A new hypothesis of drug refractory epilepsy: Neural network hypothesis

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Drug refractory is an important clinical problem in epilepsy, affecting a substantial number of patients globally. Mechanisms underlying drug refractory need to be understood to develop rational therapies. Current two prevailing theories on drug refractory epilepsy (DRE) include the target hypothesis and the transporter hypothesis. However, those hypotheses could not be adequate to explain the mechanisms of all the DRE. Thus, we propose another possible mechanism of DRE, which is neural network hypothesis. It is hypothesized that seizure-induced alterations of brain plasticity including axonal sprouting, synaptic reorganization, neurogenesis and gliosis could contribute to the formation of abnormal neural network, which has not only avoided the inhibitory effect of endogenous antiepileptic system but also prevented the traditional antiepileptic drugs from entering their targets, eventually leading to DRE. We will illustrate this hypothesis at molecular and structural level based on our recent studies and other related researches.

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Introduction

Epilepsy is the most prevalent chronic neurological disorder characterized by an enduring predisposition to generate epileptic seizures [1], affecting up to 50 million people worldwide [2]. Although many patients with epilepsy have their seizures controlled effectively by antiepileptic drugs (AEDs), around 30% of patients continue to have seizures, despite trying a range of appropriate AEDs (mono- or poly-therapy) [3,4]. Drug refractory epilepsy (DRE) is established when there is inadequate seizure control despite at least two potentially effective AEDs taken in tolerable doses for 1–2 years [5]. The consequences of DRE can be quite severe, including substantial deleterious effects on individual health, life quality, and a heavy burden on society [6,7]. Although clinicians have been aware of the problem of pharmacoresistance in epilepsy for years, the mechanisms underlying its development are still not understood.

Several putative mechanisms underlying DRE have emerged in recent years. Two main hypotheses have been proposed to account for DRE, the target hypothesis and the transporter hypothesis [8]. Based on the target hypothesis, inherited or acquired alterations in the molecular targets of AEDs lead to reduced pharmacodynamic effects of the drugs, whereas the transporter hypothesis claims that there is inadequate access of AEDs to epileptic tissue because they are removed by multidrug transporters that are pathologically over-expressed [9,10]. However, valuable as each of these hypotheses is, none could be adequate to explain the molecular and cellular mechanisms of pharmacoresistance in epilepsy for the following reasons: (1) The phenomenon of multidrug resistance does not exist in all the cases with DRE; (2) Multidrug resistance can not be adequate to explain some pathological changes in epilepsy brain tissue, such as neuronal loss, sprouting, gliosis, and the recurrent seizures induced by those pathological changes; (3) The drug designed for multidrug resistance gene (probenecid, flunarizine, etc.) have made only part of the treatment effect in DRE; (4) The target hypothesis lack of electrophysiological and morphological evidence. All above indicate there may be other unknown mechanisms involved in mechanisms of DRE.

In recently years, the theory of abnormal neural network in epilepsy has attracted more attention [11,12]. Neural network hypothesis suggests that, under the influence of gene and microenvironment, pathological disorders with recurring episodes of excessive neural activity can induce neuronal degeneration and necrosis, gliosis, axonal sprouting, synaptic reorganization and remodeling of neural network. Under the guidance of the error message, the residual neurons in injured brain induced by seizures will extend toward non-physiological direction, and form abnormal connection with the inferior synapse. Thus, the novel neural network under pathological conditions has come into being and the origin or pathway of epileptiform discharges has been changed. The formation of abnormal neural network has not only avoided the inhibitory effect of endogenous antiepileptic system but also prevented the traditional antiepileptic drugs from entering their targets, eventually leading to the DRE.
Although the neural network hypothesis has been concerned much more, its participation factors, crucial genes, direct and indirect pathways, and also its role in DRE are still unclear. The molecular biology evidence in supporting this hypothesis is, however, still lacking. Here, we will illustrate the hypothesis at molecular and structural level based on our recent studies and other related researches.

