Does Acquired Epileptogenesis in the Immature Brain Require Neuronal Death?

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Because epilepsy often occurs during development, understanding the mechanisms by which this process takes place (epileptogenesis) is important. In addition, the age-specificity of seizures and epilepsies of the neonatal, infancy, and childhood periods suggests that the processes and mechanisms that culminate in epilepsy might be age specific as well. Here we provide an updated review of recent and existing literature and discuss evidence that neuronal loss may occur during epileptogenesis in the developing brain, but is not required for the epileptogenic process. We speculate about the mechanisms for the resilience of neurons in immature limbic structures to epileptogenic insults, and propose that the type, duration and severity of these insults influence the phenomenology of the resulting spontaneous seizures.

Does Epileptogenesis in the Developing Brain Require Cell Loss?

Loss of specific neuronal populations in hippocampal areas CA1 and CA3/4, and the hilus of the dentate gyrus, is a common feature in hippocampi resected from patients with TLE (1–9). TLE often follows early-life prolonged febrile and other seizures, and it is currently unclear if the neuronal loss found in epileptic tissue preceeds or is a result of the TLE itself (7, 8, 16). In adult models of TLE in which status epilepticus (SE) is the inciting insult, cells loss is common and is plausibly required for the epileptogenic process. Because of these associations, cell loss was extensively investigated in several developmental epilepsy models. These include immature rodent models of neonatal seizures (17, 18), prolonged febrile seizures (19, 20) and febrile status epilepticus (21), and pediatric nonfebrile status epilepticus (22–24).

In the model of prolonged febrile seizures or febrile status epilepticus (FS/FSE), initial studies found no increase in acute apoptotic cell death anywhere in the hippocampal formation at 4 to 24 hours after the seizures. However, injury to pyramidal cells in hippocampal CA1 and CA3 was observed in a pattern reminiscent of human mesial temporal sclerosis (MTS). This injury, manifest as augmented Golgi-staining (19), was also apparent in the hilus, yet resolved within weeks without progressing to cell loss. Delayed cell loss was also systematically explored in vulnerable hippocampal regions in four additional animal cohorts (21, 25–27). Methods employed included Cresyl Violet augmented by neurochemical markers for interneurons and pyramidal cells as well as for glia (25). The possibility that the failure to find an appreciable reduction of cell numbers might be a result of neurogenesis was also considered using BrDU cell-birth dating (25). In all cases, blinded analyses using stereologic principles failed to show significant neuronal loss in vulnerable hippocampal regions in rats that sustained the seizures, including those that developed limbic epilepsy (21, 26). Notably, even hippocampi of rats that developed spontaneous motor seizures lasting over 100 seconds were devoid of appreciable neuronal dropout (21). Looking at potentially vulnerable regions elsewhere in the brain (e.g., dorsomedial thalamus [28]), apparent cell loss was absent, whereas injury was found also in limbic cortices (19). These data suggest that significant acute or delayed cell death was not required for the onset of TLE after experimental prolonged febrile seizures. Whether the resulting TLE, that is, the spontaneous seizures in a subgroup of FS/FSE rats, could eventually lead to cell loss in a distribution similar to the loss in MTS and to the injury observed by Toth et al. (19) remains to be determined.

Because neonatal seizures may be followed by the onset of epilepsy, several models have been developed to examine these seizures. These include seizure induction by hypoxia as well as by chemoconvulsants including kainate and repeated flurothyl exposure (17, 18, 29, 30). Among these paradigms that employed postnatal day-7 to day-10 rats, the hypoxia-induced seizure model generated later-life spontaneous seizures assessed by behavioral and EEG parameters (31). Recently, Rakhade et al. (31) have reported that over 90% of rat pups experiencing early-life hypoxic seizures develop recurrent spontaneous seizures by P100, as assessed by video-EEG long-term monitoring with intracranial depth electrodes. Moreover,
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Collectively, these studies demonstrate that some acquired epilepsy can arise without appreciable cell loss in the developing brain. Consistent with recent opinions (35), the studies do not imply that cell loss is not an important factor in some cases of pediatric epilepsy. Stroke, ischemia, trauma, or severe infection may lead to cell death during development, and to associated epilepsy. In addition, not all seizures cause epileptogenesis, and, in most children, seizures result in neither apparent structural changes nor later epilepsy. Further, clinical observations suggest that more severe brain insults, associated with severe brain injury, might be more likely to provoke epilepsy in children, and probably also in immature animals. Indeed, diverse etiologies can produce epilepsy after early-life insults, and in both humans and animals, there is no reason to assume that the mechanisms leading to epilepsy would be the same in each etiology. Thus, it is quite conceivable that cell loss occurs in some models of developmental epilepsy, such as in hypoxia-ischemia or neonatal stroke, and plays an intrinsic role in epileptogenesis. In other clinical situations and animal models, cell loss may not occur, or, if it is observed, this cell loss may be neither causal nor necessary for epilepsy development. In summary, epileptogenesis early in life may be associated with cell loss in vulnerable hippocampal regions and in other brain areas. However, epileptogenesis may arise also in the absence of appreciable cell loss: it is not required for acquired epileptogenesis, at least in the developing brain (and potentially in some instances of epileptogenesis taking place in the mature brain).

