortex were harvested for RNA isolation (Pusic et al., 2014). RNA was isolated from tissues using TRIzol reagent, followed by miRNeasy Mini Kit (Qiagen, Inc.-USA, Germantown, MD). Primer sequences were as follows: GFAP: [GenBank accession#: NM_017009.2; (forward): GCCTCTCCCTGTCTCGAATG and (reverse): TCATCCGCCTCCTGTCTGTA]; TNF α [GenBank accession#: NM_012675.3; (forward): ACCACGCTCTTCTGTCTACTGA and (reverse): CTGATGAGAGGGAGCCCATTTG]; iNOS: [GenBank accession#: NM_012611.3; (forward): AGAGACGCTTCTGAGGTTCC and (reverse): GTTGTGGGCTGGGAATAGC]; Rpl13a [GenBank accession#: NM_173340.2; (forward): TTGCTTACCTGGGGCGTCT and (reverse): CCTTTTCCTTCCGTTTCTCCTC] (Pusic et al., 2014). For each target gene, biological samples were run in duplicate, normalized to reference gene Rpl13a and analyzed using the $\Delta\Delta\text{Ct}$ method. Briefly, Ct values were averaged for each biological replicate and Ct calculated ($\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{Rpl13a}}$). The average Ct value of vehicle-treated control samples were then used to calculate Ct values for individual vehicle-treated control samples and IGF-1-treated experimental samples ($\text{Ct} = \text{Ct} - \text{Ct}_{\text{avg vehicle-treated control}}$). This allows us to determine standard error between individual vehicle-treated samples in the same manner as IGF-1-treated samples. Finally, fold change in target gene expression was calculated (fold change = $2^{-\text{Ct}}$). By this method, fold changes of 2 are considered significant (Pfaffl, 2001; Pusic et al., 2014; Pusic et al., 2015).

4.3. IGF-1 administration

We administered IGF-1 intranasally, as this route has been determined to be effective for delivery of IGF-1 to the brain (Lin et al., 2009; Liu et al., 2001; Thorne et al., 2004), at a dose and preparation that has been shown to confer protection in a rodent model of stroke (Liu et al., 2001). Animals were anesthetized in a fume hood via a rat nasal mask (model 906; Kopf, Tujunga, CA) with isoflurane (5% induction, 2–3% maintenance) in oxygen while in a recumbent position. Animals were kept warm ($37 \pm 0.5^{\circ}\text{C}$) with a heat lamp and a Digi-Sense thermoregulator (TC5000; Vernon Hills, IL) controlled with a rectal thermometer. Specifically, 150 μg human recombinant IGF-1 (#291-G1-01M; R&D Systems, Minneapolis, MN) in 50 μL vehicle (10 mM sodium succinate, 140 mM sodium chloride, pH 6.0) was administered to each animal intranasally by delivering 5 μL to alternating nostrils every 2 minutes over 20 minutes as previously done for interleukin-11 (Pusic et al., 2014) and interferon gamma (Pusic and Kraig, 2015). Sham controls received vehicle alone to control for any effects of anesthesia and animal handling. Throughout this study, each set of IGF-1-treated animals was compared to vehicle-treated age-matched and treatment schedule-matched shams, in order to control for any unwanted effects from anesthesia or animal handling.

4.4. Toxicity testing

Subsets of animals were used to probe for potential adverse effects after recurrent (every third day for five treatments over 13 days) nasal administration of IGF-1 (or vehicle) as described above. For serum analyses, animals were deeply anesthetized with progressive exposure to 100% carbon dioxide and decapitated with collection of truncal blood. Blood samples were incubated at room temperature for 20 minutes to allow complete clot formation without hemolysis. Cotton tipped wood sticks were used to scrape clotted blood away from glass tube walls and samples were centrifuged ($2,000 \times g$) for 15 minutes. Resultant serum samples were stored at -20°C in polypropylene 1.5 mL centrifuge tubes before shipment on dry ice to IDEXX Radil (Columbia, MO) for analyses using their standard liver and renal panels (Table 1).

