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Effects of small conductance calcium activated potassium channel agonists on seizure like events in *in vitro* slices and slice cultures

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Table of contents

List of abbreviations

4-AP	4-Aminopyridine
ACSF	Artificial cerebrospinal fluid
AEDs	Antiepileptic drugs
Ca^{2+}	Calcium
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
CBZ	Carbamezapine
Cl^-	Chloride
CNS	Central nervous system
CO_2	Carbondioxide
DG	Dentate gyrus
FIRES	Febrile infection-related epilepsy syndrome
fEPSP	Field excitatory post-synaptic potential
FP	Field potential
GABA	γ-Aminobutyric acid
GWX	GW-542573X
HBSS	Hank's balanced salt solution
i.p.	Intraperitoneal
K^+	Potassium
KA	Kainate
Kir	Inward rectifying potassium channel
mAHP	Medium afterhyperpolarization
mECm	Medial entorhinal cortex
MEM	Minimal essential medium
MEST	Maximal electroshock test
Mg^{2+}	Magnesium
NMDA	n-methyl-D-aspartic acid
OHSCs	Organotypic hippocampal slice cultures
P7 – P14	Postnatal 7 - 14 day old rat
PPF	Paired-pulse facilitation
PTX	Picrotoxin

PTZ	Pentylenetetrazole
RMP	Resting membrane potential
s.c.	Subcutaneous
SK-channel	Small conductance potassium channel
STZ	Strychnine
SUDEP	Sudden unexpected death in epilepsy
VPA	Valproic acid

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Abstract:

The problem of pharmacoresistance associated with anti-epileptic drugs (AEDs) is affecting one third of epilepsy patients. If the patient does not respond to 2-3 AEDs of primary choice then they are most likely to develop resistance. Surgical intervention is an ultimate choice in these patients, however, only a small proportion can profit from such treatment. It is therefore necessary to find better drugs exploiting novel drug targets. SK-channels regulate neuronal excitability, and their agonists were recently reported to be potentially effective in animal models of epilepsy. I therefore tested subunit specific SK-channel agonists in pharmacosensitive and pharmacoresistant models of epilepsy. This also involved evaluation of the potential use of SK-channel agonists in human tissue from epileptic patients who showed pharmacoresistance to AEDs.

I firstly tested the following three SK-channel agonists namely, GW-542573X (SK1 agonist), SKa-31 (SK 2 agonist) and CyPPa (SK 2 and 3 agonist) in acute slice preparations from adult rats. Seizure-like events (SLEs) were induced in slices by application of 4-aminopyridine (4-AP; 100 µM) and local field potentials were recorded in an interphase slice chamber. CyPPa (n=8) and SKa-31 (n=8), blocked SLEs in acute slice preparation whereas, GW-542573X (n=6), significantly increased the interval between SLEs (n=6, $p< 0.05$). To evaluate the effect of SK-channel agonists in the chronic epilepsy model, I used brain slices from pilocarpine treated chronic epileptic rats. CyPPa and SKa-31, blocked 4-AP induced SLEs in 88% of slices (n=8). GW-542573X was also found to be effective in this model as suppression of SLEs was found in 50% of slices compared to control group (n=6, $p< 0.05$) .

In pharmacosensitive organotypic slice culture (OHSC), CyPPa (n=6) was found to be superior over SKa-31 (n=9). SLEs blockade was achieved in 100% and 66% of studied OHSCs respectively. Even a, low dose of CyPPa was effective at 20 µM (n=7). Although, results of % SLEs blocked in pharmacoresistant OHSCs were not as good as in pharmacosensitive OHSCs, as CyPPa (n=7) and SKa-31(n=6) blocked 66% and 33%

SLEs in these OHSCs respectively. Significant results were obtained by these compounds on duration (*p< 0.05) and interval of SLEs (**p< 0.005).

The most important finding in this study was the positive result from human tissue. Both CyPPa and SKa-31 blocked 100% SLEs induced by 4-AP in human tissue, while GW-542573X failed to block it. Suppression of SLEs induced by high potassium combined with bicuculline was achieved in 88%, 33% and 50% slices by CyPPa (n=9), SKa-31 (n=7) and GW-542573X (n=4) respectively.

These findings suggest that SK-channel agonists might be useful for the treatment of pharmacoresistant seizures. I therefore concluded that SK-channels may serve as a new AEDs drug target for pharmacoresistant epilepsies. However, before translating SK-channel agonists into clinical phase, their possible side effect should be judged.

Abstrakt:

Das Problem der Pharmakoresistenz im Zusammenhang mit Antiepileptika (AED) tritt bei gut einem Drittel aller Epilepsiepatienten auf. Wenn ein Patient auf 2-3 AED nicht reagiert, dann liegt wahrscheinlich eine Resistenz vor. Für diese Patienten ist die Ultima Ratio/letzte Behandlungsoption der chirurgische Eingriff, von dem aber nur wenige Patienten profitieren. Es wäre also konsequent, bessere Angriffspunkte für Medikamente zu finden, um die Problematik der Pharmakoresistenz zu überwinden bei gleichzeitig verbessertem Nebenwirkungsprofil. SK-Kanäle regulieren die neuronale Erregbarkeit und - wie jüngst berichtet – sind SK Kanal Agonisten potentielle Antikonvulsiva. Ich habe deshalb Untereinheiten-spezische SK Agonisten in verschiedenen Anfallsmodellen untersucht, die teils pharmakosensitiv, teils pharmakoresistent sind.

Ich untersuchte die drei folgenden sK Kanal Agonisten: GW-542573X (SK1-Agonist), SKa-31 (SK2 Agonist) und CyPPa (SK2 und 3 Agonist) nach Induktion epileptiformer Aktivitäz in akuten Hirnschnittpräparaten von erwachsenen Ratten. Anfallartige Ereignisse (Eng: Seizure-like events; SLEs) wurden durch 4-Aminopyridin (4-AP; 100 µM) induziert und durch Messung lokaler Feldpotentiale in Interface Kammern verfolgt. SLEs in akuten Schnittpräparaten wurden durch die Anwendung von CyPPa (n=8) und SKa-31 (n=8) blockiert, wohingegen GW-542573X nur die Intervalle zwischen SLEs (n=6, p <0,05) erhöhte. Die Wirkung der SK-Kanal agonisten wurden dann im Hirngewebe von epileptischen Ratten nach Pilokarpinbehandlung untersucht. CyPPa und SKa-31 blockierten ebenfalls die 4-AP induzierten SLEs in 88% der Hirnschnitte (n=8). In diesem Modell war auch GW-542573X wirksam mit Unterdrückung der SLEs bei 50% der Gehirnschnitte im Vergleich zur Kontrollgruppe (n=6, p <0,05).

Die Anwendung von CyPPa zeigten eine besseres Wirksamkeit über SKa-31 in pharmakosensitiven organotypischen Kulturen (OHSC). SLE - Blockade wurde jeweils in 100% und 66% der Präparate erreicht. In diesem Paräparat waren auch niedrigere Dosen von CyPPa (20 uM; n=7) wirksam. Die Ergebnisse waren in pharmakoresistenter OHSCs nicht ganz so gut da CyPPa (n=6) und SKa-31 (n=9) jeweils nur 66% bzw 33% der SLEs blockierten. Dauer und Intervall der SLEs waren signifikant verändert (* p <0,05; ** p <0,005).

Weiterhin wurde die Wirksamkeit von CyPPa und SKa-31 am menschlichem Gewebe an durch 4-AP induzierten SLEs untersucht. Es wurden in allen Fällen Anfallsunterdrückung erreicht, wohingegen GW-542573X keine Wirkung zeigte. In Experimenten in denen SLE durch Kombination von Bicucullin und hoch-Kalium induziert worden war, waren alle drei Substanzen wirksam wobei CyPPa (n=9), SKa-31 (n=7) und GW-542573X (n=4) jeweils in 88%, 33% und 50% der Hirnschnittpräparate unterdrückte.

Diese Ergebnisse deuten auf eine Wirksamkeit der SK-Kanalagonisten besonders in pharmakoresistenten Modellen hin. Es besteht daher die Möglichkeit, dass SK-Kanal-Agonisten als neue Antiepileptika Medikamente in der Behandlung pharmakoresistenter Epilepsien dienen könnten. Vor Durchführung von klinischen Studien zu SK-Agonisten sollten deren mögliche Nebenwirkungen beurteilt werden.

Introduction:

Epilepsy is a frequent neurological disorder affecting between 0.5 and 1 % of the population worldwide. Epilepsies present with transient stereotypic changes in behavior which cannot be willingly controlled. Most epilepsies involve neuronal hyperexcitation. It may present at any age from birth to childhood and also among adults, to the older age groups (Rados 2005; Kramer 2001). There is now consensus that epilepsies are not just a single disorder. Because of variability in seizure type there is extensive debate among researchers to diagnose the exact seizure type. Seizures can occur in a number of diseases as symptoms. In order to diagnose epilepsy it is suggested that a person newly diagnosed with epilepsy must have shown at least two unprovoked seizures more than 24 h apart, or one unprovoked seizure with a chance of more seizures occurring over next ten years (Fisher et. al. 2014). There are various types of epilepsies based on site of origin and factors that contribute to it. According to classification by Commission of the International League Against Epilepsy (1989) it is divided mainly as generalized, localization related (focal, local, partial), and uncategorized. In generalized epilepsies both hemispheres of the brain are involved, although it is not necessary that the whole cortex undergo seizing state. In contrast to generalized epilepsies, localization related epilepsy occurs within one of the hemispheres. Such patients may present with aura, autonomic signs or having motor involvement. If symptoms present are due to alterations in a specific part of the brain or subcortical area it is classified as local, and if present at different parts within the hemisphere, is known as partial epilepsy (Commission, 1985 & 1989). There are also various epileptic syndromes such as Lennox-Gastaut syndrome, West syndrome, Rasmussen's encephalitis, Dravet's syndrome, Ohtahara syndrome, Ring chromosome 20 syndrome, Febrile infection-related epilepsy syndrome (FIRES). The seizures are of various types and can be categorized as tonic, clonic, tonic-clonic, absence or myoclonic (Commission, 1989).