The Important Questions About Epileptogenesis in the Immature Brain: Why Are Neurons Resilient to Excitotoxicity, and What Are the Key Mechanisms of Epileptogenesis?

Lack of an absolute requirement for cell loss in early-life acquired epileptogenesis is an important conceptual point, because it brings up two important gaps in our current understanding of epileptogenesis in general. These gaps hamper the development of interventional and therapeutic approaches.

The first question relates to the mechanism of the resilience of neurons in the immature hippocampus, limbic cortices, and thalamus to status epilepticus-induced cell death (36–38). Why don’t neurons die, when the inciting seizures are prolonged and severe? Can we exploit the underlying mechanisms to protect neurons in adults from similar insults? The mechanisms that contribute to the resilience are not fully understood; two candidates include mitochondrial uncoupling and the relative paucity of inflammation. During adult SE, metabolic demand in neurons results in the overwhelming of mitochondrial function and, hence, accumulation of reactive oxygen species (ROS), with eventual mitochondrial decompensation and runaway cell death (39). In immature brain, a fat-rich diet promotes augmented expression of the mitochondrial uncoupling protein 2 (UCP2). This protein reduces mitochondrial membrane gradient, prevents ROS accumulation and protects from SE-induced neuronal death (40, 41). A second, perhaps complementary protective element is the attenuated inflammatory response to SE during development (42). This is in contrast to the adult hippocampus, where cytokines and related mediators are both released from injured cells and contribute to neuronal death (42). Obviously, numerous other factors, potentially including augmented levels of growth factors such as brain-derived neurotrophic factor (BDNF), might protect neurons in the immature brain from excitotoxic and oxidative injury.

The second question is the logical extension of refuting the hypothesis that cell death is the sine qua non of epileptogenesis during development. Namely, if cell death is not the principal mechanism for epileptogenesis, then what is required for the epileptogenic process during infancy and childhood? If obvious structural defects are not required, then how does the developing brain become epileptic after insults? The mechanisms that truly govern the changes in neuronal phenotype and network properties that, in turn, result in epilepsy, must then be sought. Indeed, the study of these mechanisms is at the forefront of our efforts to understand epileptogenesis in the developing brain.

Results that have already been obtained in the models described above as well as by other groups are beginning to answer these questions. For example, neonatal seizures...
induced by hypoxia led to dramatic changes in subunit expression of the AMPA subtype of the glutamate receptors, as well as kinase and phosphatase activation and subsequent posttranslational modification of glutamate and GABA receptors (34, 43, 44). Long-lasting changes in the expression patterns of GABA\_\text{\textalpha} receptor subunits have been described after pilocarpine-provoked status epilepticus (45, 46). Similarly, a number of gene-expression changes, including early and enduring reduction of the expression of the hyperpolarization-activation cyclic nucleotide gated (HCN) channel type 1 and augmented endocannabinoid receptors were found after prolonged experimental FS (47–49). These molecular changes have been shown to be associated with changes in ionic currents that promote hyperexcitability and vulnerability to subsequent seizures (43, 44, 49–52). The fundamental basic transcriptional and posttranslational regulatory mechanisms governing the major and persistent changes in gene expression that promote epileptogenesis during development are under intense investigation (53–56).

**Does Temporal Lobe–Like Epilepsy Actually Result From Experimental Developmental Seizures?**

If cell loss is minimal or absent after some developmental seizures, and if cell loss is considered crucial for epileptogenesis, then it is reasonable to question whether acquired epileptogenesis actually takes place after experimental FSE, chemical SE, or neonatal hypoxia-related seizures. The clinical diagnosis of epilepsy requires the documentation of two or more seizures. Notably, in the clinical situation, prolonged monitoring using video-EEG to capture and document seizures is not required for a diagnosis of epilepsy and is rarely used for this purpose. Using the same criteria (i.e., documentation of two seizures), epilepsy was documented after developmental seizures in the models described here. In addition, prolonged video-EEG monitoring was carried out in all to address additional questions. When does epilepsy arise? How frequent are the seizures? Does the frequency vary? When does abnormal (interictal) brain activity commence? Unlike the confirmation of the presence of epilepsy and the phenotype of seizures, these types of questions may require continuous long-term monitoring in both patients and animal models.

