Pharmacokineti, pharmacodynamic, and neurochemical investigations of lamotrigine-pentylenetetrazole kindled mice to ascertain it as a reliable model for clinical drug-resistant epilepsy

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Abstract

Background: Pentylenetetrazole kindling has long been used for the screening of investigational antiseizure drugs. The presence of lamotrigine, at a very low dose, does not hamper kindling in mice; rather it modifies this epileptogenesis process into drug-resistant epilepsy. The lamotrigine-pentylenetetrazole kindled mice show resistance to lamotrigine, phenytoin, and carbamazepine. It may also be possible that other licensed antiseizure drugs, like the mentioned drugs, remain ineffective in this model; therefore, this was the subject of this study.

Methods: Swiss albino mice were kindled with pentylenetetrazole for 35 days in the presence of either methylcellulose vehicle or lamotrigine (subtherapeutic dose, ie, 5 mg/kg). Vehicle vs lamotrigine-kindled mice were compared in terms of (a) resistance/response toward nine antiseizure drugs applied as monotherapies and two drug combinations; (b) lamotrigine bioavailability in blood and brain; (c) blood-brain barrier integrity; and (d) amino acids and monoamines in the cerebral cortex and hippocampus.

Results: Lamotrigine vs vehicle-kindled mice are similar (or not significantly different $P > .05$ from each other) in terms of (a) response toward drug combinations; (b) lamotrigine bioavailability; and (c) blood-brain barrier integrity except for, significantly ($P < .05$) reduced taurine and increased glutamate in the cerebral cortex and hippocampus. Aside from these, lamotrigine-kindled mice show significant ($P < .05$) resistant to lamotrigine (15 mg/kg), levetiracetam (40 mg/kg); carbamazepine (40 mg/kg), zonisamide (100 mg/kg), gabapentin (224 mg/kg), pregabalin (30 mg/kg), phenytoin (35 mg/kg), and topiramate (300 mg/kg).

Conclusion: Lamotrigine-pentylenetetrazole kindling takes longer to develop (~5 weeks) in comparison to lamotrigine-amygdal (~4 weeks) and lamotrigine-corneal (~2 weeks) kindling models. However, drug screening through this model may yield superior drugs with novel antiseizure mechanisms.
INTRODUCTION

Epilepsy is characterized by recurrent disabling seizures. Around 50 million people worldwide have epilepsy, making it one of the most common neurological diseases across the world. To date, 42 antiseizure drugs are licensed for use in epilepsy; however, these medications remain ineffective in 15%-30% cases. Drug resistance substantially reduces the quality and expectancy of life. This mandates the requirement of novel, safer, and more efficacious drugs in the present era.

Animal model of human diseases provides basis to develop newer therapies (ie, preclinical screening) and help to better understand disease mechanisms in a way that is not possible in humans. Drug-resistant epilepsy, being a heterogeneous disorder, is highly unlikely that single animal model will suffice to predict the full therapeutic potential of an investigational antiseizure drug.

When an investigational compound targeting drug-resistant epilepsy enters into the preclinical screening stage, it passes through various acute and chronic rodent seizure models arranged in a special workflow, as described by Epilepsy Therapy Screening Program (ETSP). Animal models in the current workflow for drug-resistant epilepsy are as follows: 6-Hz electric stimulation, lamotrigine-amygdale kindling, intrahippocampal kainite injection, spontaneously bursting of hippocampal slice, and pilocarpine-induced status epilepticus, etc.

Animal models of ETSP have played an important role in preclinical evaluation of many licensed antiseizure drugs (ie, felbamate, topiramate, rufinamide, lacosamide and retigabine); however, most of these drugs remain ineffective in drug-resistant epilepsy. It is likely that same animal models may not yield novel antiseizure drugs with superior mechanism of action, which is important for the treatment of drug-resistant epilepsy. According to epilepsy benchmarks Area-III (NINDS), the development of new approaches can be advanced by better animal model.

To date, three variants of lamotrigine kindling model have shown resistance to antiseizure drugs: (a) lamotrigine-amygdale kindling in rats (electric stimulation); (b) lamotrigine-corneal kindling in mice (electric stimulation); and (c) lamotrigine-pentylenetetrazole kindling in mice (chemoconvulsant administration). The lamotrigine amygdale kindling model was first described by Postma et al in 2000 and soon after it was adopted by the ETSP. Other two models (ie, lamotrigine-corneal and lamotrigine-pentylenetetrazole) have never been implemented to ETSP.

The pharmacological profile of lamotrigine-corneal kindling model has been recently published. However, drug resistance profile and its underlying mechanism of lamotrigine-pentylenetetrazole kindling model are largely unknown. According to this model, the presence of lamotrigine, at a very low dose, does not hamper pentylenetetrazole kindling in mice; rather, it modifies this epileptogenesis process into drug-resistant epilepsy. The lamotrigine-pentylenetetrazole kindled mice show resistance toward lamotrigine, phenytoin, and carbamazepine; however, resistance beyond these three drugs is unknown and investigation of its underlying mechanism is recommended by previous studies.