A separate group of animals was treated with recurrent IGF-1 or vehicle (five treatments over 13 days). On the 14th day, animals were anesthetized with isoflurane (5% in oxygen) and maintained with intraperitoneal injections of ketamine and xylazine. Fully anesthetized animals were euthanized by perfusion-fixation with 10% buffered formalin followed by decapitation. Brains were extracted, post-fixed and processed for glial cell-specific immunostaining using 20 μm slide mounted sections. Brain midway between the where the anterior and posterior microelectrode sites would otherwise have been placed was chosen. Immunostaining for astrocytes used anti-GFAP IgG [(1:1,000) from Santa Cruz Biotechnology, Dallas, TX] and for microglia used anti-Iba1 IgG [(1:1,000) from Wako Pure Chemical Industries, Richmond, VA] plus AlexaFluor 488-conjugated (Millipore, Darmstadt, GM) secondary antibodies (1:1000)] as previously described (Pusic et al., 2014) and quantified using computer-based digital imaging strategies using MetaMorph software (ver. 7.5.4.0; Molecular Devices, Sunnyvale, CA) as previously described.

Briefly, glial fluorescence images were quantified (image average intensity) using a self-calibrating sensitive CCD digital imaging system consisting of: a QuantEM-512SC camera (500×500 pixels; Photometrics, Tucson, AZ), an electronic shutter (Lambda SC Smart Shutter; Sutter Instruments, Novato, CA) and a 200 W Xenon light source (Lumen 200, Prior Instruments, Inc. Rockland, MA) on a DMIRE2 inverted microscope (Leica, Buffalo Grove, IL) at 20× gain using stabilized electrical power (#Pro 1500; APC, West Kingston, RI) for both the light source and imaging system (Grinberg et al., 2012; Grinberg et al., 2013; Pusic et al., 2014). A stage micrometer was used to calibrate images. Four to five technical replicates were randomly acquired from neocortex for each biological replicate and the average fluorescence intensity registered. Imaging system parameters were uniformly set for all image acquisition sessions. Representative images of glial cells were inverted and uniformly enlarged by cropping and resizing in Photoshop (CC 2017; Adobe System, Inc., San Jose, CA) to improve visualization (Fig. 3).

A third subset of animals was treated with recurrent IGF-1 or vehicle (five treatments over 13 days). On the 14th day, animals were anesthetized with isoflurane (5% in oxygen) and maintained with intraperitoneal injections of ketamine and xylazine. Fully anesthetized animals were euthanized by perfusion-fixation with 10% buffered formalin followed by decapitation. Soft tissues were removed from heads which were then shipped to IDEXX Radil for deboning, histological processing and evaluation of nasal mucosa by a certified veterinary pathologist. Representative images of hematoxylin and eosin stained nasal mucosa were captured with a HV-D20 digital color camera (795×582 pixels; Hitachi Kokusai Electric, Inc., Rodgau, Germany) at 63× gain on a Leica (LMD6000) microscope. A stage micrometer was used to calibrate images. Representative Images were cropped, resized and Auto LUT applied in Photoshop.

4.5 Statistical Analysis

Data were analyzed using SigmaStat software (v.12.5, Systat Software, San Jose, CA). All data were subject to normality testing (p -value to reject: 0.05), equal variance testing (p -value to reject: 0.05), and power ($1-\beta$: >0.8). Sham control SD threshold measurements and vehicle-treated controls in molecular biology measurements were set to 1.00 with experimental data scaled proportionally to better allow inter-experiment comparisons with results expressed as mean $\pm$ SEM. SD threshold data is also presented as the nL range of 0.5 M KCl needed to evoke initial SD for each of the experimental groups. Molecular biological data analyses included two technical replicates per experimental measurement. All experimental groups consisted of biological replicates of $n = 3$ and comparisons were made between age-matched and treatment schedule-matched controls. Pairwise comparisons were made with Student's t -test and ANOVA for multiple comparisons.

Electrophysiological data was transferred from Axon Instruments software to OriginPro (ver. 8.1; OriginLab, Wellesley Hills, MA) for creation of manuscript figures. CorelDRAW X7 (Corel Corporation, Ottawa ON, Canada) and Photoshop CC 2017 (Adobe System, Inc., San Jose, CA) were used to construct manuscript figures.