A second point is the definition of a seizure. This is a complex and controversial issue, and conservative approaches consisting of the employment of both EEG and behavioral measures of seizures are preferred in both patients and animals. Galvan et al. (57) and Nissinen et al. (58) defined seizures as EEG abnormalities consisting of rhythmic discharges involving doubling of voltage and lasting over 6 seconds, associated with behavioral phenomena. D’Ambrosio et al. (59) suggested that a minimal time limit might not be needed. Both of these definitions exclude events found on EEG alone. In addition to the video-EEG criteria, seizures can be defined on behavioral criteria alone; for example, when overt motor phenomena consisting of the classic Racine progression (60) are observed. Because behavioral phenomena of limbic seizures in both humans and animal models are often subtle, behavioral approaches alone, as well as the requirement for behavioral change in association with EEG criteria may underestimate the prevalence of seizures.

After experimental long FS (lasting ~20 minutes), spontaneous short seizures consisting of Racine stage 0 to II behaviors and EEG discharges longer than 6 seconds were observed (26). These FS are induced in postnatal day-10 to day-12 rats, where the stage of hippocampal development is roughly equivalent to that of human infants (a comparative table can be found in Avishai-Eliner et al. [36]). Similarly, in the hypoxia-induced seizure model in postnatal day-10 rats, electrographic seizures were always associated with abnormal behavioral activity or motor phenomena. In both cases, these relatively subtle seizures were distinguishable from theta bursts seen in control animals, which were generally shorter and associated with exploratory behavior (26, 31).

In the model of prolonged FS, when the duration of the inciting FS was increased, generating experimental febrile status epilepticus, long-term video-EEG monitoring using hippocampal and cortical electrodes documented the appearance of longer spontaneous seizures (mean duration 137 seconds; median, 91 seconds) on EEG. Notably, these epileptic events were associated with motor phenomena (Racine stages III–V; 60), including unilateral clonus, bilateral clonus, and rearing and falling (21). These are the classical behavioral sequences observed in limbic seizures that generalize.

In a developmental model of pilocarpine-induced status epilepticus in weaning postnatal day-20 rats, EEG and intracranial video recording demonstrated spontaneous seizures defined as discrete alterations in behavior accompanied by rhythmic electrographic discharges that evolved over time and lasted for 8 or more seconds (22). Sixty-seven percent of the rats (12/18) subjected to lithium-pilocarpine–induced SE at P20 went on to develop spontaneous seizures in adulthood with a latency to spontaneous seizure onset of 45.2 ± 9.0 days. The behavioral seizure phenotypes included wild running, facial clonus, staring, head bobbing, and tail stiffening lasting from 8 to 20 seconds (stages II–III) as well as generalized tonic-clonic activity with falling (Racine stage V seizures) lasting up to 43 seconds. All behavioral seizures had EEG correlates.

Together, these findings show a spectrum of the behavioral and electrographic seizures incited by early-life insults and enable speculation about the basis of the often more subtle nature of the spontaneous seizures that are provoked by these early-life insults. The duration or severity of the inciting event might be an important factor (21). In addition, unlike the consequences of status epilepticus that provokes TLE in adult models, catastrophic injury to regions that are known to gate seizure propagation, such as the dentate gyrus, is not observed after developmental seizures and status epilepticus. Hence, it is possible that seizures that result from more subtle injury in the context of a relatively preserved hippocampal circuitry may not readily generalize. The limbic phenomenology of freezing, staring, and facial automatisms, as well as focal motor activity, may thus be more typical of TLE that follows developmental inciting events.

In summary, epilepsy, defined as more than two spontaneous seizures (using a conservative definition of seizures), was documented in several models of pediatric epileptogenesis. More work is required to define precisely the temporal evolution of this epileptogenesis.
Are We Using Appropriate Developmental Models to Study Acquired Epileptogenesis in the Immature Brain?