To end this, Swiss albino mice were kindled with pentylenetetrazole in the presence of either methylcellulose vehicle or lamotrigine, and the suitability of this model has been evaluated through therapeutic, pharmacokinetic, and neurochemical investigations because reduced bioavailability of antiseizure drugs, impairment in the blood-brain barrier integrity, and alterations in monoamine and amino acid levels have implicated in drug-resistant epilepsy.

MATERIALS AND METHODS

2.1 Study design

This study was carried out in strict accordance with the guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals. The protocol was approved by the Institutional Animal Ethics Committee of Punjabi University (Protocol Number: 107/GO/ReBi/S/99/CPEA/2017-46). One hundred Swiss albino mice of either sex were procured from Disease Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences, India. The mice were housed in groups (6 mouse/cage) under controlled temperature (22 ± 3°C), humidity (50 ± 5), and light-dark cycle (12 hours light: 12 hours dark, lights on at 8:00 am) with free access to standard chow and water ad libitum. The mice were randomized into three groups: naïve (n = 18), vehicle kindling (n = 41), and lamotrigine kindling (n = 41). The kindling groups were exposed to a similar type of chemoconvulsant (ie, pentylenetetrazole) at a similar subconvulsive dose (ie, 40 mg/kg) at a similar time interval (ie, every 48 ± 2 hours) for the same duration (ie, for 35 days) but in the presence of different pretreatments, that is, either vehicle (0.5% methylcellulose) or lamotrigine at a low dose (5 mg/kg). In the vehicle kindling group, 36 out of 41 mice achieved a stable kindled state except for 3 unexpected deaths and 2 that remain unkindled. Similarly, 37 out of 41 mice achieved a stable kindled in the lamotrigine kindling group except for 2 unexpected deaths and 2 unkindled. Successfully kindled mice were enrolled for therapeutic, pharmacokinetic, and neurochemical investigations in which separate cohort of mice were used in every steps (ie, step 2-6 in Figure 1). In step 2, nine antiseizure drugs were administered to a vehicle vs lamotrigine-kindled mice (n = 6) with a time gap of 48 h for each drug (ie, starting from day 37 up to 53) with an objective to characterize whether lamotrigine-kindled mice are responsive or remain resistant to antiseizure drugs. Similarly, in step 3, various antiseizure drug combinations were administered to another group of...
a vehicle vs lamotrigine-kindled mice (n = 6). In step 4 (ie 48 hours after kindling), Evans blue dye was aspirated into the right lateral tail vein of another group of naïve, vehicle kindled and lamotrigine-kindled mice (n = 6), and were sacrificed after 30 minutes by cervical separation for collecting brain tissues with an objective to test the integrity of blood-brain barrier. In step 5, that is, 48 hours after kindling development, the lamotrigine (15 mg/kg) was administered to naïve, vehicle kindled, and lamotrigine-kindled mice (n = 6) mice and were sacrificed after 45 minutes by cervical separation for collecting brain tissues with an objective to test the bioavailability of lamotrigine. In the final step 6, naïve, vehicle kindled, and lamotrigine-kindled mice (n = 6) were sacrificed in post-ictal phase, that is, 48 hours after stable kindled state, by cervical separation for collecting brain tissues with an objective to better understand the neurochemical milieu of the cerebral cortex and hippocampus.

2.2 Vehicle and lamotrigine kindling

Kindling groups were treated with a subconvulsive dose of pentylenetetrazole (40 mg/kg dissolved in normal saline) on alternate days (every 48 ± 2 hours) in the presence of either methylcellulose vehicle (ie, 0.5% methylcellulose, ip) or a subtherapeutic dose of lamotrigine (5 mg/kg lamotrigine suspended in 0.5% methylcellulose, ip) injected 30 minutes before pentylenetetrazole injection. After every pentylenetetrazole injection, the mice were placed individually in transparent Plexiglas cages (20 × 20 × 30 cm) where the visual convulsive seizures were recorded for a time period of 30 minutes as per modified according to Racine's scale: Stage 0: no response; Stage 1: hyperactivity, restlessness, and vibrissae twitching; Stage 2: head nodding, head clonus, and myoclonic jerks; Stage 3: unilateral or bilateral limb clonus; Stage 4: forelimb clonic seizures; Stage 5: generalized tonic-clonic seizures with falling; Stage 6: hind limb extension; Stage 7: mortality. The kindling continued until each mice had achieved criteria of three consecutive stage 5 seizures, whereby it was considered as “Stable and fully kindled.”