Pathological factors in epilepsy:

Pathophysiological factors of epilepsy are multiple and include genes, trauma, brain tumor, metabolic disturbances, infections, autoimmune reactions, and drug induced

seizure (Pauschek et. al. 2016; Cendes et. al. 2014; Khurana et. al. 2008; Engelborghs et. al. 2000).

Treatment of Epilepsy:

Antiepileptic drugs are divided into 1st and 2nd generation antiepileptics, first generation antiepileptics include phenobarbital, phenytoin, carbamazepine, primidone, clonazepam, valproic acid and ethosuximide. Among the second generation antiepileptic drugs levetiracetam, gabapentin, topiramate, lamotrigine, zonisamide, oxcarbazepine, vigabatrin, pregabalin, rufinamide, tiagabine, felbamate, lacosamide, fosphenytoin, lorazepam, clobazam and stiripentol are widely used (Reimers 2012; McCabe 2000; Perucca 1996). The newest ones include eslicarbazepine acetate, lacosamide and perampanel which are placed by some as third generation antiepileptics (Johannessen Landmark & Patsalos 2010).

The major target of available anticonvulsants involves either blocking voltage-gated sodium channels or enhancing GABA inhibition as both results in reduction of hyper excitation thus providing for the desired effect in epilepsy patients (Theile & Cummins 2011). Some of available anticonvulsants act also by blocking voltage gated calcium channel, such as gabapentin, valproate and ethosuximide. Few anticonvulsants also reduce glutamate effects thereby potentiating GABAergic neurotransmission (felbamate, parampanel) see review of Patsalos 2005. Although some anticonvulsants act by multiple mechanisms, the classical example is valproate that possesses all the above mentioned action of mechanism (Johannessen and Johannessen, 2003).

Treatment problems with current antiepileptic drugs:

Although there are many available anticonvulsants used in clinical practice, still nearly 35% of patients are resistant to these anticonvulsants by not providing for acceptable seizure control (Kobau et. al. 2008). Another outcome of these medications are unwanted side effects that frequently affect these patients (Jacoby et. al. 2015). Besides this, there is economic burden with high cost medication that not everyone can afford (Jacoby et. al. 2015; Whiteford et. al. 2015) particularly people from poor countries

(Ibinda et. al. 2014; 2014). Mostly, patients are maintained on more than one drug that results in further non-compliance, especially in kids or elderly patients. Finding new avenues for epilepsy therapy is therefore required including the exploration of mechanisms underlying resistance to clinical used anticonvulsants.

Pharmacoresistance:

Pharmacoresistance or drug resistance remains a challenging factor for researchers and pharmaceutical industries to deal with and come up with potential drug candidates that address pharmacoresistance. It is seen with numerous medication classes and in various diseases and more commonly with antibiotics. Pharmacoresistance is defined as persistent seizures in spite of satisfactory medication. As mentioned before, around 30-35% patients face drug resistance with clinically used anticonvulsants. Reasons for pharmacoresistance may include severity in disease state. Seizure severity might be indicated by the duration of SLEs and the amplitude of rises in extracellular potassium concentration.

There are numerous other factors that may contribute to pharmacoresistance. Widely discussed is upregulation of drug transporters (Baars et. al. 2006; Baltes et. al. 2007; Löscher & Potschka 2002). In general, drug transporters are mainly responsible for absorption, distribution, metabolism and elimination of drugs as well as their related toxicity. Drug transporters presumably involved in pharmacoresistance, are P-glycoprotein, MRP, BCRP etc. (Barry 2014van Vliet et. al. 2007 & 2005; Aronica et. al. 2005 see review Löscher 2002) However, blockade of drug transporters in human tissue from pharmacoresistant patients did not really improve efficacy of AEDs (Sandow et. al. 2015) . Another recently reported factor for resistance is damage to the blood brain barrier (Salar et. al. 2014). Furthermore, genetic factors that result in mutation of various genes lead towards non-responsiveness to drug therapy (Dombrowski et. al. 2001; Miceli et. al. 2014; Wang et. al. 2014). For the development of AEDs effective in the treatment of pharmacoresistant epilepsy models of pharmacoresistance have to be employed. These include slices from very young animals, slice cultures, human tissue slices and models of prolonged status epilepticus.

This strategy is not yet realized in antiepileptic drug discovery programs. Presently a battery of tests is employed as proposed in the anticonvulsant drug discovery program (ADDD) (Smith et. al. 2007). In my master's thesis I have used in vivo acute screening tests such as scPTZ, scPTX, scSTN tests and MES test. Further I have used chronic model of epilepsy known as kindling to test bioactive natural products (Raza et. al. 2010). In my doctorate project I have utilized in vitro models of epilepsy to screen for potentially active anticonvulsant compound. Here I am describing them briefly.

Screening of anticonvulsant and anti-epileptogenic activity

According to antiepileptic drug development program a potential drug candidate undergoes a series of screening tests which is based on acute seizure as well as chronic seizure tests. For acute screening, namely maximal electroshock test - MES Test as well as subcutaneous pentylenetetrazole (scPTZ) tests are commonly used. The kindling model of epilepsy is considered a chronic model of epilepsy in which also epileptogenesis can be studied.

Maximal Electroshock Seizure (MES) Test:

Maximal electroshock seizure (MES) test is the most universally employed test for the screening of AEDs, structure-activity research, rational drug design and other approaches to antiepileptic drug development (Löscher et. al. 1991a). The MES test is the simplest to perform and provides predictive values for detecting new clinically helpful AED (White 2003; Löscher & Schmidt 1988; Krall et. al. 1978; Swinyard 1969). This test is thought to evaluate the ability of a testing material to prevent seizure spread through neural tissue, and a model for generalized tonic-clonic (grand mal) seizures (Piredda et. al. 1985; Löscher & Schmidt 1988; White 1997). Moreover, it also enables to identify the usefulness of the test drug against the partial seizure (Löscher 2002a). Many currently used anticonvulsants i.e. carbamazepine, felbamate, lamotrigine, phenobarbital, phenytoin, primidone, oxcarbazepine, topiramate, valproic acid, and zonisamide were successful in this test, and thus they are effective against generalized tonic-clonic seizures. However, ethosuximide showed a negative result in MES screening and is thus ineffective against generalized tonic-clonic seizures in human

beings (Browning 1992; Löscher & Schmidt 1994; Macdonald & Kelly 1995; White 1999).

In epilepsy, type and severity of the generalized seizures are related to the intensity of the applied stimulation current (Tedeschi et. al. 1956; Laffan et. al. 1957; Swinyard 1972; Piredda et. al. 1985). Based on this fact, in MES test, a supramaximal current strength (e.g., 50 mA/>50 mA) in mice or 150 mA/>150 mA in rats) i.e., with a stimulus 5-10 times higher than the individual electrical seizure threshold of the animals is used (Swinyard 1949; Swinyard et. al. 1952; Woodbury & Davenport 1952; Swinyard 1972) so that variation in seizure threshold do not alter tonic seizures in this model (Löscher et. al. 1991a). It should be noted that MES test is not a perfect discovery model against the partial seizures since vigabatrin and levetiracetam were ineffective in MES test but showed effectiveness in kindling model. Similarly, tiagabine which is not effective in MES test at various doses showed effectiveness at the dose of 2-3 folds than normal dose (Holland et. al. 1992). At present, due the problem of pharmacoresistant epilepsies it is important to screen the new compound to be effective in resistant forms of epilepsies, but MES test is not the right test to evaluate new compounds for pharmacoresistant epilepsies (Löscher 2002a; Löscher 2002b).

Subcutaneous pentylenetetrazole (scPTZ) seizure model:

As mentioned earlier the scPTZ test is an acute test which is extensively used in AED primary screening (White 2003; Löscher & Schmidt 1988; Krall et. al. 1978; Swinyard 1969). This test is used to identify the potential of a given drug against non-convulsive absence or myoclonic seizures (White 1997; Löscher & Schmidt 1988).

The type and severity of the generalized seizures are related to the dose and route of injection of PTZ (Löscher et. al. 1991b; Mitchell & Keasling 1960). AEDs such as ethosuximide, benzodiazepines, felbamate, gabapentin, barbiturates, trimethadione, tiagabine and valproic acid were found active in this test, while carbamazepine, phenytoin, and lamotrigine failed to show effectiveness (White 1999; Macdonald & Kelly 1995; Löscher & Schmidt 1994).

According to early observations, trimethadione may possibly block the PTZ-induced seizures (Everett & Richards 1944) and we know clinically that trimethadione is effective

in reducing petit mal seizures (Lennox 1945). Such clinical correlation suggested that this test would be useful for identifying drugs which are effective in the treatment of absence seizures. Almost all the medication proven active in the scPTZ test were found to be clinically useful against myoclonic seizures, suggesting that the PTZ test might have larger implication in the identification of medicine effective in myoclonic instead of absence seizures (Löscher & Schmidt 1988; White 1997 & 1999).