It is an a priori assumption that no single animal model can capture the full breadth of the clinical condition (35). Suitable models focus on salient elements of the condition that is being modeled, and on the questions that are being studied. In addition, because acquired epilepsy in infancy and childhood is heterogeneous and can result from numerous etiologies, varied animal models of developmental epilepsies induced by different methods will undoubtedly be needed to fully understand the breadth of mechanisms that may underlie developmental epileptogenesis.

Because prolonged FS and FSE may be a risk factor described in a significant proportion of individuals with TLE (61, 62), it is important to study if and how these developmental seizures might cause epilepsy. However, unlike in young children, fever cannot be induced in immature rats. Therefore, as stated by Reid et al., “the reason that all models employed to date rely on more than just a true fever is that, at least at the ages tested, even in immature common laboratory rat strains, FSS are difficult to evoke....” This has led to a number of models of FS, using hyperthermia (19, 21, 25, 27, 49, 51, 52, 63–68), or lipopolysaccharide combined with kainic acid at ambient temperature of 30°C (20, 69). Whereas the mechanisms for the elevation of brain temperature might differ in these models, hyperthermia per se induces the release of endogenous fever mediators and specifically interleukin 1 beta (IL1-β) in the brain. Indeed, binding of IL1-β to its receptor may be required for “febrile” seizure generation, as found by studying IL1-β receptor null mice (70, 71). To exclude the possibility that hyperthermia itself may provoke epilepsy, hyperthermic controls have been used (26, 51). In addition, core and brain temperatures during these seizures have been monitored throughout their duration (21, 71, 72), and refinement of the model has lowered these temperatures to ~39 to 40°C. Thus, temperatures employed in these models do not exceed those in children with high fever. In addition, dehydration, a marker of heat stroke or shock does not take place (weight loss is ~2%, whereas a clinically relevant change is >5%).

Neonatal seizures may precede epilepsy in children and are most commonly caused by hypoxic/ischemic encephalopathy (17). Similar to the human, the immature rat responds to hypoxia and hypoxia/ischemia with seizures that can be refractory to conventional anticonvulsants (73, 74) and may thus prove useful for preclinical investigative and therapeutic studies (75).

Whereas neonatal and febrile seizure models are intrinsically age specific, chemical convulsant-induced status epilepticus has been extensively used in the study of adult epileptogenesis. The most common chemoconvulsants include pilocarpine (with or without lithium) (76–79), and kainic acid. Developmental models have relied on these chemoconvulsants as well (22, 28, 80, 81), and it is reasonable to propose that, in this case, the validity of these models does not vary with age.

Conclusions

In summary, epilepsy affects primarily children and young adults, so that studying how it arises is of paramount clinical importance. Models have been established and validated for several of the many and varied insults to the developing brain that can lead to epilepsy, including neonatal hypoxia, hypoxia-ischemia, fever/hyperthermia, and status epilepticus. The resulting data indicate that epileptogenesis in the developing brain has features that are distinct from those in the adult. Therefore, extrapolating principles established from the study of adult epileptogenesis (including cell loss) to developmental epileptogenesis may not be justified. In addition, careful consideration of the state of maturation of specific brain regions across ages and across species might be warranted (a comparative table can be found in Avishai-Eliner [36]). The emerging consensus further suggests that epileptogenic insults early in life might act by changing the gene expression repertoire and functional phenotype of neurons, rather than by killing them. These data are exciting because if we understand these mechanisms and identify the “master switches” that govern them, we will have made a major advance in preventing developmental epileptogenesis.

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References


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<td>X</td>
<td></td>
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<td>Add</td>
</tr>
<tr>
<td>5. Grants/grants pending</td>
<td>X</td>
<td></td>
<td>NIH, CURE AES, EF</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>Add</td>
</tr>
<tr>
<td>6. Payment for lectures including</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>service on speakers bureaus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>7. Payment for manuscript preparation.</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>8. Patents (planned, pending or issued)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>9. Royalties</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>10. Payment for development of</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>educational presentations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Stock/stock options</td>
<td>X</td>
<td></td>
<td>We won stock in Johnson and Johnson and Warner Chilcott</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>12. Travel/accommodations/meeting expenses</td>
<td>X</td>
<td></td>
<td>AES Board of Directors</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>unrelated to activities listed.**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>13. Other (err on the side of full disclosure)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
</tbody>
</table>

* This means money that your institution received for your efforts.
** For example, if you report a consultancy above there is no need to report travel related to that consultancy on this line.