2.3 Characterization of seizures by antiseizure drug therapies

Vehicle-kindled mice (n = 6) and lamotrigine-kindled mice (n = 6) were treated with nine standard antiseizure drugs (administered as monotherapies and two drug combinations) with the objective to characterize the nature of convulsive seizures, that is, drug resistant or drug responsive. The first monotherapy was applied on day 37, that is, when mice achieved a stable kindled state. Briefly, lamotrigine (15 mg/kg suspended in 0.5% methylcellulose) was injected into vehicle kindled (n = 6) and lamotrigine-kindled (n = 6) mice through intraperitoneal route. After 30 minutes, the chemoconvulsant was injected (40 mg/kg pentylenetetrazole dissolved in normal saline, ip) for seizure induction. Immediately after chemoconvulsant injection, the mice were placed in transparent Plexiglas cages (20 × 20 × 30 cm) and visual convulsive seizures were recorded for a time period of 30 minutes as per modified Racine’s scale (detailed in Section 2.2). The mean seizure severity score of vehicle kindled group vs lamotrigine-kindled group was compared. Similarly, following monotherapies and two drug combinations were applied: levetiracetam (40 mg/kg dissolved in normal saline, ip) on day 39; carbamazepine (40 mg/kg suspended in 0.5% methylcellulose) on day 41; zonisamide (100 mg/kg suspended in 0.5% methylcellulose) on day 43; gabapentin (30 mg/kg dissolved in normal saline) on day 45; pregabalin (30 mg/kg dissolved in normal saline) on day 47; phenytoin (35 mg/kg dissolved in normal saline) on day 49; topiramate (300 mg/kg suspended in normal saline) on day 51.
on day 51; valproate (300 mg/kg dissolved in normal saline) on day 53; 7.5 mg/kg lamotrigine + 20 mg/kg levetiracetam on day 37; 7.5 mg/kg lamotrigine + 20 mg/kg carbamazepine on day 39; 7.5 mg/kg lamotrigine + 50 mg/kg zonisamide on day 41; 7.5 mg/kg lamotrigine + 112 mg/kg gabapentin on day 43; 7.5 mg/kg lamotrigine + 15 mg/kg pregabalin on day 45; 7.5 mg/kg lamotrigine + 17.5 mg/kg phenytoin on day 47; 7.5 mg/kg lamotrigine + 150 mg/kg topiramate on day 51; 7.5 mg/kg lamotrigine + 150 mg/kg valproate on day 53. Monotherapies and two drug combinations were applied on two separate cohorts of mice (steps 2 and 3; Figure 1).

2.4 Testing the integrity of blood-brain barrier

The integrity of the blood-brain barrier was assessed by the Evans blue method. Briefly, 200 µL of 0.5% sterile solution of Evans blue was aspirated into the right lateral tail vein of naïve, vehicle kindled, and lamotrigine-kindled mice (n = 6). After 30 minutes, mice were sacrificed using cervical dislocation. The brain was carefully dissected, weighed, and homogenized in formamide (5 mg brain/50 mL formamide). This homogenate was transferred into the water bath maintained at 55°C for 30 minutes and then incubated at room temperature for 2 days. After 48 hours, the homogenate was centrifuged at 10 000 g for 10 minutes to pellet tissue fragments and the absorbance of supernatant was recorded at 610 nm. The comparative extravasation of Evans blue in naïve vs vehicle kindled vs lamotrigine is expressed in ng per mg tissue.

2.5 Quantification of lamotrigine in plasma and brain

Quantification of lamotrigine was carried out using high-performance liquid performance chromatography coupled with ultraviolet detector. Briefly, 15 mg/kg lamotrigine was administered to naïve, vehicle kindled, and lamotrigine-kindled mice (n = 6) through intraperitoneal route. Mice were sacrificed after 45 minutes, brain tissue was carefully dissected, homogenized in sodium saline in a proportion of 5 mL/g. 10 µL of 20% trichloroacetic acid was added to 1 mL brain homogenate, centrifuged at 1585 g for 10 min at 15°C, and supernatant was carefully separated. Plasma was carefully separated from the collected blood. A quantity of 100 µL of brain homogenate or plasma was taken to a microcentrifuge tube to which 100 µL 2 mol/L NaOH was added to ensure all LTG in molecular form. This sample was mixed briefly by vortex (10 sec) and 500 µL ethyl acetate was added. The sample was centrifuged at 1585 g for 10 minutes at 15°C. The upper supernatant was transferred to clean microcentrifuge tube and evaporate to dryness. The residue was reconstituted with 100 µL mobile phase (0.1 mol/L potassium dihydrogen phosphate solution: HPLC-grade methanol and trimethylamine: 64.7:35:0.3) and injected through a Rheodyne manual injector in reverse-phase column (C18 4.6 mm, 150 mm, 5 µm Waters, XBridge) and detected at 305 nm (2489 UV detector, Waters, USA).