Tests for elucidation of mechanism of action (scBicuculline, scPicrotoxin, scStrychnine Tests):

After testing the effectiveness of a new compound in acute tests i.e. MES test and scPTZ, it is required to elucidate its possible mechanism of action. Various tests are employed to further characterize the anticonvulsant profile of the substance. For this purpose, test compounds are evaluated against seizures induced by subcutaneous administration of strychnine (STN), a known glycine receptor antagonist (Rajendra et. al. 1997; Curtis et. al. 1971; Larson 1969), bicuculline (BIC), a known $GABA_A$ receptor competitive antagonist (Vicini et. al. 1986; Meldrum & Nilsson 1976; Curtis et. al. 1970), and picrotoxin (PTX), a known $GABA_A$ receptor non-competitive antagonist that blocks the chloride channel (Olsen 1981; Takeuchi & Takeuchi 1969).

Chronic epilepsy models - the kindling model:

Kindling is a model of epileptogenesis, it was first described by Graham Goddard in 1967, who was initially investigating amygdaloid complex by stimulating it electrically to study mechanisms of learning. During this examination, he noticed that a number of experimental rats developed seizures after repeated stimulation. He further recognized that the brain was changing in response to a constant stimulus, and this change resulted in a plasticity that can be good model of neural plasticity. In the late sixties he first reported this phenomenon and defined it as a process whereby continual supply with subconvulsive electrical or chemical stimuli consequently decreases neuronal thresholds for induction of seizures which became eventually generalized seizure (Goddard et. al. 1969; Goddard 1967). Goddard demonstrated kindling phenomenon in rats, but other investigators demonstrated that kindling development occurs also in different animals such as mice, rabbits, monkeys, cats, dogs, gerbils and baboons (McNamara et. al. 1980; McNamara 1986).

To date, tremendous efforts have been made in order to understand the underlying mechanisms of kindling that allows the enhanced understanding of epilepsy. Over the years many investigators have reported the involvement of various neurotransmitter systems, as well as much of the intracellular mechanisms (Corcoran 1988; Racine 1978; Racine & Burnham 1984). The changes that occurred due to kindling also remain over a longer period of time, and are commonly viewed as being semi-permanent (Dennison et. al. 1995; Wada et. al. 1974).

Chemical kindling is one of the models of epilepsy in which periodic administration of chemoconvulsants in a subconvulsive dose, induces change in seizure threshold resulting in progressive seizure activity (Mason & Copper 1972). Pentylenetetrazole is one of the commonly used chemoconvulsant which was first used by Sacks and Glaser in 1941 (Sacks & Glaser 1941). Beside pentylenetetrazole the researchers used other chemicals for the induction of kindling such as lindane, endusalfan, chlordimeform, amitraz and chlorpyrifos (Wurpel et. al. 1993; Gilbert 1992; Gilbert & Mack 1989; Joy et. al. 1983). These are pesticides with known convulsant effects, when delivered repeatedly at low doses (Gilbert 2001; Gilbert 1995). This model is now a very good screening parameter for developing the new drug due to its universality across species and parameters throughout the brain, thus its usefulness as a model of human epilepsy is well established (Racine 1978).

Like every other model, kindling model has limitations. An example is that kindling results in increased sensitivity of rats to adverse effects of certain antiepileptic drugs because of alteration in their metabolism. (Löscher & Honack et. al. 1992).

Kindling Scores

Racine was the first scientist to define the seizure patterns which develop during kindling acquisition and described the mechanism of kindling progression in detail, discussing the seizure patterns during the gradual development of kindling and classified these seizure patterns into 5 distinct behavioral stages as shown in **table 1** (Racine 1972):

Score	Observations
Stage 0	no response
Stage 1	twitching of ears and face
Stage 2	convulsive wave all the way through body
Stage 3	myoclonic jerks
Stage 4	clonic tonic convulsions, proceeds into side position
Stage 5	generalized clonic-tonic seizures, failure t control posture

Table 1: Kindling score based on behavioral observations in animals according to Racine.

Although by principle the 5th stage of kindling is considered to be its end state, kindling can be pushed ahead of this point towards the appearance of spontaneous seizures (Pinel & Rovner 1978). Later some scientists have modified scoring scale, for example, by Axel Becker (Becker & Grecksch 1995).

Chronic epilepsy models - The Pilocarpine model of temporal lobe epilepsy or epileptogenesis:

Infusing pilocarpine (which is a muscarinic receptor 1 agonist) in rat produces consistent seizures and a status epilepticus. Normally continuous status is ended with benzodiazepine, for example, since continued status epilepticus will otherwise cause death of the animal. The mortality rate associated with pilocarpine injection is around 30-40% as reported by others (Curia et. al. 2008) and comparable to our own data. Pilocarpine induced status causes damage to the brain. A latent phase is observed during which network reorganization takes place and results in spontaneous seizures due to development of epileptic foci. EEG analysis from hippocampus shows interictal spikes and also theta rhythm. These are then changing into full limbic seizures (Turski et. al. 1989). It is thus a widely accepted model to study pathomechanism of temporal lobe epilepsy as well as to investigate the process of epileptogenesis (Lévesque et. al. 2016; Löscher 2002). It is reported by Soukupova and others groups that there is persistent increase in the level of glutamate with in hippocampus of pilo-treated epileptic

rats (Soukupova et. al. 2015). Another study reported activation of glial cells along with hippocampal loss (Chakir et. al. 2006). Also, GABA levels seem to be altered after pilocarpine injection in rats along with the GABA cell loss (Soukupová et. al. 2014). In pilocarpine treated animal changes in inward rectifying potassium channel have recently been reported. One of its subunit i.e. Kir 4.1 is expressed in astrocyte (Nagao et. al. 2013) and important for determining resting membrane potential in these cells and thereby affecting transport capacity for glutamate and GABA as well as other agents. Another important consequence during seizure activity is ischemic-hypoxia that generally leads to the neuronal cell loss in brain and specifically the hippocampus (Lucchi et. al. 2015).

All these consequences are also found in humans whether there is seizure activity going on or in later phase of epilepsy i.e. ischemia, astrocytic activation, neuronal cell loss of various brain regions and variation in all chief neurotransmitter of nervous system namely GABA, glutamate and dopamine and thus endorses the usefulness of pilocarpine model for the developing of new drug candidate (Meurs et. al. 2008; Smolders et. al. 1997).

In vitro testing of new compounds:

Besides series of acute screening tests and chronic models as mentioned above i.e. kindling or pilocarpine treated chronic epilepsy, potential new compounds are further subjected to in vitro testing that may utilize brain slices from a chronic epileptic rat or normal rat. Slices under research are infused with chemoconvulsants for the induction of epileptiform activities and then the test compound is applied to check whether it is capable to block seizure like activity in that slice or not. There are several different chemoconvulsants in use for induction of epileptiform activity in vitro. Among the most widely used chemicals is the 4-aminopyridine (4-AP), which is a non-selective potassium channel blocker acting in low concentrations on members of the Kv 1 and Kv 3 family and produces stable seizure activity in the hippocampal formation and particularly in entorhinal cortex (Rutecki et. al. 1987; Perreault & Avoli 1991). Seizure like events induced by 4-AP in slices express the following components (if the electrophysiological recoding is performed in DC mode): a) sharp negative DC shift

superimposed by b) low amplitude with high frequency spikes termed as tonic phase followed by c) clonic phase which shows low frequency and high amplitude signals as shown in **Fig. 1**. (Brückner & Heinemann 2000).

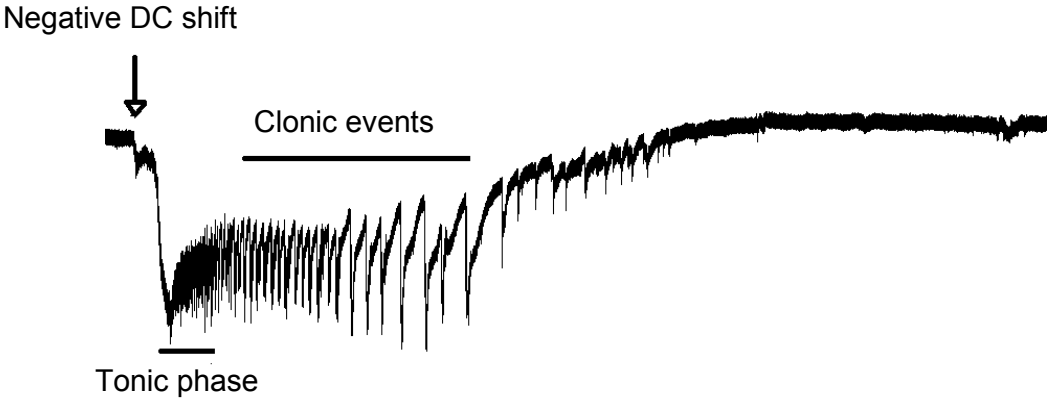


Figure 1: Components of classical seizure-like event in rat entorhinal cortex hippocampal slice induced by 4-AP (100 µM) in vitro. Showing initial negative -ve DC shift potential superimposed by tonic and then clonic like activity.

Similar seizure like events can also be induced by omitting magnesium in aCSF (Anderson et. al. 1986; Walther et. al. 1986; Gloveli et. al. 1995; Buck et. al. 1978). In slices from adult rats short recurrent discharges initiate in CA3 from which they spread to CA1, whereas, in entorhinal cortex and subiculum low magnesium induces seizure like events with a tonic and subsequent clonic phase along with negative DC shift (Gloveli et. al. 1995). If slice is perfused with Mg^{2+} free aCSF for longer duration then seizure activity turned into late recurrent discharges which are resistant to current antiepileptics (Dreier et. al. 1998, Albus et. al. 2008). Molecular mechanism of low Mg^{2+} induced SLEs involved the NMDA receptors (Heinemann et. al. 2006) and sodium channel gating due to alteration in the surface charge screening (Mody et. al. 1987, Isaev et. al. 2012). Studies revealed that lowering of magnesium concentration in slices from chronic epileptic rats was weaker compared to normal rats (Simpson et. al. 1991). Long term exposure with low magnesium is also causing reduction in GABAergic activity (Whittington et. al. 1995).