Section #4 Other relationships
Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

___ No other relationships/conditions/circumstances that present a potential conflict of interest.
___ Yes, the following relationships/conditions/circumstances are present:
Thank you for your assistance.
American Epilepsy Society

Epilepsy Currents Journal
Disclosure of Potential Conflicts of Interest

Section #1 Identifying Information

1. Today's Date: 1/11

2. First Name France Last Name Jensen Degree MD

3. Are you the Main Assigned Author? Yes No

If no, enter your name as co-author France Jensen

4. Manuscript/Article Title: Does acquired encephalopathy influence brain function or neural death?

5. Journal Issue you are submitting for: Epilepsy Currents Vol 11, No. 1, Feb 2011 pp 12-18

Section #2 The Work Under Consideration for Publication

Did you or your institution at any time receive payment or services from a third party for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Complete each row by checking “No” or providing the requested information. If you have more than one relationship just add rows to this table.

<table>
<thead>
<tr>
<th>Type</th>
<th>No</th>
<th>Money Paid to You</th>
<th>Money to Your Institution</th>
<th>Name of Entity</th>
<th>Comments**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Grant</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Consulting fee or honorarium</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Support for travel to meetings for the study or other purposes</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4. Fees for participating in review activities such as data monitoring boards, statistical analysis, end point committees, and the like</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Payment for writing or reviewing the manuscript</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Provision of writing assistance, medicines, equipment, or administrative support.</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Other</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* This means money that your institution received for your efforts on this study.

** Use this section to provide any needed explanation.
Section #3 Relevant financial activities outside the submitted work.
Place a check in the appropriate boxes in the table to indicate whether you have financial relationships (regardless of amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the “Add” box. You should report relationships that were present during the 36 months prior to submission.

Complete each row by checking “No” or providing the requested information. If you have more than one relationship just add rows to this table.

<table>
<thead>
<tr>
<th>Type of relationship (in alphabetical order)</th>
<th>No</th>
<th>Money Paid to You</th>
<th>Money to Your Institution*</th>
<th>Name of Entity</th>
<th>Comments**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Board membership</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>2. Consultancy</td>
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<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>3. Employment</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>4. Expert testimony</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>5. Grants/grants pending</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>6. Payment for lectures including service on speakers bureaus</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>7. Payment for manuscript preparation.</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>8. Patents (planned, pending or issued)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>9. Royalties</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>10. Payment for development of educational presentations</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
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<tr>
<td>11. Stock/stock options</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>12. Travel/accommodations/meeting expenses unrelated to activities listed.**</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
<tr>
<td>13. Other (err on the side of full disclosure)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>Add</td>
</tr>
</tbody>
</table>

* This means money that your institution received for your efforts.
** For example, if you report a consultancy above there is no need to report travel related to that consultancy on this line.

Section #4 Other relationships
Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

☐ No other relationships/conditions/circumstances that present a potential conflict of interest.
☐ Yes, the following relationships/conditions/circumstances are present:

Thank you for your assistance.
Instructions
The purpose of this form is to provide readers of your manuscript with information about your other interests that could influence how they receive and understand your work. Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. The form is in four parts.

1. Identifying information.
   Enter your full name. If you are NOT the main contributing author, please check the box “no” and enter the name of the main contributing author in the space that appears. Provide the requested manuscript information.

2. The work under consideration for publication.
   This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking “No” means that you did the work without receiving any financial support from any third party — that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check “Yes”. Then complete the appropriate boxes to indicate the type of support and whether the payment went to you, or to your institution, or both.

3. Relevant financial activities outside the submitted work.
   This section asks about your financial relationships with entities in the bio-medical arena that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work. For example, if your article is about testing an epidermal growth factor receptor (EGFR) antagonist in lung cancer, you should report all associations with entities pursuing diagnostic or therapeutic strategies in cancer in general, not just in the area of EGFR or lung cancer.

   Report all sources of revenue paid (or promised to be paid) directly to you or your institution on your behalf over the 36 months prior to submission of the work. This should include all monies from sources with relevance to the submitted work, not just monies from the entity that sponsored the research. Please note that your interactions with the work’s sponsor that are outside the submitted work should also be listed here. If there is any question, it is usually better to disclose a relationship than not to do so.

   For grants you have received for work outside the submitted work, you should disclose support ONLY from entities that could be perceived to be affected financially by the published work, such as drug companies, or foundations supported by entities that could be perceived to have a financial stake in the outcome. Public funding sources, such as government agencies, charitable foundations or academic institutions, need not be disclosed. For example, if a government agency sponsored a study in which you have been involved and drugs were provided by a pharmaceutical company, you need only list the pharmaceutical company.

4. Other relationships
   Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.