2.6 Analysis of neuroactive amino acids and monoamines

Naïve, vehicle kindled, and lamotrigine-kindled mice (n = 6) were sacrificed in the interictal phase, that is, 24 hours after a stable kindled state. Brain tissue was carefully isolated from individual mice and dissected into the cerebral cortex and hippocampus. Dissected parts were weighed, homogenized in freshly prepared ice-cold 10% w/v perchloric acid, and centrifuged at 14 000 g for 30 minutes at 4°C (REMI C-24BL, Cooling Centrifuge, REMI, India). For quantification of amino acids, such as alanine, arginine, β-serine, gamma-aminobutyric acid (GABA), glutamate, glutamine, taurine, and tryptophan, the clear supernatant was derivatized with O-phthalaldehyde and β-mercaptoethanol (10 µL supernatant + 40 µL derivatizing solution) and loaded into a reverse-phase column (C18 4.6 mm, 150 mm, 5 µm Waters, XBridge) for separation at a flow rate of 1.1 mL/min (515 binary pumps, Waters, USA) and detected using an electrochemical detector (2465 electrochemical detector, Waters, USA). For estimation of monoamines, such as norepinephrine (NE), kynurenine (KYN), dopamine (DA), and 5-hydroxytryptamine (5-HT), the clear supernatant was directly injected without derivatization for UV detection (2489 UV detector, Waters, USA as described earlier. The acquired data were processed using the Empower Pro® UJ Operating system (Waters®, Milford, USA).

3 RESULTS

3.1 Kindling development

The mean seizure severity score of the vehicle vs lamotrigine kindling groups (n = 41) did not reach statistical significance (P > .05) throughout the kindling (Figure 2). In the vehicle kindling group, 36 out of 41 mice achieved the kindling criteria within 33 days except for 3 unexpected deaths and 2 that remain unkindled. Similarly, 37 out of 41 mice were kindled within 33 days in the lamotrigine kindling group except for 2 unexpected deaths and 2 that remained unkindled. Based on this result (Figure 2), the presence of lamotrigine at low dose, that is, 5 mg/kg does not hamper pentyletenetrazole kindling development or does not increase mortality rate or the number of unkindled mice.

3.2 Resistance extends beyond the lamotrigine

The mean seizure severity score of the vehicle kindled group (n = 6) was significantly (P < .001) reduced following treatment with monotherapies (Figure 3A) and a combination of antiseizure drugs (Figure 3B). However, mice kindled in the presence of lamotrigine displayed significant (P < .001) resistance toward lamotrigine (15 mg/kg), levetiracetam (40 mg/kg); carbamazepine (40 mg/kg), zonisamide (100 mg/kg), gabapentin (224 mg/kg), pregabalin (30 mg/kg), phenytoin (35 mg/kg), and topiramate (300 mg/kg), except valproate and combination of antiseizure drugs. Based on this result, lamotrigine
resistance is not exclusive to lamotrigine; rather, it extends over many antiseizure drugs with the varied mechanism of actions with few exceptions such as valproate and combinations of antiseizure drugs.

3.3 | Kindling in the presence of lamotrigine does not impair blood-brain barrier and lamotrigine remains within therapeutic range irrespective to its resistance

Evans blue is an azo dye that has a very high affinity for serum albumin that cannot cross the blood-brain barrier. In our observation (Figure 4A), there was no significant difference in the extravasation of Evans blue dye in the brain of naïve vs vehicle kindled vs lamotrigine-kindled mice. Based on this result, it is unlikely to consider a disturbing blood-brain barrier as an underlying cause of lamotrigine resistance.

Poor response to drug treatment in patients with drug-resistant epilepsy is believed to be secondary to a reduction in the penetration of the drugs to the central nervous system, that is, modification in the drug pharmacokinetics causing an inadequate concentration of antiepileptic drugs in brain tissue. Considering this hypothesis as a suspected underlying cause of drug resistance, in the case of mice kindled in the presence of lamotrigine, we have analyzed the levels of lamotrigine in blood circulation and brain tissue. However, there was no significant difference in the level of lamotrigine in systemic circulation and brain tissue. This indicates that change in pharmacokinetics is unlikely to produce the drug resistance described herein.

3.4 | Lamotrigine-kindled mice exhibits specific neurochemical alterations

Tryptophan is the only brain precursor of serotonin, and based on our results (Figure 5), its level was significantly reduced in the cerebral cortex and hippocampus of vehicle-kindled mice. This is consistent with the previous report. In line, clinical reports also suggest beneficial effects of tryptophan in suppressing drug-resistant epilepsy. However, based
Kynurenine was significantly increased in the cerebral cortex ($F_{(2, 15)} = 8.193, P < .05$) and hippocampus ($F_{(2, 15)} = 7.635, P < .05$) of vehicle-kindled mice and lamotrigine-kindled mice. However, no significant difference was observed between the vehicle vs lamotrigine-kindled groups (Figure 5). Thus, deficiency in serotonin may not be considered as a key neurochemical feature of drug resistance resulting from lamotrigine kindling.