Hippocampal organotypic slice culture (OHSC):

Slice culture often express pharmacoresistance and are therefore another tool to study anticonvulsant potential of a new compound. Normal slices from new born pups are cultured and provided with essential nutrition at controlled temperature to keep them alive. Interestingly, it was observed that nerve cells continued to grow and new neuronal connections are formed within cultured slices, (Gähwiler et. al. 1997; Gutierrez et. al. 1999) while most other structural aspects of the hippocampus are preserved (Zimmer & Gahwiler 1984). Slice cultures are usually prepared from animals with an age of less than 2 weeks. Work by Jiang and Swann, showed that if the rat pups are over 14 days development, neuronal connections are impaired (Jiang & Swann 2005). Slice culture prepared from P6 showed higher incidence of seizure compared to P14, whereas, the optimal age range for the preparation of slice culture may range between P7-P14 days (Kovács et. al. 2011). Composition of media also interferes with the behavior of the culture.

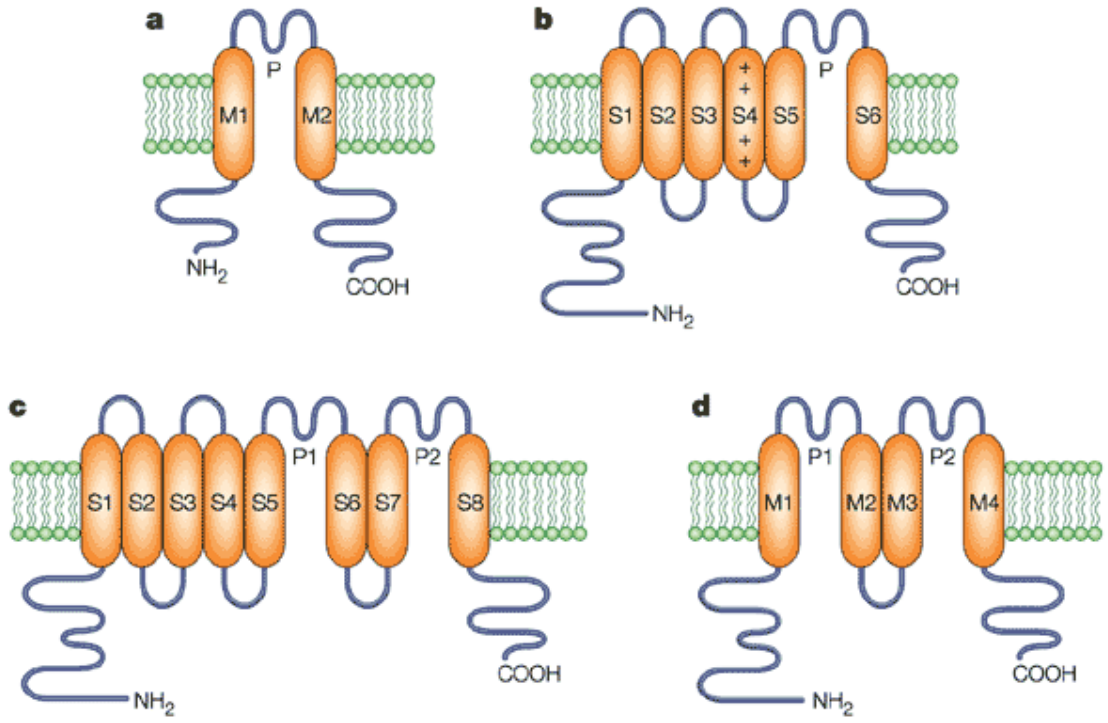
Organotypic slice cultures are being used routinely by researchers to address various pathological questions including epilepsy. It is easy to induce epileptiform activity within slice culture as reported by Kovács (Kovács et. al. 2002). Our lab has previously reported and validated pharmacoresistance properties of hippocampal slice cultures Several AEDs that are employed in different types of seizures in clinical setting were tested and presented with resistance (Wahab et. al. 2010; Albus et. al. 2009).

Potassium channel and Epilepsy:

According to compendium on ion channel classifications, potassium channels are extremely diverse both in terms of structure, expression and function (Gutman et. al. 2005). Potassium channels have variable nomenclature based on genome name HUGO Gene Nomenclature Committee (HGNC) or IUHPAR name. Three main families of potassium channels according to sequence of their sub-unit amino acid arrangement are shown in figure (**Fig. 2**), i.e. a) two transmembrane regions with one pore, b) four transmembranes with two-pore and c) six transmembrane regions (S1-S6) having single pore; the first cloned channels were voltage-gated K^+ channels called Shaker-like channel. Kv channels are diversified and comprises 12 families ranging from Kv 1 to Kv

12. There are 40 genes associated to Kv channels that make this channel family more diversified than other K^+ channel types. The voltage gated potassium channels further includes KCa (calcium-activated potassium channel, (Gutman et. al. 2005).

Potassium channels are thought to be involved in a variety of diseases due to their wide expression throughout the human body and in particular the CNS. Among many pathologies associated with potassium channel defect or abnormalities, epilepsy is one of them (Miceli et. al. 2015; Parrock et. al. 2014; Newitt et. al. 1991). Potassium channels are involved in multiple functions such as in maintaining resting membrane potential or threshold for induction of action potentials, as well as in discharge pattern of neurons. These channels also take part in regulation of conduction of electrical signals along dendrites and axons. Another important role of potassium channels is in the regulation or limiting the firing rate of neurons (Li et. al. 2013; Johnston et. al. 2010; Traub & Llinás 1979). It is of interest if any potential drug keeps the membrane potential at resting state or reduces the conductance or diminishes the neuronal firing. Here I would like to mention examples of two drugs, one of which is already in clinical use i.e. Retigabine or Ezogabine which was approved in 2011 for treating partial seizure as an adjunct therapy, importantly it is found effective when seizures are not controlled by other medication (Brodie et. al. 2010). Retigabine is a potassium channel opener, this provided me with the idea to target potassium channel openers for investigating new drugs in epilepsy models. The second example is an experimental drug, 1-EBIO; an SK-channel agonist. Recent studies reported its in vitro and in vivo anticonvulsant activity. (Anderson et. al. 2006; Garduño et. al. 2005). SK-channel regulates the neuronal firing by limiting its rate because it maintains the state of slow afterhyperpolarization (Pedarzani et. al. 2001). I therefore tried new SK-channel agonists in various in vitro preparations.



Senyon C, 2002, pg 115-121 **Nature Reviews | Neuroscience**

***Figure 2**: Structure describing various potassium channels according to their domains; where a) channel having 2 transmembrane domain and single pore b) 6 transmembrane domain based channel; c) 8 transmembrane domain (found in yeast) and d) 4 transmembrane domain containing potassium channel.*

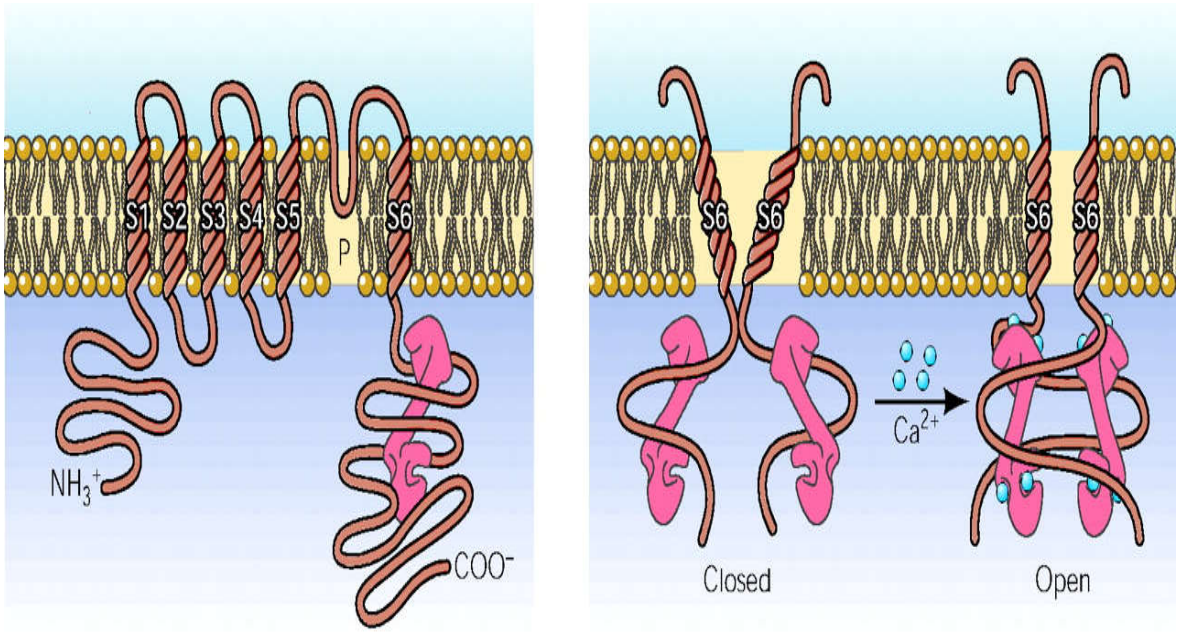
SK (Small conductance calcium-activated potassium) channels:

SK-channels that were recently discovered (Liegeoos et. al. 2003 review) are the class of potassium channel which become activated upon entry of calcium into cells thereby resulting in hyperpolarization. They mainly act on mAHP and therefore are involved in reducing neuronal excitability and firing frequency (González et. al. 2012; Pedarzani et. al. 2005; Pedarzani et. al. 2001; Storm 1990).

