The Role of Hyperexcitable Existing Granule Cells in Seizure-Induced Ectopic Granule Cells

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Abstract:

In adult neurogenesis, where new functional neurons are generated from precursor cells, certain neurological conditions like seizures can disrupt the normal production, migration, and integration of these neurons. This disruption is characterized by ectopic granule cells (GCs) appearing in locations other than the granule cell layer, particularly following seizure-induced neurogenesis. These ectopic cells form abnormal connections and contribute to the hyperexcitability of hippocampal circuits. Although the precise molecular mechanisms behind this phenomenon remain unclear, the interplay between hyperexcitable old GCs and ectopic seizure-induced GCs is a critical factor in understanding epileptic pathology. This research proposal focuses on the hypothesis that seizure-induced neuroblasts migrate as expected but fail to connect with old GCs due to heightened excitability, resulting in abnormal connections in incorrect locations. This investigation seeks to shed light on this complex interplay and its implications for epilepsy and potential therapeutic targets.

Keywords: Adult neurogenesis, dentate granule cell neurogenesis, seizures, ectopic granule cells, seizure-induced ectopia

1. Introduction

Adult Neurogenesis is a currently widely accepted phenomenon that refers to the rise of new neurons from precursor cells, such as stem cells and lineage-determined progenitor cells (Cope & Gould, 2019; Kempermann et al., 2004; Ming & Song, 2005). In the adult mammalian brain, the major neurogenic regions encompass the subventricular zone (SVZ), from which newborn neurons migrate to the olfactory bulb, and the subgranular zone (SGZ), which is located between the granule cell layer (GCL) and the hilus of the hippocampal dentate gyrus. (Carleton et al., 2003; Cope & Gould, 2019; Kempermann et al., 2004; Ming & Song, 2005). The process of neurogenesis in the SGZ commences with the division of radial glial stem cells. These stem cells transform into amplifying progenitor cells that subsequently progress into neuroblasts, which are dedicated to differentiate into granule cells (GCs). The cell body of these neuroblasts migrates a short distance from SGZ to GCL and undergoes differentiation into GCs (Cope & Gould, 2019), projecting dendrites and axons to the inner molecular layer and the CA3 pyramidal cell layer respectively (Abbott & Nigussie, 2020).

Despite this orchestrated process, neurological conditions like epilepsy (Beghi et al., 2015) can disrupt the normal production, migration, and integration of new neurons. Epilepsy is defined by unprovoked recurrent seizures stemming from uncontrollable neuronal electrical activity. Animal models featuring pilocarpine-induced status epilepticus (SE) are applied in the study of epilepsy, particularly in the context of exploring mesial temporal lobe epilepsy (TLE). (Jessberger & Parent, 2015). Research on these models has revealed that SE-associated continuous seizures prompt substantial and continuous cell proliferation in the SGZ (Parent et al., 1997). Interestingly, ectopic GCs located in the hilus and the inner molecular layer rather than the GCL are observed during such seizure-induced neurogenesis. (Jain et al., 2023; Jessberger & Parent, 2015; Kokaia, 2011; Overstreet-Wadiche et al., 2006; Parent et al., 1997; Parent & Murphy, 2008)an area that contains neurons which are vulnerable to insults and injury, such as severe seizures. Previous studies showed that increasing adult neurogenesis reduced neuronal damage after these seizures. Because the damage typically is followed by chronic lifelong seizures (epilepsy. These ectopic cells project aberrant axons to both the dentate inner molecular layer and the CA3 pyramidal cell region, resulting in likely excitatory connections that contribute to the reorganization and hyperexcitability of hippocampal circuits (Parent et al., 1997). Consequently, this could trigger increased hippocampal seizure activity and the development of epilepsy (Parent et al., 1997).