Serotonin is a monoamine neurotransmitter. Increasing its level in the brain has an anticonvulsive effect, especially in drug-resistant epilepsy.\textsuperscript{15} Compared to the naïve group (Figure 5), the level of serotonin was significantly reduced in the cerebral cortex ($F_{(2, 15)} = 8.688, P < .05$) and hippocampus ($F_{(2, 15)} = 7.796, P < .05$) of vehicle-kindled mice and lamotrigine-kindled mice. However, no significant difference was observed between the vehicle vs lamotrigine-kindled groups (Figure 5). Thus, the role of dopamine in the generation of drug resistance in lamotrigine-kindled mice seems unlikely. Although dysregulation of dopaminergic neurotransmission has been reported in drug-resistant epilepsy, its involvement has been majorly influenced by the type of epilepsy syndrome ie predominantly in case of limbic seizures.\textsuperscript{29}

Dopamine is a monoamine that acts as a neurotransmitter in the brain. Dysregulation of dopamine has been reported in the brain of epilepsy patients and epileptic animals.\textsuperscript{28} Compared to the naïve group (Figure 5), the level of dopamine was significantly reduced in the cerebral cortex ($F_{(2, 15)} = 6.063, P < .05$) and hippocampus ($F_{(2, 15)} = 7.550, P < .05$) of vehicle kindled and lamotrigine-kindled mice. However, no significant difference was observed between vehicle vs lamotrigine-kindled mice (Figure 5). Thus, deficiency in serotonin may not be considered as a key neurochemical feature of drug resistance resulting from lamotrigine kindling.

β-Alanine is an endogenous amino acid that functions as an inhibitory neurotransmitter in the central nervous system and also regulates the transport of taurine across the blood-brain barrier.\textsuperscript{30} Compared to naïve mice (Figure 6), the level of β-alanine was significantly increased in the cerebral cortex ($F_{(2, 15)} = 14.68, P < .05$) and hippocampus ($F_{(2, 15)} = 8.525, P < .05$) of vehicle-kindled mice, as reported previously.\textsuperscript{31} Its level was further increased in lamotrigine-kindled mice; however, this difference was insignificant as compared to vehicle-kindled mice (Figure 6).

\textit{L}-Arginine is a basic amino acid and it could decrease the seizure frequency in drug-resistant epilepsy through biosynthesis of nitric oxide, glutamic acid, and agmatine in the brain.\textsuperscript{27} Compared to naïve mice (Figure 6), the level of arginine was significantly reduced in the cerebral cortex ($F_{(2, 15)} = 4.669, P < .05$) and hippocampus ($F_{(2, 15)} = 7.796, P < .05$) of vehicle-kindled mice. The anticonvulsant role of arginine has been reported in pentylentetrazole kindling.\textsuperscript{32} However, the difference between the vehicle vs lamotrigine kindled groups was insignificant (Figure 6). This indicates that alteration arginine levels are unlikely to produce drug resistance in lamotrigine-kindled mice.

\textit{D}-Serine is an endogenous co-agonist of N-methyl-o-aspartate receptors and it has been implicated in epileptogenesis.\textsuperscript{33} Particularly in animal models of epilepsy, the levels of \textit{d}-serine are reduced.\textsuperscript{27} In
line with these reports, the \( \delta \)-serine level was significantly reduced in the cerebral cortex \((F(2, 15) = 5.633, P < .05)\) and hippocampus \((F(2, 15) = 8.558, P < .05)\) of vehicle-kindled mice and lamotrigine-kindled mice in comparison to naïve mice (Figure 6). However, there was no significant difference between vehicle vs lamotrigine-kindled mice. Thus, it is likely that the drug resistance observed in lamotrigine-kindled mice may arise from other neurochemical alterations irrespective of increased \( \delta \)-serine levels in the cerebral cortex and hippocampus.

Norepinephrine is a catecholamine neurotransmitter with anti-convulsant property in human and epileptic animals.\(^\text{34}\) Compared to the naïve group (Figure 6), the level of norepinephrine was significantly reduced in the cerebral cortex \((F(2, 15) = 10.89, P < .05)\) and hippocampus \((F(2, 15) = 8.706, P < .05)\) of vehicle kindled and lamotrigine-kindled mice. However, no significant difference was observed between vehicle vs lamotrigine kindled groups (Figure 6). Based on this result, it is unlikely to consider deficiency of norepinephrine as a cause of drug resistance in lamotrigine-kindled mice.