**Growth cone is an important structure potentially involved in DRE**

DNA microarrays, which provide global insight into transcriptional events occurring in a studied phenomenon, are now widely used tools for large-scale studies of gene expression in the brain of neuronal disorders [13–15]. There are also some available studies describing large-scale analysis of gene expression in conditions relevant to epilepsy [19,20]. One function of DNA microarrays analysis is the generation of new hypotheses to guide future research, and one such hypothesis can be proposed based on these data.

Using a complementary DNA microarray representing 4096 human genes, we have analyzed differential gene expression in the anterior temporal neocortex of DRE patients relative to controls [16–18]. Novel genes involved in cytoskeleton, synaptic plasticity, and structural/cellular reorganization have been identified in the brains of DRE patients. Our gene chip data are generally in agreement with the published results on epilepsy [19,20]. We had verified those novel genes by real-time fluorescence-quantitative polymerase chain reaction as well as immunohistochemistry, immunofluorescence and western blot analysis. After analysis of the results, we have found an interesting phenomenon that most of those abnormal expressed genes are associated with growth cone (Table 1).

Growth cone at the tip of each axon is a highly motile structure that can help each neuron to extend an axon and find its ultimate destination amongst a complex environment during nervous system development [21,22]. Growth cone completes its movement function relying on filopodia, lamellipodia, and the internal cytoskeleton [23]. There are two cytoskeletal filaments present in growth cone: microtubules and actin filaments. Cytoskeletal proteins including microtubule-associated proteins (MAPs), tubulin, myosin etc. have been reported to play an important role in growth cone steering [24]. Coincidentally, those cytoskeletal genes were also abnormally expressed in the brains of DRE patients in our research (Table 1) [16]. It is complicated for the intracellular and extracellular signaling pathways that regulate the cytoskeletal reorganization during growth cone pathfinding. Cytoskeletal effectors in Rho-family GTPase signaling such as Cdc42, RhoA, N-WASP and actin-related protein 2/3 are known to regulate all aspects of the actin cycle which affect growth cone steering [21,25], and they have also been reported to be abnormally expressed in DRE brains [26,27].

Intriguingly, growth cone plays its role primarily during nervous system development [28,29], but why it remains active under pathologic condition of DRE? Elliot et al. [20] have used DNA microarray analysis to characterize gene expression in the dentate gyrus of the hippocampus and identify genes exhibiting similar patterns of regulation during epileptogenesis and development. In their study, 37 genes had an altered level of expression during both epileptogenesis and development. Most of the genes shared between the two groups are implicated in cell morphology and axon outgrowth. Therefore, the authors hypothesized that epileptogenesis shares the same features with normal development of nervous system. Based on this intriguing hypothesis, we speculate that the cell-biological events taking place in growth cone during development could also occur during epileptogenesis or development of DRE.

### “Integrin – growth cone system” for neuronal network hypothesis

The formation of neural circuits requires proper connectivity that is established between neurons during development. Failure to achieve correct connectivity would result in dysfunction of nervous system, which might be associated with disorders. Growth cone is a crucial structure in central nervous system that is relevant to circuit formation and plasticity, including cell positioning and migration, synapse formation and plasticity, dendrite development, axon outgrowth and regeneration [30]. Local protein synthesis in growth cone has been described in the adult, and more interestingly, associated with neurological disorders and neural repair [31–33]. Therefore, our studies investigated the neural network hypothesis primarily focused on the growth cone.