Nevertheless, the exact molecular mechanisms behind seizure-induced neurogenesis (Kokaia, 2011) and ectopic newborn GCs (Parent et al., 1997, 2006) are not fully understood. Previous studies have demonstrated that the migration of newborn GCs is disrupted by enhanced

excitatory GABA(A)-R signaling after febrile seizures, further contributing to prolonged granule cell ectopia (Koyama et al., 2012). Moreover, continuous seizures lessen the immunoreactivity of Reelin, a secreted migration guidance cue of adult neurogenesis. Therefore, downstream Reelin signaling molecule Disabled 1 (Dab1) in ectopic neuroblasts in the hilus increases (Gong et al., 2007). While the molecular mechanisms behind the aberrant migration and integration of seizure-induced GCs are complicated, the intricate interplay between the proper migration and the reestablishment of functional connections has garnered increasing attention as a critical factor in epileptic pathology and potential therapeutic targets. This research proposal, distinct from prior studies, delves into the interplay between hyperexcitable old GCs and ectopic seizure-induced GCs. The hypothesis posits that seizure-induced neuroblasts migrate to the granule cell layer as expected but fail to connect with the old GCs due to the latter heightened excitability induced by the sudden electrical burst during seizures, resulting in abnormal connections in incorrect locations like the inner molecular layer as well as hilus.

2. Literature Review

2.1 *Seizures increase Dentate Granule Cell Neurogenesis and Contributes to Aberrant Network Reorganization in the Adult Rat Hippocampus*

It is widely acknowledged that the dentate GCs exhibit postnatal neurogenesis (Altman and Das, 1965, 1967; Gueneau et al., 1982; Eckenhoff and Rakic, 1988) that continues throughout adulthood in the rodent (Kaplan and Hinds, 1977; Bayer and Yackel, 1982; Kaplan and Bell, 1984; Cameron et al., 1993b; Seki and Arai, 1993; Kuhn et al., 1996). Apart from their distinct developmental pattern, these cells contribute to the development of temporal lobe epilepsy. (Houser, 1992; Manford et al., 1992; Weiser et al., 1993; Engel, 1996).

Building upon the context above and additional details, researchers in this paper proposed that the plasticity of the hippocampal network due to chronic seizures mainly arises from new granule neurons, not mature preexisting ones. BrdU was administered systemically to examine this goal first to label the mitotically active cells. Results from the experiments have demonstrated that cell proliferation in the SGZ increases during pilocarpine-induced SE. Next, BrdU was injected seven days after pilocarpine or saline treatment to examine the ultimate destiny of these mitotically active cells. BrdU immunostaining displayed a progressive distribution of labeled cells throughout the GCL in rats treated with pilocarpine, significantly surpassing that of the control group with saline. Other experiments further suggest that newly generated GCs in dentate gyrus exhibit ectopic migration modes and atypical organization of mossy fiber following seizures.

All the above findings in this research validate the fundamental theoretical framework of the hypothesis in this research proposal. The research question in this proposal is only logical and viable if seizures or SE will increase adult neurogenesis and contribute to ectopic newborn GCs.

2.2 *GABAergic Excitation after Febrile Seizures Induces Ectopic Granule Cells and Adult Epilepsy*

Neurotransmitter GABA and its receptors play an imperative role in the migration and integration of newborn GCs, as GABAergic inputs are the initial functional synaptic connections established in young GCs (Lopez-Rojas & Kreutz, 2016). Therefore, this paper concentrates on GABA receptors, examining the mechanisms that govern the appearance and role of ectopic GCs in the aftermath of experimental febrile seizures.

The primary finding of this study underscores the pivotal role of GABAA-R signaling in modulating the localization of GCs. Administration of the GABAA-R antagonist picrotoxin effectively counteracted the migration of ectopic GCs induced by seizures in rats. Conversely, applying a GABAA-R positive modulator amplified the number of ectopic GCs within the hilus. This observation gains added significance when combined with discovering excitatory GABAA-R inputs received by migrating hilar GCs. Consequently, the intricate regulation of this GABA receptor substantiates the hyperexcitable microenvironment to which migrating GCs are exposed.

When GABAA-R is blocked, according to the above conclusion, the excitatory inputs migrating neurons receive reduce, indicating that the inhibitory effects on those moving neurons are stronger. Under such a premise, hypothesizing the existing GCs are hyperexcited enough to disrupt their normal connection with the migrating new ones is feasible since the paper doesn't address where those excitatory GABAA-R inputs come from. Thus, this paper proves the feasibility of the hypothesis in this research proposal.