GABA is a principal inhibitory neurotransmitter in the brain and its deficiency contributes to seizure generation and epilepsy state.\(^\text{14}\) Compared to naïve mice (Figure 7), the level of GABA was significantly reduced in the cerebral cortex \((F(2, 15) = 7.399, P < .05)\) and hippocampus \((F(2, 15) = 4.666, P < .05)\) of vehicle-kindled mice. The level of GABA was reduced a step further in lamotrigine-kindled mice.
mice (Figure 7); however, this reduction did not reach statistical significance, consistent with previous reports.\textsuperscript{14} Thus, it is likely that reduced GABA may contribute to drug resistance up to a certain extent; however, it may not be considered as a key neurochemical deficiency that produces drug resistance in lamotrigine-kindled mice.

Glutamate is a principal excitatory neurotransmitter in the brain. Abnormality of glutamate regulation is one of the most consistent findings in seizure and epilepsy state.\textsuperscript{14} Compared to naive mice (Figure 7), the level of glutamate was significantly increased in cerebral cortex ($F(2, 15) = 15.04, P < .05$) and hippocampus ($F(2, 15) = 10.03, P < .05$) of vehicle-treated mice. Interestingly, the glutamate level was drastically increased in the lamotrigine kindled group as compared to the vehicle kindled group (Figure 7). This dramatic increase in the level of glutamate indicates that kindling in the presence lamotrigine results in significant dysregulation of glutamate, and it may be considered as a key neurochemical deficiency of this model. Similar dysregulation has also been reported in humans whereby elevated glutamate underlies features of drug-resistant epilepsy\textsuperscript{14} and most of the available antiseizure drugs are not efficacious in reducing the abnormally elevated glutamate levels enhanced through synthesis and release by glial cells.\textsuperscript{35} Thus, it is likely that kindling in the presence of lamotrigine mimics one of the key neurochemical features of human drug-resistant epilepsy.

Glutamine is a critical component in the metabolic pathways of glutamate and GABA although it does not, by itself, function as a neurotransmitter. Compared to naive group (Figure 7), the level of glutamine was significantly reduced in cerebral cortex ($F(2, 15) = 4.618, P < .05$) and hippocampus ($F(2, 15) = 4.929, P < .05$) of vehicle-kindled group. However, no significant difference was observed between vehicle vs lamotrigine kindled groups (Figure 7). This indicates that the level of glutamine is highly regulated in mice kindled in the presence of vehicle and lamotrigine as well as in human patients of drug-resistant epilepsy.\textsuperscript{14}

Taurine is the most abundant inhibitory amino sulfonic acid in the brain. It has been used widely for the treatment of epilepsy as it crosses the blood-brain barrier.\textsuperscript{36} Its level (Figure 7) was significantly reduced in cerebral cortex ($F(2, 15) = 15.28, P < .05$) and hippocampus ($F(2, 15) = 16.84, P < .05$) of vehicle-kindled mice with dramatically lowest level found in lamotrigine-kindled mice. Thus, it may be considered as a key neurochemical deficiency which underlies feature of drug resistance in lamotrigine-kindled mice. It is also consistent with previous reports in humans that suggest that taurine is useful for the treatment of epilepsy.\textsuperscript{36}

**4 | DISCUSSION**

Limitations in the drug screening program have not been resolved to any significant extent by the recent introduction of a new animal model of drug-resistant epilepsy, although alternate strategies based on modifications of the kindling paradigm has been developed.\textsuperscript{7-9} These reports and our results (Figure 2) indicate that the presence of lamotrigine does not hamper kindling development at low doses in mice. Lamotrigine is a second-generation anticonvulsant that produces inhibitory action by reducing the release of the excitatory neurotransmitter glutamate through inhibition of voltage-gated calcium channels\textsuperscript{37} and sodium channels.\textsuperscript{38} Additionally, in rodent studies, it was found that lamotrigine blocks potassium channels presynaptically, which causes an enhanced release of inhibitory neurotransmitter GABA in synaptic cleft,\textsuperscript{39,40} where its inhibitory action on GABA\textsubscript{A} receptor complex is blocked by pentylenetetrazole (non-competitive inhibitor of GABA).\textsuperscript{41} Although not the focus of this study, by this mechanism, the presence of lamotrigine does not hamper pentylenetetrazole kindling, and it remains a testable hypothesis.