An axon grows mainly at its tip, and the growth cone at its foremost senses and integrates signaling from multiple guidance cues [34], guiding the growth of neural fibers toward specific directions to gradually form neuronal networks. The growth cone is guided by extracellular guidance cues. Some axon guidance cues that play important role during embryonic development have also been reported to be possibly involved in epilepsy, such as netrin-1 [35], ephrin-A3 [36], the class 3 semaphorins [37–39]. In our previous study of epileptic patients and experimental animals, we observed axon guidance cues slit2 and netrin-G2 were abnormally expressed in the epileptic brains [40,41]. We found some cell adhesion

<table>
<thead>
<tr>
<th>Genbank_ID</th>
<th>UniGene</th>
<th>Definition</th>
<th>cy5/ cy3* Ratio</th>
</tr>
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<tr>
<td>NM_002291</td>
<td>Hs.489646 Homo sapiens laminin, beta 1 (LAMB1), mRNA</td>
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<td>NM_014325</td>
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<td>AB003592</td>
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<tr>
<td>NM_004540</td>
<td>Hs.177691 Homo sapiens neural cell adhesion molecule 2 (NCAM2), mRNA</td>
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<tr>
<td>NM_016261</td>
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<tr>
<td>X17033</td>
<td>Hs.482077 Human mRNA for integrin alpha-2 subunit</td>
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<tr>
<td>BC031051</td>
<td>Hs.317632 Homo sapiens cadherin 18, type 2, mRNA (cDNA clone MGC:33908 IMAGE:528717), complete cd.</td>
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<td>NM_005909</td>
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<tr>
<td>NM_183387</td>
<td>Hs.325846 Echinoderm microtubule-associated protein like 5 (EML5)</td>
<td>3.427</td>
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Cy5/Cy3 refer to the ratio of the fluorescent signals of patient/control groups, and were analyzed by GenePixPro3.0 software. Gene expression in epileptic tissue was considered to be over-expressed if the intensity value was double that of the control group (Cy5/Cy3* > 2). Conversely, gene expression was considered down-expressed when Cy5/Cy3* < 0.5. [16,83].
molecules such as LAMB1 [42], Cadherin18, NCAM2 and NB-3 (Table 1) which do not exist in normal physiological conditions, and some molecules such as integrin α2 which mediates integrin signal transduction molecules, were significantly abnormal in the brains of DRE patients comparing with the controls. All these evidence confirm that there exist guidance and signaling molecules which could guide axons to grow toward inappropriate direction and form aberrant nerve connections.

Integrins are heterodimeric cell surface receptors that act as cell-extracellular matrix and cell-cell adhesion molecules. Studies on animals have shown that some integrin subunits are possibly involved in epilepsy [43,44]. Here, we take “Integrin – growth cone system” for example to illustrate neural network hypothesis in DRE (Fig. 1). The ectodomain of integrin in the surface of growth cone receives the error signals from altered extracellular environment caused by seizures, and adhere growth cone to extracellular matrix with the action of cell adhesion molecules (such as LAMB1, Cadherin18, NCAM2 and NB-3, etc.). At the same time, transmembrane domain of integrin interacts with CORO1C and MYO1E in microfilament of growth cone to make the microfilament extense and retract continuously. On the other hand, transmembrane domain of integrin interacts with microtubule-associated proteins (such as MAP1A, MAP1B, MAP2) and tubulin (such as SNX17, TUBD1, TUBG1) to evoke the motility of microtubules. In addition, other signals such as phosphorylated tau protein loop may also participate in this “integrin – growth cone system”. Interaction of these molecules and protein may amplify the signals from seizure disorder, and steer the growth cone towards non-physiological direction, then cause plastic changes including abnormal axon outgrowth, sprouting, synaptic reorganization and formation of aberrant neural network. The plastic changes that follow recurrent seizures may aggravate the seizure condition, and contribute to the DRE.

Brain plasticity in neural network hypothesis

Why consider the plastic changes in neural network hypothesis are associated with DRE? The premise is that the state of recurrent uncontrolled seizures in DRE is associated with underlying structural and functional abnormalities, that is the alteration of brain plasticity [45], including axonal sprouting, synaptic reorganization, neurogenesis and gliosis. Growth cone is an important structure participating in those plastic alterations.

Synaptic reorganization and neural network

Synaptic reorganization of the mossy fiber pathway has received considerable attention recently as a potential mechanism that increases the excitability of the neural network through the formation of new recurrent excitatory collaterals. Poorly controlled seizures may induce progressive sprouting and synaptic reorganization, eventually forming aberrant neural network and leading to DRE.