3. Materials and Methods

3.1 *Animals*

3.1.1 *General information*

To investigate whether existing GCs play an important role in newborn granule cell ectopia, a chemogenetic

approach in conjunction with transgenic experimental models of mice (Zhu et al., 2016) is applied in this research. Synthetic variants of human muscarinic receptors, hM3Dq and hM4Di, coupled to Gi and Gq proteins, respectively (Armbruster et al., 2007; Tunc-Ozcan et al., 2019)"container-title":"Proceedings of the National Academy of Sciences","DOI":"10.1073/pnas.0700293104","ISSN":"0027-8424, 1091-6490","issue":"12","journalAbbreviation":"Proc. Natl. Acad. Sci. U.S.A.","language":"en","page":"5163-5168","source":"DOI.org (Crossref, are exclusively activated by the exogenous ligand clozapine-N-oxide (CNO) (Armbruster et al., 2007)"container-title":"Proceedings of the National Academy of Sciences","DOI":"10.1073/pnas.0700293104","ISSN":"0027-8424, 1091-6490","issue":"12","journalAbbreviation":"Proc. Natl. Acad. Sci. U.S.A.","language":"en","page":"5163-5168","source":"DOI.org (Crossref.

Mice with floxed hM3Dq and hM4Di alleles are crossbred with mice carrying tamoxifen-inducible Cre recombinase controlled by a specific promoter, specifically targeting pre-existing GCs in the GCL. This breeding leads to the generation of double-transgenic progeny (Tunc-Ozcan et al., 2019). The mice's genotyping is performed by PCR using their genomic DNA (Tunc-Ozcan et al., 2019) and primers. Tamoxifen administration helps achieve conditional expression of HA-tagged hM4Di and hM3Di (Tunc-Ozcan et al., 2019). The control group consists of mice that do not carry Cre and/or DREADD genes and are littermates of heterozygote breeding. (Tunc-Ozcan et al., 2019).

3.1.2 *Suppressing Existing Granule Cells During Seizures*

hM4Di, the inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADD), is controlled to express in the first cohort of mice in their existing GCs in the dentate gyrus to prevent these neurons from hyperexcitability when seizure is induced. After tamoxifen administration, HA-Tagged hM4Di is expressed in these old GCs in the double-transgenic progeny, +hM4Di mice. Following binding with CNO, hM4Di triggers the canonical Gi pathway, causing hyperpolarization of the newly generated neural cells, which can selectively reduce the excitability of pre-existing neural cells in the GCL.

3.1.3 *Activating Existing Granule Cells During Seizures*

Another double-transgenic mouse line, called +hM3Dq mice, is created in the second group of mice. This line expresses HA-tagged hM3Dq in the old neurons following tamoxifen administration. Similar to the expression of the inhibitory DREADD hM4Di, hM3Dq, following CNO administration, activates the canonical Gq pathway and thus causes the increased firing of the old GCs. (Rogan & Roth, 2011; Tunc-Ozcan et al., 2019).

3.2 *Drug Administration*

3.2.1 *Pilocarpine-induced status epilepticus*

Two days before the pilocarpine injection, the mice are pinched in the lower back at the intended injection site. This approach is designed to minimize stress during pilocarpine treatment, as stress can influence both seizures and adult neurogenesis. (Cain & Corcoran, 1984; Maguire, 2014; Sawyer & Escayg, 2010; (Jain et al., 2019).