Structure of SK-channel:

SK-channel is a tetramer, each subunit contains six hydrophobic domains that are designated as S1 to S6, number corresponding to each domain within subunit. The P-loop in the structure forms selective filter that faces the center of channel. This P-loop is present between S5 and S6 (González et. al. 2012; Tuteja et. al. 2010) Since they are activated by entry of calcium and the calcium sensing is due to the presence of calmodulin (Cui et. al. 2014) Calmodulin is bound to cytoplasmic C terminus (González et. al. 2012) as shown in figure (Fig. 3). Beside this calmodulin can further regulate sensitivity to calcium by a kinase (CK2) and phosphatase (PP2A) (Weatherall 2010; Sun et. al. 2013).

A B.



Ledoux et. al. 2006, pg 69-78, Physiology (Bathesda)

Figure 3: Structure of SK-channel showing 6 transmembrane domains along with amino and carboxy terminals. B. refers an open and close state of SK-channel upon calcium binding.

Types of SK-channel:

SK-channel family contains four subtypes although the nomenclature is different in various literatures. SK-1, SK-2, SK3 and SK-4 are also termed as Kca2.1, K_{Ca}2.2, K_{Ca}2.3 and K_{Ca}3.1 respectively (Weatherall 2010). Genes associated with these four types are as follows: KCNN1, KCNN2, KCNN3 and KCNN4 (Shmukler et. al. 2001). Other subfamilies of SK-channel include K_{Ca}4.1, K_{Ca}4.2 and K_{Ca}5.1 (IUPHAR/BPS Guide to pharmacology).

Expression of SK-channels:

SK-channels are widely expressed throughout the body including heart (Hancock et. al. 2015; Hilgers et. al. 2006), pancreas (Fatherazi & Cook 1991) smooth muscles (Lu et. al. 2014) and uterus (Rahbek et. al. 2014). Regarding the nervous system, SK-channels are distributed widely in different brain parts (Gymnopoulos et. al. 2014). The channels are expressed in human as well as in rat cortex with regional moderate down regulation of SK2 channels in some models of chronic epilepsy (Oliveira et. al. 2010). Therefore, they might serve as a useful target for controlling hyperexcitability as seen in epilepsy. I showed here that CyPPa and SKa-31 were particularly effective in controlling seizure like events in all used preparations and epilepsy models suggesting that the SK2 channel is a particularly useful seizure control target.

SK2 and 3 channels are highly expressed in the amygdala, cortex, hippocampus, septum and olfactory system while SK1 channels are highly expressed in entorhinal cortex and the CA1 and CA3 regions of hippocampus (Stocker and Pedarzani 2008). Expression of SK3 is also high in entorhinal cortex, basal nuclei and in septum (Stocker and Pedarzani 2000). SK1 and SK2 channels seem to be more often co-expressed in the same neuron than SK2 and SK3 (Sailer et. al. 2002). In the neurons activation of SK-channel causes frequency adaptation of action potentials, which is rarely seen in interneurons that exhibits fast repetitive firing. It is therefore assumed that SK-channel expression is poor on inhibitory interneurons in cortical structures and therefore might not prevent release of GABA by fast spiking inhibitory cells (Rossignol et. al. 2013) Within substania nigra SK2 and SK3 were both expressed in dopaminergic neurons

(Deignan et. al. 2012), with further SK3 selectively controlling affects the firing frequency by expression in the soma region but not the regulation of action potential, whereas, SK2 seems to merely involve in the precision or timing regulation of action potential of DA neuron by expression in apical dendrites (Deignan et.al. 2012).

Therapeutic potential of SK-Channels:

SK-channel modulation was also studied for treatment of various other disorders including depression, Parkinson's disease, ataxia, memory disturbances, and disturbances of cognition and schizophrenia (see review of Blank et. al. 2004; Lam et. al. 2013). Recently a study highlighted the protective role of SK-channel in Alzheimer's disease (Kuiper et. al. 2012). Also, researchers studied effectiveness of SK-channel in stroke (Cipolla et. al. 2009), hypertension (Diness et. al. 2011, Hilgers & Webb 2007), diabetes (Tamarina et. al. 2000), cancer (Kallarackal et. al. 2013, Weaver et. al. 2006) etc. These diseases were investigated with respect to SK-channel modulation as an effective treatment. Indeed, beside nervous system various types of SK-channels are also present in the heart (Diness et. al. 2015a,b), bladder smooth muscle (Lee et. al. 2013, Morimura et. al. 2006, Soder et. al. 2013), endocrine system (Jacobson et. al. 2010, Traut et. al. 2009) and intestine (Fujita et. al. 2001).

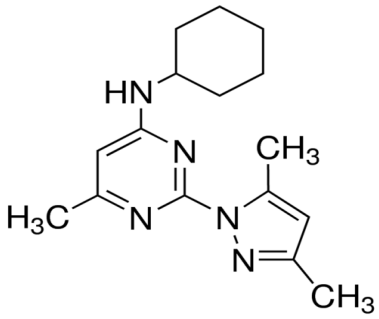
My working hypothesis was that SK-channel agonists may be useful in controlling seizures and particularly seizure like events which are pharmacoresistant. I therefore planned to compare effects of different subtype specific SK-channel enhancers, with GW-542573X (chemical structure given below) acting preferentially on SK1, with SKa-31 (chemical structure given below) preferentially acting on SK 2 and with CyPPa (chemical structure given below) acting preferentially on SK3. In order to have some insight whether SK-channel agonism may also be helpful in pharmacoresistant models of epilepsy I have employed both pharmacosensitive and pharmacoresistant seizure models. In most of these models we used 4-AP to induce seizure-like events (SLEs) (Avoli et. al, 2002; Brückner & Heinemann 2000; Perreault & Avoli 1992). 4-AP in concentrations of 100 µM affects members of the Kv 1 and the Kv 3 family (Streit et. al, 2011; Kiss et. al, 2002) with highest sensitivity on Kv 1.4 and 1.5 members. These are strongly expressed in presynaptic terminals (Geiger & Jonas 2000) and increase

transmitter release by increasing presynaptic Ca^{2+} influx (Schubert & Heinemann 1986; Wu & Saggau 1997). I have used three SK-channel agonists to study their effects on 4-AP induced seizure-like events in entorhinal cortex of normal and of pilocarpine treated epileptic animals. There is particular need for efficacy studies in models of pharmacoresistant epilepsy we employed the agents in slice cultures (Albus et. al. 2008) which we tested for pharmacosensitivity enabling us to differentiate between pharmacosensitive and pharmacoresistant slice cultures. These present a model of trauma induced epileptogenesis (Dudek & Staley 2011; Simonato et. al, 2012). To get further information in condition of pharmacoresistance we have employed zero-magnesium model in slices from normal adult rats and slice cultures. Finally we used potassium elevation combined with bicuculline or if possible application of 4-AP alone or combined with elevation of extracellular potassium concentration to induce seizure-like events in human temporal cortex slices in which evoked seizure like events are frequently pharmacoresistant (Sandow et. al. 2015).

A: **<u>CyPPa</u>**

Chemical name: N-Cyclohexyl-N-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-4-pyrimidinamine.

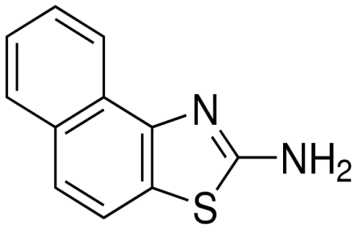
Molecular formula: $C_{16}H_{23}N_5$.



B: **<u>SKa-31</u>**

Chemical name: Naphtho[1,2-d]thiazol-2-ylamine.

Molecular formula: $C_{11}H_8N_2S$.



C: **GW-542573X**

Chemical name: 4-[[[[(2-Methoxyphenyl)amino]carbonyl]oxy]methyl]-piperidinecarboxylic acid-1,1-dimethylethyl ester

Molecular formula: $C_{19}H_{28}N_2O_5$

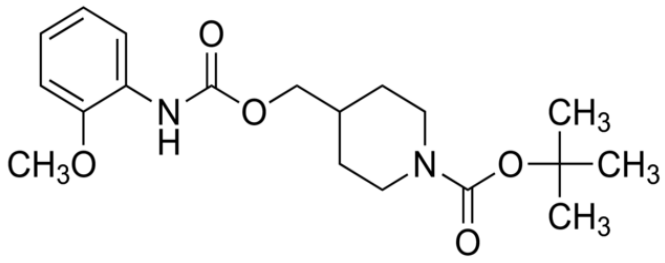


Figure 4: Chemical structure and formula of SK-channel agonists. A) CyPPa, B) SKa-31 and C) GW-542573X (Adopted from Sigma-Aldrich)

I've divided the work into three parts:

1. In the first part of the project I've applied different SK-channel agonists after inducing seizure like events using 4-AP (100 µM) in brain slices from normal rats. Studies on acute slices do not provide information about chronic changes. I therefore planned to test them in chronic model.

2. In the second part of my research question, SK-channel agonists were tested in slice culture (pharmacosensitive). Slice culture provides an important platform to test anticonvulsant potential of drugs in vitro, particularly if children epilepsies are modeled. It is convenient to manipulate exposure time period of test compound by adding it within media over days. It is therefore, very easy to evaluate the drug's action over a longer time frame (Albus et. al. 2013). It is important to mention that two different types of slice cultures were used i.e. pharmacoresistance and pharmacoresistant. Similarly, pilocarpine model presents an understanding to find the usefulness of a new compound in chronic phase of disease and also its antiepileptogenic potential. SK-channel agonists were tested in brain slices from a pilocarpine treated chronic epileptic rat.