One type of DRE that can serve as a useful example is temporal lobe epilepsy (TLE). TLE is the most common form of adult focal epilepsy and is often refractory to AEDs [46]. Mossy fibers sprouting is a widely studied and remarkable characteristic of TLE [47]. Mossy fibers are the axons of granule cells (GCs) in dentate gyrus which target to pyramidal cells in cornu ammonis (CA3) region. After epileptic insults, mossy fibers lose their original targets due to massive neuronal death in hilus area and CA3, and form incorrect synaptic connections on the dendritic and somatic sites of GCs, causing abnormal neuronal circuits and hyperexcitability [48,49].

Both the pyramidal cell axons and the GC axons sprout new collaterals in response to brain injuries caused by seizures. Pyramidal

Fig. 1. Integrin – growth cone system for neural network hypothesis. The ectodomain of integrin in the surface of growth cone receives the error signals from altered extracellular environment caused by seizures, and adhere growth cone to extracellular matrix with the action of cell adhesion molecules (such as LAMB1, Cadherin18, NCAM2 and NB-3, etc.). At the same time, transmembrane domain of integrin interacts with CORO1C and MYO1E in microfilament of growth cone to make the microfilament extense and retract continuously. On the other hand, transmembrane domain of integrin interacts with microtubule-associated proteins (such as MAP1A, MAP1B, MAP2) and tubulin (such as SNX17, TUBD1, TUBG1) to evoke the motility of microtubules.
Neurogenesis and neural network

Neurogenesis in the brain of adult mammals is considered to occur throughout lifetime, and has been clearly demonstrated at two places under normal conditions: the subgranular zone (SGZ) of the dentate gyrus in hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Seizure activity increases proliferation in both SGZ and SVZ, but most studies focus on dentate gyrus SGZ because the hippocampus is one of the areas of the brain that is quite susceptible to seizures. TLE is emphasized, because it is characterized by various pathophysiological changes in the hippocampus such as neurogenesis. In the dentate gyrus, the early proliferative response appears to be mediated by both progenitors and neuroblasts [56]. The two major abnormalities of dentate GCs neurogenesis in TLE include the formation of hilar basal dendrite and the ectopic migration of newborn GCs into the polymorphic cell layer [57].

The newborn GCs in the normal adult dentate gyrus often have a transient basal dendrite. Following seizures, newly generated GCs show a significantly greater percentage of hilar basal dendrites as compared to mature GCs [58]. These basal dendrites from the newborn neurons persist for long durations after seizures and are postsynaptic to axon terminals [59]. Thus, they may contribute to recurrent excitatory circuitry for newborn GCs, ultimately incorporating into the existing hippocampal circuitry [60]. Electro-physiological studies have confirmed the existence of this aberrant excitatory circuitry in the hippocampus [62,63]. Therefore, guidance cues that affect neuronal migration during development are strong candidates. As the GCs complete migration in adults, outgrowth of axons and dendrites occurs. It is possible to speculate that seizures may stimulate local protein synthesis of guidance cues and related factors in the growth cone of GCs progenitors, and guide the abnormalities of GCs progenitors migration, leading to abnormal integration of newborn GCs in the epileptic adult hippocampus. The hilar ectopic GCs generated by seizures exhibit excitatory postsynaptic potentials in response to extracellular stimulation of dentate gyrus [65], and also receive strong excitatory input from area CA3 [50]. The hilar ectopic GCs may promote seizure activity by participating in a reverberatory circuitry within the dentate gyrus. Activity within this circuitry might eventually exit the hippocampus and propagate seizure activity to the neocortex or other brain regions [66].

Electrophysiological studies also showed ectopic GCs might be a critical contributor in establishing a recurrent excitatory circuitry eventually leading to enhanced excitability in the epileptic hippocampus [67].

Overall, these processes during dentate GCs neurogenesis ultimately form the connections that become essential components of mature aberrant neural network. For the ongoing network activity increases over time [67], this circuitry could lead to the progression of pathophysiological alterations [68–70], and finally contribute to DRE.