One dose of pilocarpine is administered following an initial injection of pretreatments (Jain et al., 2019). The pretreatments are a solution of scopolamine methyl nitrate, a muscarinic antagonist aimed at minimizing the side effects of pilocarpine and thus preventing mortality, and terbutaline hemisulfate, aβ2-adrenergic receptor agonist that supports respiration during seizures (Cho et al., 2015) the effect of ablating adult neurogenesis before acute seizures is long lasting as it suppresses chronic seizure frequency for nearly 1 year. These findings establish a key role of neurogenesis in chronic seizure development and associated memory impairment and suggest that targeting aberrant hippocampal neurogenesis may reduce recurrent seizures and restore cognitive function following a pro-epileptic brain insult.","container-title":"Nature Communications","DOI":"10.1038/ncomms7606","ISSN":"2041-1723","issue":"1","journalAbbreviation":"Nat Commun","language":"en","page":"6606","source":"DOI.org (Crossref. Pilocarpine hydrochloride is injected thirty minutes after the pretreatments (Jain et al., 2019). An additional dose will be given if seizure activity does not commence within 1 hour after the initial injection of pilocarpine hydrochloride (Parent et al., 2006). Seizures are stopped using diazepam following approximately 4 hours of convulsive SE (Parent et al., 1997). Control mice are treated with 0.9% sodium chloride solution instead of pilocarpine (Parent et al., 2006) and diazepam injections simultaneously (Parent et al., 1997). No pretreatments are required for the controls.

3.2.2 *Tamoxifen and CNO administration*

For conditional hM4Di and hM3Dq expression, tamoxifen is injected for five consecutive days (Tunc-Ozcan et al., 2019). There is no tamoxifen injection in the two cohorts for the controls with Cre and/or DREADD-negative mice as a result of heterozygote breeding (Tunc-Ozcan et al., 2019).

The day after 5-day consecutive tamoxifen administration, CNO is injected in 4 cohorts of mice. Control mice received saline injections in the same time course.

3.2.3 *Immunostaining and immunohistochemistry*

Newly generated GCs are labeled by a single dose of the S-phase marker 5-bromo-2'-deoxyuridine (BrdU) one day after pilocarpine injection (Koyama et al., 2012). BrdU staining allows experimenters to observe the existence and migration of seizure-induced GCs. Mice are sacrificed at either 1, 7, 14, or 35 days after BrdU staining for immunohistochemical staining with Prospero homeobox 1 (Prox1), which is used to mark the GCs (Koyama et al., 2012; Muramatsu et al., 2008)and these "ectopic" GCs have synchronous epileptiform bursting with other hippocampal neurons. In this study, we investigated whether early-life status epilepticus (SE.

A confocal microscope will be employed to image the sections of the mice's hippocampus. The number of BrdU+ nuclei located in or near the GCL and the hilus, along with cells displaying colocalization of BrdU and Prox1, is counted.

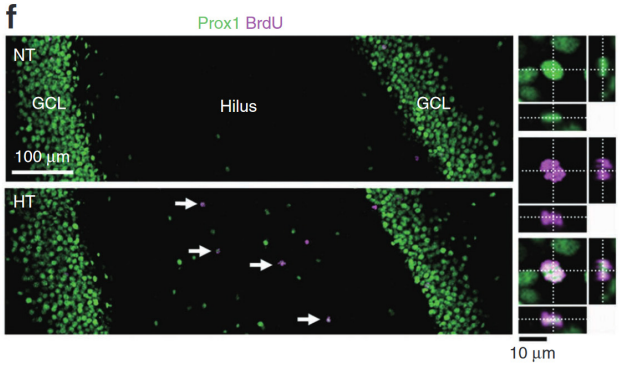

Figure 1 *BrdU and Prox1 immunostaining*

Note. Adapted from Koyama et al., 2012. Green areas in the figure represent Prox1-stained old GCs (the GCL). Purple areas represent BrdU-labeled newborn granule cells. In the hilus of the mice from the seizures cohort, newborn granule cells (Prox1+BrdU+) are shown by the white arrows.

3.2.4 *Electrophysiological analysis*

Electrophysiological analysis of patch clamp is applied to measure the electrical activity of the hippocampus and thus to confirm the hyperexcitability of the granule cell layer during seizures.

3.2.5 *Semicircular diagrams of the dentate gyrus in the hippocampus*

Semicircular diagrams are used like that in Koyama et al., 2012 to visualize how newly generated GCs are distributed. Granule cell positions were determined using angular and distance measurements in the dentate gyrus. Each cell's angle was defined by connecting the hilus center with the suprapyramidal edge (0°) and the infrapyramidal edge (180°) of the GCL. Distances were normalized to the distance from the CA3 pyramidal cell layer edge to the SGZ in the hilus. For newborn GCs in the GCL, the GCL's total width was used for normalization. These methods facilitated comparisons of granule cell locations across different dentate gyrus sections through semicircular diagrams.