Animal group sizes were based on our prior use of rats to probe for significant changes in designated SD-related experimental variables. This experience over 35 years has shown us

that we can generally use 3 – <10 animals to reach statistical significance with a Power that is always >0.80 and generally approaches or is 1.00.

All research materials used in this work are commercially available. All experimental data was included in this work.

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Abbreviations

CGRP	calcitonin gene related peptide
iNOS	inducible nitric oxide synthase
IGF-1	insulin-like growth factor-1
SD	spreading depression
TNFα	tumor necrosis factor alpha

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Highlights

- Spreading depression (SD) is the mechanism of migraine aura and perhaps pain
- Here we test whether intranasally administered IGF-1 protects from SD in adult rats
- A single dose of IGF-1 promptly protected against SD
- A single dose of IGF-1 continues to protect against SD out to 7 days
- Multiple SDs make brain more susceptible to future SD, and IGF-1 inhibits this

Figure 1. Paradigm for study of the impact of intranasal delivery of IGF-1 on SD

(A) Treatment paradigm for data shown in Figure 2A. Vehicle or IGF-1 was administered as a single dose (blue dot) and SD threshold evaluated at varying time points afterwards. (B) Experimental paradigm for data shown in Figure 2B. Multiple doses of IGF-1 or vehicle were administered (blue dots) and SD threshold subsequently measured. (C) Placement of KCl injection micropipette (2 mm caudal to bregma and 2 mm lateral to midline) and recording microelectrode (6 mm caudal to bregma and 4.5 mm lateral to midline) are shown. (D) Representative neocortical SD recorded 750 μm below the pial surface following KCl stimulus at the same depth. Voltage scale is negative down.

Figure 2. Intranasal IGF-1 protects from SD

(A) A single dose of IGF-1 protected against SD acutely and out to seven days. In each group, experimental animals were compared to age-matched and treatment schedule-matched vehicle-treated shams following the administration schedule shown in Figure 1A. The ranges of 0.5 M KCl volumes, in nL, needed to elicit SD were: 0.2 – 4.2 and 18.2 – 94.0 for acute sham and IGF-1, respectively; 0.4 – 1.6 and 4.2 – 44.6 for one day sham and IGF-1, respectively; 1.1 – 8.0 and 26.4 – 60.9 for three day sham and IGF-1, respectively; 1.1 – 5.9 and 6.5 – 29.6 nL for seven day sham and IGF-1, respectively. Each treatment paradigm resulted in a significant ($*p = 0.009, 0.029, 0.001, 0.025$, respectively) increase in SD threshold. (B) Multiple doses of IGF-1 (according to treatment schedule shown in Figure 1B) further protected from KCl-microinjection-induced SD. For multi-dosing, each group of experimental animals was compared to schedule-matched vehicle-treated shams, in order to control for repeated anesthesia exposure. The ranges of 0.5 M KCl volumes in nL needed to elicit SD were: 0.4 – 2.9 and 15.9 – 94.4 for one week multi-dose sham and IGF-1, respectively; 0.1 – 3.0 and 62.3 – 170.3 for two week multi-dose sham and IGF-1, respectively. Each treatment paradigm resulted in a significant ($*p = 0.007$ and 0.002 , respectively) increase in SD threshold.

Figure 3. Impact of nasal IGF-1 delivery on CNS glia

The impact of recurrent doses of IGF-1 or vehicle delivered nasally to rats over two weeks on glial activation was assessed. (A) Recurrent treatment with IGF-1 did not result in increased neocortical mRNA expression of GFAP (a measure of astrocyte activation), TNF α , or iNOS (both measures of microglial pro-inflammatory activation). Relative gene expression was calculated by the $\Delta\Delta C_t$ method, normalized to reference gene Rpl13a. Dotted line shows 2-fold change threshold for significance. Similarly, recurrent nasal delivery of IGF-1 (five doses given every third day for 13 days) did not activate brain glia, as evidenced by semi-quantitative immunostaining for astrocytes and microglia (see text for details). Middle row shows representative (B) naïve, (C) sham treated and (D) IGF-1 treated astrocytic GFAP immunostaining. Scale bar = 20 μ m. Bottom row shows representative (E) naïve, (F) IGF-1 treated and (G) sham treated microglial (Iba1) immunostaining.