3. In the third part of thesis work, to address the issue of pharmacoresistance. I have used neocortical tissue from pharmacoresistant epileptic patients. SK-channel agonists were tested in human tissue. SLEs were induced with either elevated potassium (8 mM) + Bicuculline (50 µM) or 4-AP (100 µM). This is an established protocol as reported by our lab (Sandow et. al. 2015; Gabriel et. al. 2004). Similarly, pharmacoresistant slice cultures were also used to further proof the anticonvulsant action of SK-channel agonists in pharmacoresistance (Albus et. al. 2008; Wahab et. al. 2011).

Methods:

Slice preparation and solutions:

Animals were purchased either from Harlan-Winkelmann (Germany) or from Janvier (France). To avoid the impact of transportation and stress, the animals were kept in the animal facility of Charite at least for one day to allow for acclimatization. In the case of experiments with normal rats, juvenile rats weighing 140-200 g were used. For preparing chronic epileptic rats and their controls, the initial weight of the rats was 120-130 g however their weight increased with time due to the long time it takes for development of the epilepsy. For preparation of slice cultures, rat pups were used with an age 5-6 days after birth.

All animals were kept in a thermostatically controlled environment to avoid affects of temperature variation. Animals were provided with food and water ad libitum. To exclude the possibilities of disturbed circadian rhythm, which might interfere with accurate results and is of concern for CNS experiments (Löscher 1990) animals were given 12h/12h light and dark cycle with lights on at 6 am.

Animal experiments were conducted as per European Communities Council's guidelines and approved by the local authority (LaGeSO Berlin: T0212/08). Adult rats were decapitated either under deep isoflurane anesthesia or under isoflurane anesthesia combined with laughing gas (70 % N_2O, 30 % oxygen with 1 % isoflurane) and head of the rat was cut under sharp-edged guillotine, the separated head put on table surface and hair with skin over skull was quickly removed. Using bone cutter the side bones of skull which are attached with lower neck region were cut for ease of removing the brain. With the help of a spoon, the whole brain was then removed by disconnecting it from brain stem using blade (number 18). The removed brain was transferred into chilled artificial cerebrospinal fluid (aCSF). To provide the brain with oxygen requirement, aCSF was already supplied with carbogen (95% O_2-5% CO_2) composition of aCSF is given in table **2**. The whole brain was kept in chilled aCSF for 90-120 s after which using a spoon, put into a petri-dish containing filter paper over the surface. As we know that brain is composed of fats it might causes stickiness on filter

paper. To avoid stickiness, filter paper was wet with chilled aCSF. Both the hemispheres were then gently and softly separated by blade (number 23). Care was taken not to give any mechanical stress to the brain as it will affect quality of electrophysiological responses. Separated hemispheres were then turned side-ward in order to prepare horizontal slices. For gluing the hemispheres firmly on cutting plate for slicing, a cut was given in such a way that gave flat surface to the hemispheres. With the help of filter paper each hemisphere was shifted and then glued onto a cutting plate. All this procedure was completed within 60- 90 s, if longer time passes then the quality of the tissue deteriorates due to hypoxia or cell loss. The chamber with cutting plate was set into the vibrotome and surrounded with small pieces of ice. The purpose of chilling temperature during slicing was to retain normal processes as much as possible by freezing. Care was taken to avoid vibration from the cutting blade that may exert stress to the slice and thus before slicing the vibrotome was calibrated to achieve a smooth run. Horizontal hippocampal slices with intact entorhinal cortex of 400 μm thickness, were prepared on a vibrotome (Model # VT 1200, Leica Microsystems, Wetzlar, Germany) more dorsal slices were not used for electrophysiological recording. Soon after slicing each slice was quickly transferred to an interface chamber, perfused with carbogenated aCSF at 36 ± 0.5°C (flow rate: ~1.8-2 ml/min, pH 7.4, osmolarity: 300 ± 5 mosmol/kg). For the easiness to move the slices or to position them for electrode placement, a double layered thin lens paper was kept inside each column of chamber and slices were placed on lens paper. The chamber was packed with socking for smooth flow rate inside the slice. Slices were left to recover for at least 2 hours before recording was started. Slices from normal rats and from pilocarpine treated epileptic rats, were obtained in the similar fashion.

Table 2: Ingredients of aCSF in mM concentration.

Ingredients	*Conc. In mM*
NaCl	**129**
$CaCl_2$	**1.6**
$NaHCO_3$	**21**
KCl	**3**
$MgSO_4$	**1.8**
NaH_2PO_4	**1.25**
glucose	**10**

Pilocarpine treatment and chronic epilepsy:

Pilocarpine treatment:

To prepare chronic epileptic rats, male Wistar's at 5 weeks of age, weighing 120-130 g were used. Animals were weighed one day before injection and food was withheld. The next day all rats received methylscopolamine (1 mg/kg) subcutaneously (Turski 1983). Pretreatment of methylscopolamine prevents the rats from having any peripheral side effects of pilocarpine injection which results from cholinergic activation (Clifford et. al. 1987). A higher dose of methylscopolamine i.e. 20 mg/kg would prevent induction of status epilepticus in rats (Turski 1983). After 30 min all animals received an intraperitoneal (i.p.) injection of pilocarpine (340-350 mg/kg) to induce status epilepticus (SE) (Wozny et. al. 2005). To compare the effect of vehicles, in place of the pilocarpine injection animals received 0.9 % NaCl. To increase the survival ratio of the rats, muscarinic antagonist i.e. atropine (1 mg/Kg) was administered (Clifford et. al. 1987). An animal was considered in status epilepticus (SE) if the seizing state remains continued after 3 consecutive seizures within a 10 min time duration. SE was stopped after 90 min with an injection of diazepam (i.p. 10 mg/kg). For rapid recovery and to prevent weight loss, each animal was injected with lactate ringer solution which covers the basic nutritional needs. A few pieces of cucumber were also kept in the home cages. The animals did not show any free movement for the next 15-20 hrs Animals

were kept under a 12 h light/dark cycle, freely provided with food and water. Two to three months after the initial injection, video recordings were performed to confirm epileptogenesis. For video purposes, animals were videoed for 48 h and these tapes were then analyzed to confirm that spontaneous seizures were present. For testing of SK-channel agonists only those animals that developed at least 3 seizures of Racine's scale stage 4-5 were sacrificed. These animals were all 6 months or older.

Preparation of organotypic hippocampal slice cultures (OHSCs):

It is crucial that cultures are prepared in a highly clean and hygienic environment otherwise during the incubation period the culture may develop contamination. Because of this reason, all instruments used in these studies were sterilized in an autoclave prior to every preparation. The inside area of the biosafety cabinet was cleaned aseptically using ethanol, this included all the dishes, pipettes, pipette box, and electric wires of instruments such as tissue chopper, microscope or lamp. To prepare organotypic hippocampal slice cultures (OHSCs), we adopted the interface culture method in which culture media is used for optimal growth during incubation phase (Stoppini et. al. 1991; Noraberg et. al. 1999). Wistar rats, 7-11 days old, were decapitated after anesthetising with isoflurane. Using the sharp scissor, the head of the pup was separated from the body and immediately moved into a sterile petri dish containing medium. The brain was taken out after removing the upper bones of the skull. After removal of the brain, the banana shaped structure i.e. hippocampus was carefully detached from the brain and fixed into the tissue chopper for slicing. Transverse hippocampal slices (320 µm) were cut and then transferred onto Millicell culture plate inserts (PICM 03050, 0.4 µm, 30 mm; Millipore GmbH, Schwalbach/Ts, Germany), and then inserted into six well culture plates. Slices were only transferred onto culture plates if they showed proper morphology. To see their gross morphology we first examined them under the microscope and picked rightly shaped slices which contained an intact entorhinal cortex. Each well contained 1.1 ml of medium comprising of HBSS - Hank's balanced salt solution, 25 ml (Biochrom AG, Berlin, Germany), MEM - modified Eagle's Minimum Essential Media, 50 ml (Opti-MEM; Invitrogen GmbH, Karlsruhe, Deutschland), and 25 ml of inactivated horse serum (Invitrogen GmbH). The culture plates were then placed in an incubator at 36.5 °C with 5 % CO_2. Old medium was replaced with fresh 1.1 ml of

a chemically defined medium (Neurobasal A; Invitrogen GmbH) every 2nd day and supplemented with 1-mm l-glutamine (Biochrom AG) and B27 (Invitrogen GmbH) (Brewer et. al. 1993). We prepared two different types of culture, one was pharmacosensitive and the other was pharmacoresistant. For making culture pharmacoresistant we used serum free medium that turned the slices into a pharmacoresistant state (Wahab et. al. 2010; Albus et. al. 2008). For the LFP recordings, 7-14 days old OHSCs were used. Culture was cut along with membrane and placed into interphase setup, supplied with carbogen and aCSF with flow rate of 1.8-2 ml/min. We allowed slices to recover for 2 h before commencing to record. A glass microelectrode filled with aCSF was placed in the entorhinal cortex to record signals.

Human tissue slices:

To obtain seizure control by AEDs or to cure the epilepsy, parts of the hippocampus and temporal neocortex have to be surgically removed in pharmacoresistant patients. Patients are considered drug resistant when seizure control can not be obtained by two AEDs administered alone and in combination at maximal tolerated dose within 2 years (Dlugos, 2001). Ethics Committee at the Charité has approved the study (EA125/2001, EA1/042/04) and studies were performed as per declaration of Helsinki. The pre-surgical analysis was performed in the Epilepsy-Centre of Berlin-Brandenburg according to the German and European guidelines (Baumgartner et. al. 2000; European Federation of Neurological Societies Task Force, 2000). Before surgery from each patient a written informed consent was taken for the study. For diagnostic purposes part of neighbouring sections of the tissue used in this study were analyzed by the Department of Neuropathology at the Charité Universitätmedizin-Berlin.