3.2.6 *Overall experimental procedure*

All mice are divided into four groups (n=5 animals per group). The first group contains double-transgenic +hM4Di mice, whose old GCs are inhibited when seizures occur. The second group consists of double-transgenic +hM3Dq mice, whose old GCs are further activated after seizures are induced. Animals in both groups received tamoxifen treatment for five consecutive days, and CNO was administered one day after tamoxifen administration. Then pilocarpine and its pretreatments are given to the animals. Immunostaining's process and time course remains the same in all four groups. The day after pilocarpine injection, BrdU immunostaining labels seizure-induced proliferating GCs. Mice are sacrificed at either 1, 7, 14, or 35 days after BrdU labeling for immunohistochemical staining with Prox1.

Mice in the third group receive identical treatment to the preceding two groups, except that they are Cre and/or DREADD-negative progeny from heterozygote breeding (Tunc-Ozcan et al., 2019). In combination with the first group, the third cohort of mice aims to investigate whether hyperexcitable old GCs contribute to seizure-induced ectopic GCs. The comparison between the second and the third cohort of animals is intended to further confirm the above hypothesis.

The fourth cohort of mice, Cre and/or DREADD-negative littermates from heterozygote breeding (Tunc-Ozcan et al., 2019), only undergoes the same immunostaining process as the above three groups without pilocarpine-induced seizures. Group 3 and Group 4 are designated to prove that seizures will increase adult neurogenesis.

4. Expected Result

4.1 *Seizures increase adult neurogenesis in the dentate gyrus*

Suppose the number of newly generated GCs in the semicircular graph is considerably larger in the third cohort of mice compared to the fourth cohort (as shown in Figure 2). In that case, seizure can be proved to increase adult hippocampal neurogenesis in the dentate gyrus. Such results will be consistent with the observations in (Koyama et al., 2012); (Parent et al., 1997) (Jessberger & Parent, 2015).

4.2 *Seizures increase granule cell ectopia in the hilus*

By comparing the number of newborn GCs within the hilus region between the third and fourth groups, experimenters can investigate whether seizures contribute to an increment in ectopic GCs. Suppose a significantly higher quantity of ectopic GCs is found in the hilus of the third cohort of mice compared to the fourth group. In that case, it can be inferred that seizures contribute to the migration of newborn GCs to ectopic locations (as shown in Figure 2 and 3).

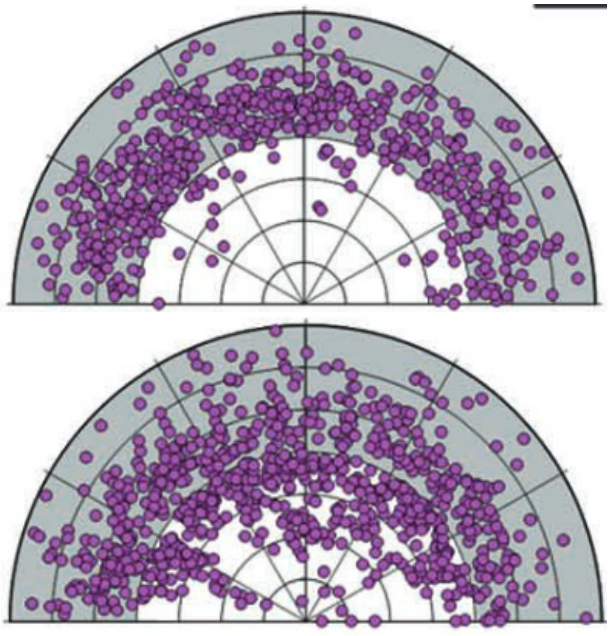

Figure 2 Semicircular graphs of mice in group 3 (right) and group 4 (left)

Note. Adapted from Koyama et al., 2012. The left semicircular graph displays results from group 4, while the right one represents group 3. The third group shows a larger number of newborn GCs, indicating that seizures enhance adult neurogenesis. Additionally, group 3 exhibits more ectopic granule cells, underscoring that seizures contribute to granule cell ectopia.