Figure 4. Normalcy of nasal mucosa histology after recurrent IGF-1 administration

The impact of five doses of IGF-1 delivered nasally to rats over two weeks on nasal mucosa histology (hematoxylin and eosin staining) was evaluated and compared to naïve controls and vehicle sham animals. An external commercial service made and evaluated histological sections. For analyses, the nasal cavity was sectioned into 3 levels [(anterior) II middle (II) posterior (III)] in accordance with guidelines from the National Toxicology Program of the Department of Health and Human Services (Miller and Cesta, 2015). No evidence of mucosal cell necrosis, hyperplasia, or inflammatory cell infiltrates was noted in any of the histological sections (n = 4 per nasal level-animal treatment group type) except a single section from one IGF-1 treated animal that and no inflammatory cell infiltrates were noted. The latter showed evidence of a few pyknotic cells at nasal cavity level I. We consider this an incidental finding given that it was not seen in other sections from the same animal or in other biological replicates. For representative purposes, only images from IGF-1-treated animals at levels I (A), II (B) and III (C) are shown. Scale bar = 50 μ m.

Figure 5. SD increased susceptibility to subsequent SD, an effect prevented by IGF-1

(A) Representative interstitial DC potential changes are shown after recurrent micro-injections of normal saline (sham) or 0.5 M KCl (SD) positioned at $t=0$ as shown in (B). The animals were allowed to recover and then SD threshold was evaluated the next day with microinjections at $t=1$ day (B). (C) The relative threshold to SD was significantly ($p < 0.001$) decreased one day after the SD exposure when compared to animals exposed to the sham stimuli. This decrease in SD threshold was prevented as shown by a significant ($p < 0.001$) increase in SD threshold induced by IGF-1 treatment immediately following SD induction at $t=0$. The ranges of 0.5 M KCl volumes in nL needed to elicit SD were: 3.8 – 8.8, 1.4 – 2.9, and 11.5 – 18.5 nL for naïve, sham and IGF-1 treated animals, respectively.

Table 1

Weight and serum values for hepatic and renal function in animals receiving five doses of IGF-1.

Measure	Normal Range [#]	Sham	IGF-1	<i>p</i> value
2 week weight gain (grams)		61.6 ± 3.0	65.4 ± 9.2	0.704
Albumin/Globulin ratio	~1	1.00 ± 0.0	0.96 ± 0.02	0.141
Urea nitrogen (mg/dL)	12 – 22	22.8 ± 0.49	25.0 ± 2.28	0.373
Creatinine (mg/dL)	0.38 – 0.8	0.24 ± 0.02	0.24 ± 0.02	1.00
Sodium (mM)	142 – 150	139.4 ± 1.29	139.8 ± 0.86	0.803
Potassium (mM)	4.3 – 6.3	7.44 ± 0.16	7.24 ± 0.49	0.709
Chloride (mM)	100 – 109	101.20 ± 1.20	100.60 ± 0.60	0.626
Total protein (g/dL)	5.9 – 7.8	5.04 ± 0.07	5.20 ± 0.08	0.159
Albumin (g/dL)	3.3 – 4.6	2.52 ± 0.04	2.54 ± 0.07	0.803
Globulin (g/dL)	2.2 – 3.5	2.52 ± 0.04	2.66 ± 0.04	0.034 [*]
Calcium (mg/dL)	7.6 – 12.6	9.08 ± 0.12	9.16 ± 0.07	0.596
Phosphorus (mg/dL)	5.3 – 8.4	9.10 ± 0.19	9.54 ± 0.36	0.308
Total Bilirubin (mg/dL)	0.23 – 0.48	0.26 ± 0.02	0.26 ± 0.06	1.00
Alanine aminotransferase (U/L)	52 – 144	89.60 ± 3.23	56.80 ± 4.36	0.620
Alkaline phosphatase (U/L)	40 – 191	210.8 ± 12.24	196.6 ± 9.47	0.386
Aspartate aminotransferase (U/L)	54 – 192	139.6 ± 8.55	122.2 ± 13.61	0.310

[#]Normal values from IDEXX BioResearch.

^{*}Significant (Student's *t*-test) elevation of serum globulin, which nonetheless remained within the normal range, after 5 every-third-day doses of nasal IGF-1 administered over 13 days.