Preparation of human tissue:

After the removal of the cortical part by a neurosurgeon tissue is collected into a container filled with oxygen bubbled transport solution and carried to the laboratory. Depending on the size of the resected cortical part, coronal sections of around 5 mm thickness were cut from that resected temporal cortex and immediately transferred to cold (0.5-1°C) carbogenated transport solution, containing (in mM) NaH_2PO_4 1.25, KCl

3, $MgSO_4$ 2, $MgCl_2$ 2, $CaCl_2$ 1.6, glucose 10, sucrose 200, $NaHCO_3$ 21, and (±) α-tocopherol 0.1 pre-dissolved in ethanol (osmolality 303 mosmol/kg, 0.005 v% ethanol, pH 7.4) as previously described (Gabriel et. al. 2004; Kann et. al. 2005; Jandova et. al. 2006). The tissue was then transported within a 30 min timeframe to the laboratory in an airtight cooling receiver. To avoid the tissue bending during slicing the dura around the cortex was removed. Then, a coronal section of the tissue was dissected into slices of 500 µm thickness with the help of a vibratome (VT 1200, Leica Microsystems, Wetzlar, Germany). The slices were then quickly transferred into interface chambers, having a perfusion rate of 1.8 - 2 ml/min with prewarmed (35 °C) carbogenated artificial cerebrospinal fluid containing following ingredients in mM: NaCl 129, NaH_2PO_4 1.25, KCl 3, $MgCl_2$ 2, $CaCl_2$ 1.6, $NaHCO_3$ 21, glucose 10, (±) α-tocopherol 0.03 (osmolality 303 mosmol/kg, 0.002 v% ethanol, pH 7.4). Local field potentials recording was started after 4-5 h of recovery phase since slicing.

Stimulation induced evoked potential in acute hippocampal slices:

To study the effect of SK-channel agonists on evoke potentials, a stimulation electrode was placed in schaffer collateral of the stratum radiatum. Recording electrodes were places in stratum pyramidale layer of CA1 and CA3 regions. Optimal current intensity was determined using input out curve. Initially pulse was given at different intensities starting from as low as 0.5 mA current intensity. Current intensity was increased in a stepwise manner to establish an intensity that resulted for maximum response. Paired-pulse was given with master aid stimulation being given at interval of 30 ms. SK-channel agonists were applied for at least 45 min. fEPSP were recorded and stored on computer disks for offline analysis.

Local Field Potential Recordings:

Extracellular field potentials (FP) were recorded with glass microelectrodes filled with 154 mM NaCl (5 - 10 MΩ) or aCSF, using interphase chamber. Slices from rats or slice culture electrodes were placed in the layer V/IV of the medial entorhinal cortex (mEC). In human tissue the electrode was placed into deep layer V of temporal neocortex. Potentials were amplified 200 times with a custom-made amplifier, low-pass filtered at 3

kHz, and digitized at 5-10 kHz and stored on computer disk for offline analysis using a 1401 CED interface and spike 2 software (CED, Cambridge, UK. The signals were amplified with a SEC 05L amplifier (NPI Instruments, Tamm, Germany).

Induction of seizure like events (SLEs) in vitro:

Slices were continuously superfused (1.8 - 2 ml/min) with prewarmed (35.5 ± 0.2°C) and oxygenated (95% O_2/5% CO_2).aCSF. SLEs were induced with application of 4-AP (100 µM, Perreault & Avoli 1991, Brückner & Heinemann 2000; Zahn et. al. 2012) in aCSF. Once SLEs became stable with respect to duration and interval between SLEs, drugs were applied for 45 min. For inducing SLEs in human tissues we have used either 4-AP (100 µM) or I applied 8 mM potassium for 15 min combined then with bath application of bicuculline methiodide (50 µM) (Sandow et. al. 2015).

Moreover, SLEs were also induced in slices by omitting magnesium from aCSF. Lowering or removing magnesium in aCSF results in seizure generation particularly in the entorhinal cortex (Walther et. al. 1986; Mody et. al. 1987). Continued application of magnesium free aCSF lead to late recurrent discharges (LRDs) and this condition is resistant to clinically used anticonvulsant (Wahab et. al. 2009; Dreier & Heinemann 1990).

Drugs & chemicals:

All drugs were purchased from Sigma-Aldrich (Taufkirchen, Germany) i.e. 4-AP, carbamezapine, phenobarbital and valproate except for CyPPa, SKa-31 and GW-542573X (Structure as appendix I) which were purchased from Tocris Bioscience (Bristol, UK). Apart from 4-AP phenobarbital and valproic acid all other drugs were dissolved in DMSO (final concentration < 0.1 %) and then added to aCSF and applied by continuous bath perfusion. All the chemicals used were of analytical grade with purity of more than 99%.

Statistics:

Data was analyzed using SPSS ver12. All values were expressed as mean value ± standard error of the mean (SEM). Statistical significance was determined by applying ANOVA selecting Dunnett test for comparative analysis. For analysis of evoked potentials, amplitudes of last 5 stimulation responses were averaged pre and post drug administration fEPSP were extracted out with the help of script in spike 2 software. *P* values <0.5 or * <0.05 or **<0 005 were considered significant.

Results:

Effects of SK-channel agonists on 4-aminopyridine induced SLEs in brain slices from normal rat:

SLEs were induced in the entorhinal cortex by application of 4-AP (100 µM) in all slices. In my experimental conditions SLEs lasted between 30 and 90 s (average of 69 ± 26 s). The initiation of SLEs was categorized by a sharp negative DC shift from the baseline superimposed by a tonic phase followed by clonic like discharges. These SLEs are sensitive to standard AEDs as reported by (Brückner & Heinemann 2000, Fueta & Avoli 1992). I have tested three different SK-channel agonists namely, GW-542573X (SK 1 channel agonist), SKa-31 (SK 2 channel agonist) and CyPPa (SK 2 and the 3 channel agonist). Examples of original recordings of the effects of CyPPa on entorhinal cortex seizure like events are illustrated in **Fig. 5A** which depicts recurrent SLEs after application of 4-AP (100 µM). Complete blockade of SLEs by CyPPa was obtained within 30 to 35 min. **Fig. 5Ab** representing SLEs on an extended time scale. Results of two different concentrations of CyPPa (50 and 100 µM) and control are summarized in **Fig. 5B**. I have selected the last 2 SLEs before application of the drug and the last 2 SLEs before complete block was achieved for comparing seizure parameters. Furthermore I have analyzed the effects of CyPPa on interval and duration of SLEs. Interval between SLEs was increased by CyPPa and a significant reduction in duration was observed as shown in **Fig. 5Ba** and **b**. However at 50 µM, SLEs were blocked in 2 out of 6 experiments while with 100 µM SLEs were blocked in all studied slices (n = 8). CyPPa works on both SK 2 and SK 3 channels (Hougaard et. al. 2007) and SK 3 channels have been reported to be persistently down regulated in the hippocampus of pilocarpine treated animals (Oliveira et. al. 2010). I have therefore selected the more specific SK 2 channel agonist, SKa-31 to study its effect on SLEs. SKa-31 also increased intervals between SLEs from 5 ± 4 min to 14 ± 5 min. Similar to CyPPa, it also decreased the duration from 110 ± 135 s to 59 ± 71 s of SLEs before complete suppression of SLEs (**Fig. 5Ca** & **5Cb**). This reduction in duration of SLEs was not dose dependent, while increment in interval was found in a dose dependent fashion (**Fig. 5Ca** & **Cb**). SKa-31 at 150 µM blocked SLEs in all studied slices (n = 8). However, some seizure like activity remained in the 6 slices studied with 50 µM SKa-31 (**Fig. 5Cc**).

I further tested the effects of a third compound, GW-542573X which acts mainly on SK 1 channel. In comparison to CyPPa and SKa-31, GW-542573X was least effective in blocking SLEs, at lower dose of 100 μM it failed to suppress SLEs, while at 200 μM it caused a block of activity only in 2 out of 6 slices (**Fig. 5Dc**). However, GW-542573X was found effective to dose dependently increase the interval between seizure like events (± 3 min to 7 ± 2 min, **Fig. 5Da**). There was no effect on duration of SLEs (**Fig. 5Db**).