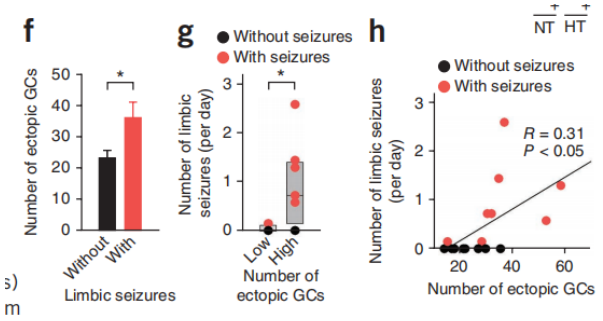

Figure 3 *Line graphs showing the number of ectopic GCs in groups with and without seizures*

Note. Adapted from Koyama et al., 2012. Line graphs depicting that ectopic GCs increase due to seizures.

4.3 *Hyperexcitable existing granule cells contribute to ectopic newborn granule cells*

Assuming that the quantity of seizure-induced ectopic GCs in group 2 is significantly higher compared to that in group 3 (where the hyperexcitability of old GCs during seizures is further activated), and the quantity in group 1 (where the hyperexcitability of old GCs during seizures is inhibited) is notably lower than that in group 3, it can be concluded that hyperexcitable pre-existing GCs indeed prompt the ectopia of newly generated GCs.

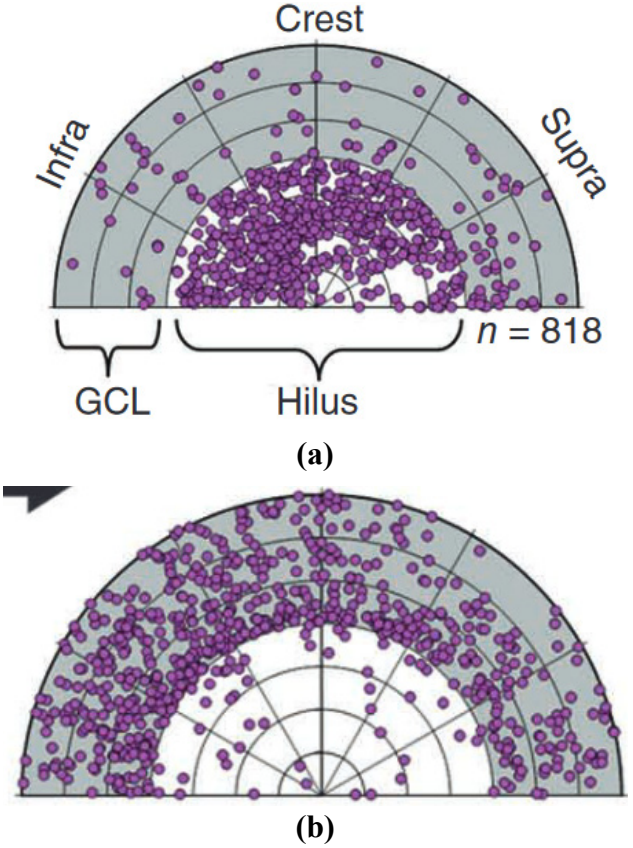

Figure 4 *S*emicircular graphs of mice in group 2 (a) and group 1 (b)

Note. Adapted from Koyama et al., 2012. Semicircular graph (a) represents results from group 2, while the graph (b) represents group 1. A detailed explanation is given above.

5. Discussion

The above-expected results first convincingly prove how seizures and adult neurogenesis correlate and can significantly increase adult hippocampal neurogenesis in

the dentate gyrus, which is fully established according to previous studies (Jessberger & Parent, 2015; Parent et al., 1997). In addition, by suppressing and enhancing the activity of the GCL during seizures, the anticipated outcomes reveal that seizures could lead to heightened excitability in pre-existing neurons, such as the GCs, influencing the migration of newly generated cells. Given the statistical relationship between misplaced GCs and epilepsy in adult rats with febrile seizures, there emerges a potential therapeutic avenue: addressing the appropriate migration of newborn GCs.