Until the early 1980s, antiseizure polytherapy was widely practiced as the first-line management of drug-resistant epilepsy for
achieving synergistic effects along with minimizing toxicities by use of smaller doses of two drugs rather than larger doses of one antiseizure drug.\textsuperscript{45} Subsequent trials led to a change in this method by validating monotherapy as a first-line treatment for drug-resistant epilepsy.\textsuperscript{43} Therefore, lamotrigine resistance in our experimental setup was characterized by antiseizure monotherapies. However, the selection of monotherapies was one of the challenging parts because 42 antiseizure drugs have been licensed since 1912, along with the recent introduction of 43rd drug, cenobamate, in January 2020. Characterization of any animal model with these drugs has never been done before because it requires a large number of experimental animals along with extensive efforts on time scale.

To overcome this experimental difficulty, we have selected nine most commonly used antiseizure drugs with the varied mechanism of actions based on the fact that failure of first antiseizure drug trial is one of the most important predictive factors of drug-resistant epilepsy in human, that is, resistance of the first drug extends over most of the other antiseizure drugs.\textsuperscript{41,44} In line, the lamotrigine resistance in our study extends beyond lamotrigine, that is, toward levetiracetam, carbamazepine, zonisamide, gabapentin, pregabalin, phenytoin, and topiramate (Figure 3A). Based on this result, lamotrigine-resistant mice replicate monotherapy failure features of human drug-resistant epilepsy.\textsuperscript{45} It is also likely that lamotrigine resistance may extend over untested licensed antiseizure drugs (approx. 33) and it remains as the subject for detailed future investigation whereby individual drugs can be tested at different doses because it requires a large number of kindled animals along with extensive efforts, and time.

Drug resistance is characterized by failure of antiseizure monotherapies or combination at low to high doses\textsuperscript{45}; however, we used only single fixed dose because the number of kindled mice was limited and it is a limitation of present study. Aside from monotherapies, a low-dose multi-drug regimen should be considered after the failure of two monotherapy trials.\textsuperscript{45} Polytherapy is not only acceptable but is standard practice nowadays.\textsuperscript{45} Theoretically, any two antiseizure drugs combination can be selected where synergistic interaction improves seizure control. However, two drug selection and its experimental testing is a major challenge for basic and clinical research because approx. 740 combinations are possible if 42 licensed antiseizure drugs are combined. In our study, lamotrigine was combined with eight antiseizure drugs with varied mechanisms of action because lamotrigine is a central drug of this model, and clinical studies shows synergistic interaction of lamotrigine with valproate, topiramate, and levetiracetam.\textsuperscript{46} Based on our results (Figure 3B), lamotrigine-kindled mice do not fulfill the criteria of drug-resistant epilepsy, that is, failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom\textsuperscript{44} due to significant antiseizure effects of combination (Figure 3B). Besides this, lamotrigine-kindled mice could be a useful tool for identification for a drug-like molecules with superior or novel antiseizure mechanisms and to study resistance mechanisms in experimental animals because it remains resistant to most of the monotherapies (Figure 3A).

The drug-resistant epilepsy is multifactorial and impacted by various underlying causes.\textsuperscript{10} According to the "drug-target hypothesis," resistance results from a structural or functional change at the site of drug action causing a change in sensitivity to antiseizure drugs.\textsuperscript{43} Resistance observed in lamotrigine-kindled mice could arise from modifications in targets of lamotrigine ie voltage-gated calcium channel,\textsuperscript{57} sodium channels,\textsuperscript{38} potassium channels,\textsuperscript{39,40} or pentyleneetrazole target, that is, GABA\textsubscript{A} receptors.\textsuperscript{41} However, the ineffectiveness of levetiracetam (through synaptic vesicle glycoprotein 2A,\textsuperscript{47} voltage-dependent N-type calcium channel subunit alpha-1B),\textsuperscript{48} zonisamide (through voltage-dependent T-type calcium channel subunit alpha-1G),\textsuperscript{49} pregabalin (through voltage-dependent P/Q-type calcium channel subunit alpha-1A),\textsuperscript{50} gabapentin (through voltage-dependent calcium channel subunit alpha-2/delta-1),\textsuperscript{51} and the effectiveness of combination of lamotrigine + phenytoin/carbamazepine (through voltage-gated sodium channel) indicates that change in drug target is unlikely to produce drug resistance in lamotrigine-kindled mice.

Leaky vessels, aberrant neurovascular, and altered morphology have been reported in the most common form of drug-resistant epilepsy viz. hippocampal sclerosis, brain neoplasm, and malformation of cortical development\textsuperscript{11,12} independent of the fact that such leakage is associated with or a result of the seizure itself. In epilepsy, recurrent seizures manifest with variable extents of blood-brain barrier dysfunctions.\textsuperscript{23} It allows albumin and glutamate to react to the neuronal parenchyma that alters neuronal excitability.\textsuperscript{52} Evans blue is an azo dye that has a very high affinity for serum albumin that cannot cross blood-brain barrier.\textsuperscript{23} When the blood-brain barrier has been compromised, albumin-bound Evans blue enters the brain that can be determined quantitatively.\textsuperscript{23} In our observation (Figure 4A), there was no significant difference in the extravasation of Evan's blue dye in the brain of naïve vs vehicle kindled vs lamotrigine-kindled mice. Therefore, it is unlikely to consider a disturbing blood-brain barrier as an underlying cause of lamotrigine resistance, which was previously suspected as a possible etiology based on literature reports.\textsuperscript{11}