Astrocytes and neural network

Astrocytes are more than the traditional supportive cells supplying only structural and metabolic support to neurons [71]. Astrocytes form a highly interconnected network not only with other astrocytes but also neurons [72]. A single astrocyte contacts multiple dendrites of a single neuron and a single neuron is associated with multiple astrocytes [73]. Astrocyte-to-neuron communication could modulate neuronal plasticity at the level of single synapse and neural network [74–76]. Brain insults caused by prolonged seizures result in reactive gliosis, which is characterized by severe morphological and biochemical alterations of pre-existing astrocytes [77] as well as the generation of newborn astrocytes from stem cells [78]. Studies on the autopsy and surgical resection specimens from post-traumatic seizures and intractable TLE have shown astrogliosis is a prominent feature of the epileptic brain.

In our recent studies, we have found some proteins were abnormally expressed in astrocytes of DRE brains. Nestin, a typical marker of neural, which has active functions in neurogenesis and gliosis, was upregulated in intractable epilepsy brain [79]; Aquaporin-1, a osmotic water channel, involved in maintenance water and ion homeostasis in the brain, was increased in astrocytes, but not in neurons or oligodendrocytes [80]; TGFbeta type I receptor, involved in pathogenesis of epilepsy via mediating albumin uptake into astrocytes, was mainly accumulated in the cytoplasm of astrocytes and increased in the patient group [81]; Slit2, one of repellent guidance molecule, was mainly expressed in neurons in human controls but in both neurons and astrocytes in the brains of DRE patients, and the expression was significantly higher in patients [40]. These results indicate that the expression of those proteins in astrocytes may be activated under some pathologic injuries, such as seizures or DRE. On one hand, considering the critical role of astrocytes in modulation of excitatory synapses and maintenance of neuronal microenvironment, astrogliosis has been implicated in mechanism of neural network hyperexcitability underlying DRE. On the other hand, astrogliosis is involved in the formation of a glial scar which has some deleterious effects, including inhibition of axonal sprouting in damaged brain and...
prevention of access of drugs to a lesion [82]. Thus, astrogliosis in epileptic brain may participate in pharmacoresistance in epilepsy.

In a word, astrogliosis may interfere the conformation of normal neuronal connections, make axonal growth steer away from gliosis foci, and disrupt the fabric of pre-existing neural networks. Therefore, astrocyte-to-neuron communication would influence neural network at structural and functional level, promoting the development of DRE.

Conclusion

Intractability to currently available AEDs may reveal the fact that there are many mechanisms for seizure generation in a given patient, and if one is inhibited other mechanisms still exist. The alterations of brain plasticity including axonal sprouting, synaptic reorganization, neurogenesis and gliosis would not be the etiological factors, but rather adaptive changes in response to seizures. However, poorly controlled seizures may induce progressive alterations of brain plasticity, eventually forming aberrant neural network and causing DRE. Thus, the formation of aberrant neural network may be a potential contributing and etiological factor for DRE.

Neural network hypothesis also has some limitations, e.g. (1) As we all know, the alterations of brain plasticity do not exist exclusively in DRE but also exist in other types of epilepsy that are well controlled by AEDs. So are there any differences between the two? Or are the plastic alterations in DRE more severe than that in other epilepsy? There may be some underlying molecular mechanisms involved in plastic alterations which make epilepsy become pharmacoresistance in some patients while others can achieve control with medication. The morphological and molecular biological evidence are still needed; (2) Since the biologic basis of pharmacoresistance is multifactorial and varies from one patient to another, any single hypothesis could not be adequate to explain mechanisms of pharmacoresistance in all patients, and so does neural network hypothesis.

Finally, further studies of the molecular and structural mechanisms governing formation of aberrant neural network in DRE should lead to the identification of novel molecular targets that might open new avenues for the development of alternative antiepileptic therapies.

Conflict of interest statement

None declared.

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