When it comes to the functional significance of hilar ectopic GCs, as reviewed (Parent & Murphy, 2008) respectively (Bengzon et al., 1997; Parent et al., 1997, 2002; Scott et al., 1998, some evidence suggests that hilar ectopic granule cells (HEGCs) integrate in an aberrant fashion. These seizure-generated HEGCs exhibit excitatory postsynaptic potentials when exposed to extracellular stimulation within the outer molecular layer in the dentate gyrus (Scharfman et al., 2003). Furthermore, anatomical evidence indicates that pyramidal neural cells in area CA3 form recurrent synapses and sprouting of mossy fibers induced by seizures within CA3 and the dentate gyrus, respectively, which help establish the assumption that HEGCs can presumably lead to seizures through joining in a reverberatory loop (Buckmaster & Dudek, 1997; Parent & Murphy, 2008; Scharfman, 2007) Paul S. and F. Edward Dudek. Network properties of the dentate gyrus in epileptic rats with hilar neuron loss and granule cell axon reorganization. J. Neurophysiol. 77: 2685–2696, 1997. Neuron loss in the hilus of the dentate gyrus and granule cell axon reorganization have been proposed as etiologic factors in human temporal lobe epilepsy. To explore these possible epileptogenic mechanisms, electrophysiological and anatomic methods were used to examine the dentate gyrus network in adult rats that had been treated systemically with kainic acid. All kainate-treated rats, but no age-matched vehicle-treated controls, were observed to have spontaneous recurrent motor seizures beginning weeks to months after exposure to kainate. Epileptic kainate-treated rats and control animals were anesthetized for field potential recording from the dentate gyrus in vivo. Epileptic kainate-treated rats displayed spontaneous positivities ("dentate electroencephalographic spikes".

However, the mechanisms behind hilar ectopic GCs are not fully understood. Several factors such as migration guidance cue Reelin, GABAergic hilar neurons, and hyperexcitable granule cell layer, which will be deeply discovered in this research, may work together to contribute to the ectopic phenomenon. Future research focusing on understanding the intricate interplay of various influencing factors could pave the way for more effective therapeutic approaches to address seizures and similar brain injuries.

6. Conclusion

The extensive study presented in this research proposal delves into the intricate relationship between seizures, adult neurogenesis, and their role in epilepsy. The expected results confirm that seizures significantly impact adult hippocampal neurogenesis in the dentate gyrus. These results are consistent with existing studies and provide further evidence of the complex interplay between neurological conditions and neurogenesis.

One of the key findings is the increased excitability in pre-existing granule cells (GCs) due to seizures, which, in turn, affects the migration of newly generated cells. The presence of ectopic GCs in the hilus and inner molecular layer, rather than the granule cell layer (GCL), highlights the abnormal connections formed during seizure-induced neurogenesis. This phenomenon is crucial in understanding the reorganization and hyperexcitability of hippocampal circuits, which are strongly associated with epilepsy development.

Despite these advancements, the molecular mechanisms underlying seizure-induced neurogenesis and ectopic GC formation remain complex and not fully elucidated. Studies indicate the role of factors like enhanced GABA(A)-R signaling and changes in Reelin signaling in the process. This research proposal offers a new perspective on the interplay between hyperexcitable old GCs and ectopic seizure-induced GCs. It suggests that while seizure-induced neuroblasts migrate to the granule cell layer, they struggle to connect with old GCs due to heightened excitability caused by sudden electrical bursts during seizures. This results in abnormal connections in incorrect locations, such as the inner molecular layer and hilus.

In conclusion, this research deepens our understanding of the relationship between seizures and adult neurogenesis and sheds light on the potential mechanisms that lead to epilepsy. It underscores the importance of studying the complex interactions between various factors in the brain. These findings open new avenues for therapeutic approaches aimed at addressing seizures and similar neurological conditions, potentially improving the quality of life for those affected by epilepsy. Further research in this direction is crucial for developing effective treatments and interventions.

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