Persistently low plasma levels of carbamazepine, phenytoin, and valproic acid have been detected in patients with drug-resistant epilepsy.\textsuperscript{10} It is believed that the overexpression of efflux transporters in peripheral organs decreases the level of antiseizure drugs in blood, thereby reducing the amount of drug crossing blood-brain barrier to epileptic focus.\textsuperscript{10} Considering pharmacokinetic alterations as a suspected underlying cause of drug resistance, the level of lamotrigine were analyzed the levels of lamotrigine in blood circulation. However, there was no significant difference observed across naïve vs vehicle kindled vs lamotrigine-kindled mice (Figure 4A,B). Clinical reports also suggest that poor drug response in epilepsy result from poor drug penetration into the brain despite adequate plasma levels within the therapeutic range due to overexpression of P-gp at the blood-brain barrier.\textsuperscript{10} However, no significant difference observed across naïve vs vehicle kindled vs lamotrigine-kindled mice. By this result (Figure 4A,B), change in pharmacokinetics is unlikely to produce
the drug resistance in lamotrigine-kindled mice because the level of lamotrigine was similar or not significantly different in blood and brain across lamotrigine responsive vs resistant mice.

Aberrant neurotransmission contributes to seizure development and epilepsy state. Clinical reports indicate aberrant neurotransmission of tryptophan, kynurenic, seratonin, dopamine, alanine, arginine, serine, norepinephrine, GABA, glutamate, glutamine, and taurine. Contrary to these reports, most neurochemical comparisons between vehicle vs lamotrigine-kindled mice did not reach statistical significance (Figures 5 and 6) except drastically comparisons between vehicle vs lamotrigine-kindled mice did not reach statistical significance (Figures 5 and 6) except drastically decreased taurine and elevated glutamate (Figure 7). Inside our observation, glutamate levels were abnormally elevated in the epileptogenic cortex and hippocampus of patients with drug-resistant epilepsy and taurine deficiencies have been reported in various animal models and human studies. By these two key neurochemical alterations along with some of the non-significant alterations, such as reduced GABA, serine, and norepinephrine, the lamotrigine-kindled mice replicate neurochemical features of human drug-resistant epilepsy.

To date, three variants of lamotrigine kindling have shown resistance to licensed antiseizure drugs: (a) lamotrigine-amygdale kindling in rats (electric stimulation); (b) lamotrigine-corneal kindling in mice (electric stimulation); and (c) lamotrigine-pentylenetetrazole kindling in mice (chemoconvulsant administration). The lamotrigine-amygdale kindling model is a part of ETSP, and it is different from other two models (ie, lamotrigine-corneal and lamotrigine-pentylenetetrazole) in terms of time, skills and resources required for model development.

The lamotrigine-amygdale kindling model requires special surgical skills, access to stereotaxis apparatus, electrical stimulator, social isolation of chronically-implanted rats, and electric stimulation of amygdale for ~4 weeks. Second variant of electrical kindling (ie, lamotrigine-corneal) can be generated in ~2 weeks without any surgical procedure or social isolation; however, access to electrical stimulator and mild topical anesthesia is still required. However, lamotrigine-pentylenetetrazole kindling model can be generated by repetitive chemoconvulsant injections only (ie, pentylenetetrazole administration for ~5 weeks), without any surgery, electric stimulation, or social isolation of mice.

By these differences, lamotrigine-pentylenetetrazole kindling may be considered as a simple, resource free, and cost-effective alternative to electric lamotrigine kindling models (ie, lamotrigine-amygdale and lamotrigine corneal).

5 | CONCLUSION

The lamotrigine-resistant mice may better delineate the disease mechanism in comparison to the traditional pentylenetetrazole-kindling model. Chronic nature of this model renders it unsuitable for frontline drug discovery. Albeit, drug screening through this model may mitigate the existing issue ie it may yield superior drugs with a novel antiseizure mechanism of action.

ACKNOWLEDGMENTS

The corresponding author acknowledges, Indian Council of Medical Research, New Delhi, India for granting senior research fellowship to Mr Sandeep Kumar under his guidance (grant number: 45/1/2019/PHA/BMS).

CONFLICT OF INTEREST

None.

AUTHORS CONTRIBUTIONS

RKG conceptualized this study. SK conducted the experiments, collected and analysed the data and wrote the manuscript. RKG coordinated the entire study and revised the manuscript for final submission.

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