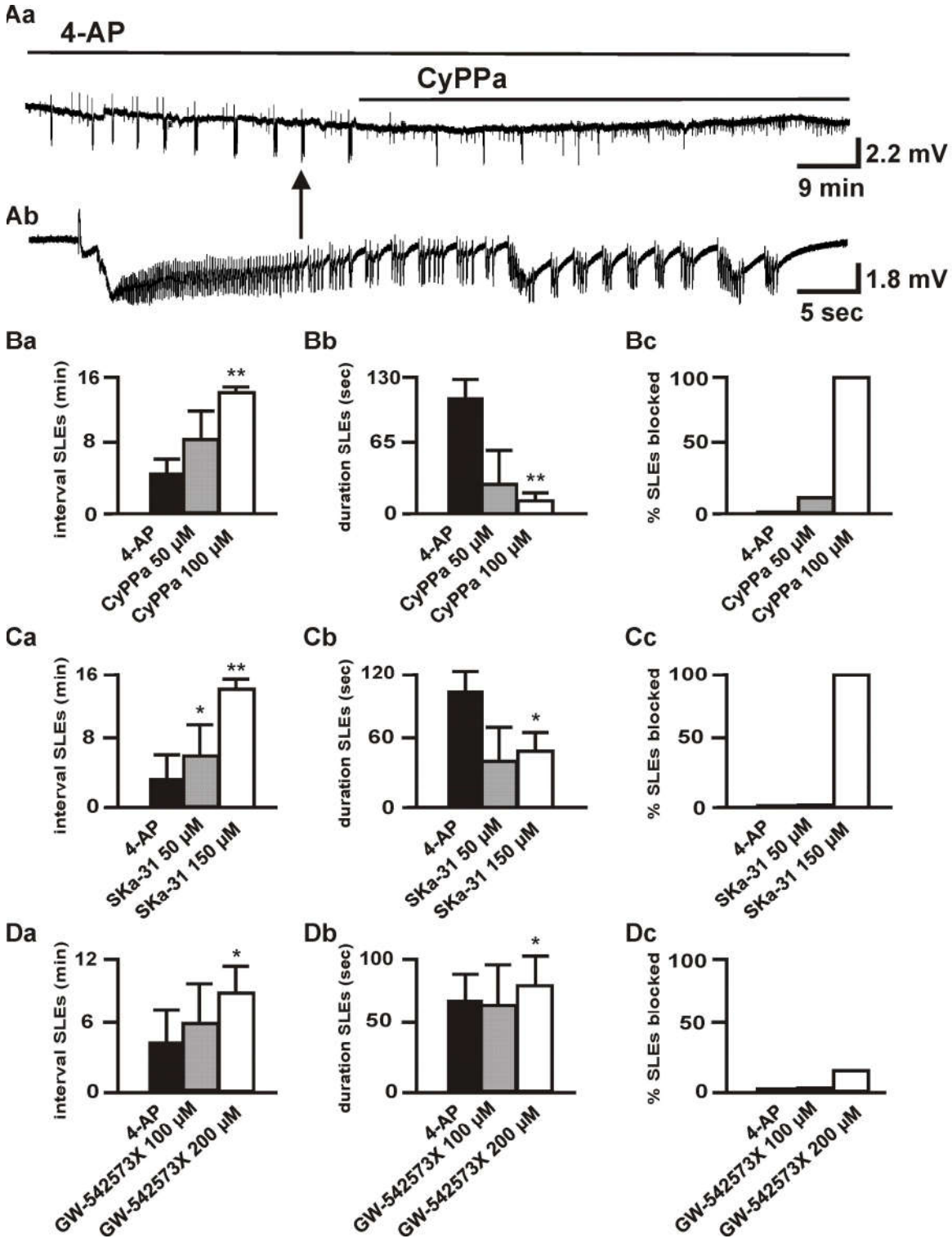


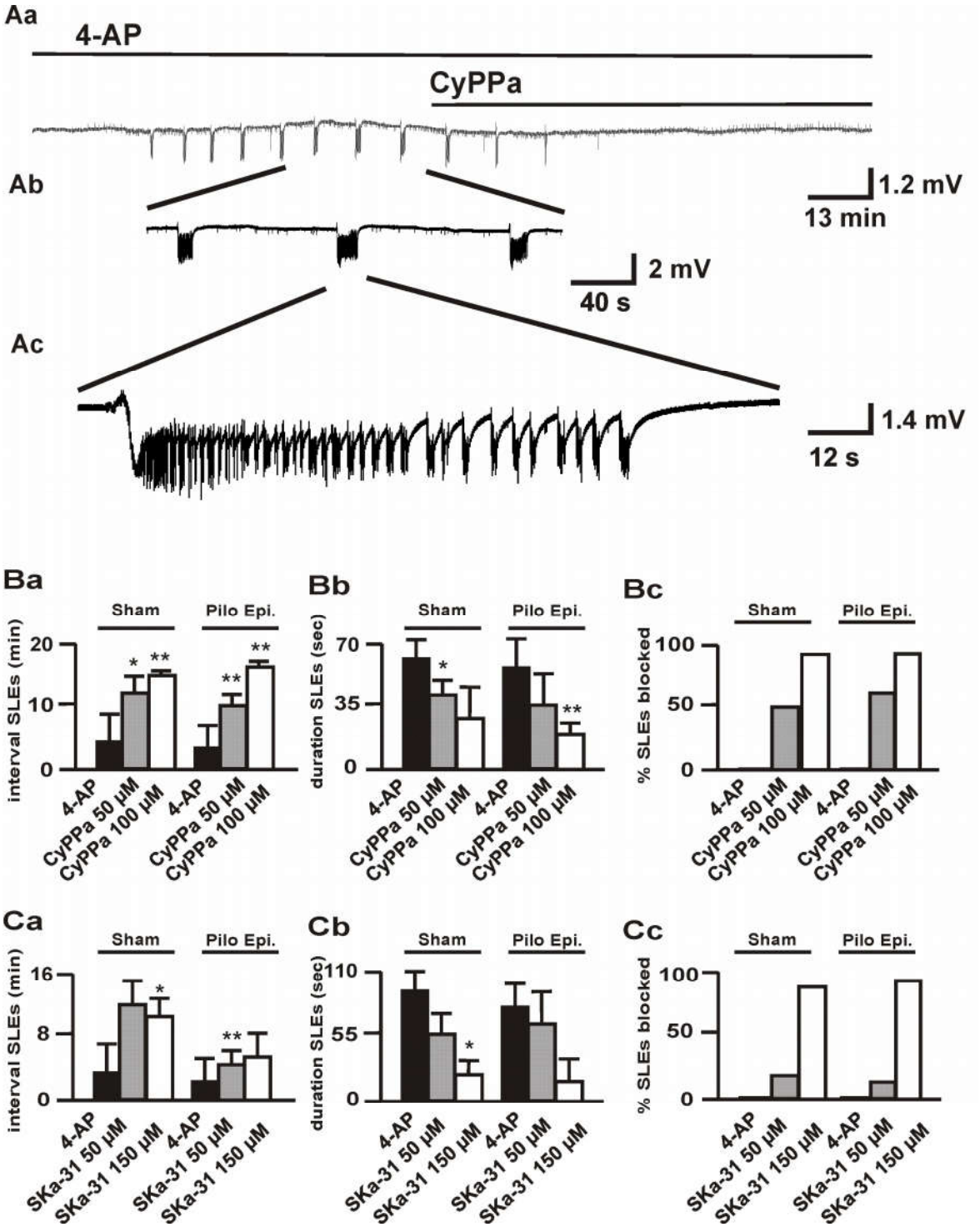
Figure 5: *Effects of SK-channel agonists on SLEs induced by 4-AP in acute slices. A) An original trace of field potential recording from entorhinal cortex of slice from normal rat's brain. Application of CyPPa (100 µM, n=8) blocked SLEs effectively in acute slice. Ab) Single SLE on extended time scale. (Ba,b,c) Effect of CyPPa as bar graphs*

*representing the % SLEs blocked, effect on duration of SLEs (**p<0.005) and interval in between SLEs (**p<0.005) respectively. Ca,b,c) Bar graphs displayed results of SKa-31 (n=8) on the % SLEs blocked, effect on duration of SLEs and interval (**p<0.005) in between SLEs, respectively (Da,b,c) showed bar graph representing the % SLEs blocked, effect on duration of SLEs (*p<0.05) and interval in between SLEs (*p<0.05) respectively by GW-542573X (n=6). Values given as standard error of the mean (SEM), *p<0.05 and **p<0.005.*

Effects of SK-channel agonists on 4-AP induced SLEs in brain slices from pilocarpine treated chronic epileptic rats:

SLEs were observed after bath application of 4-AP (100 μM) in all slices from both pilocarpine treated as well as sham operated rats. The effect on slice after application of 4-AP and SK-channel agonist is illustrated in **Fig. 6.** The figure clearly demonstrates dose dependent effects of SK 2 channel agonist SKa-31 on 4-AP induced SLEs in sham treated and pilocarpine treated rats. Importantly, sham treated animals were the same age (about 6 month) as pilocarpine treated animals, indicating that the blocking effects of SK-channel agonists are not age dependent. There was no difference in the effect of sham operated and pilocarpine treated rats, with the exception of slices from pilocarpine treated animals having a shorter interval inbetween SELs. At a lower dose of 50 μM, SKa-31 (SK 2 agonist) blocked SLEs in 2 out of 6 slices from sham operated animals but at 150 μM blockade was achieved in 88% of studied slices (n=6) (**Fig. 6Cc**). Likewise, similar results were found in the pilocarpine treated group. SKa-31 (50 μM) blocked SLEs in 2 out of 7 slices and successful suppression of SLEs was noted with 150 μM in 88% slices (n=8). However, in the sham operated group, CyPPa at 50 and 100 μM blocked SLEs in 50% and 88% of slices (n=8) respectively (**Fig. 6Bc**). CyPPa also increased in a dose dependent manner the seizure interval (**Fig. 6Ca**). In regards to duration of the SLEs similar results were found with CyPPa as in case of SKa-31 (**Fig. 6Cb**). Compared to the sham treated group CyPPa exhibited better activity in the pilocarpine treated group. Blocked off SLEs was observed in 6 out of 8 slices with 50 μM and 7 out of 8 slices with 100 μM. This indicates that the CyPPa may be more effective in slices from chronic epileptic rats (**Fig. 6Dc**). GW-542573X,

(specific agonist of SK 1), was ineffective in acute slices at 100 μM, whereas, at a dose of 200 μM, GW-542573X blocked SLEs in 50% of slices (n=8; **Fig. 6Dc**). At 100 μM GW-542573X had no effect on the duration of SLEs, however positive action on the interval of SLEs was observed. GW-542573X, at 200 μM also prolonged the interval between SLEs from 6 ± 1 min to 14 ± 2 min (**Fig. 6Da**). At 200 μM it reduced the duration of SLEs to half i.e. from 60 ± 11 s to 26 ± 3 s (**Fig. 6Db**). This suggests that SK1 agonist is more effective in chronic pilocarpine treated epileptic rats than in normal rats.


Aa
4-AP
CyPPa
Ab
1.2 mV
13 min
2 mV
40 s
Ac
1.4 mV
12 s
Ba
Sham
Pilo Epi.
interval SLEs (min)
4-AP
CyPPa 50 µM
CyPPa 100 µM
Bb
duration SLEs (sec)
Bc
% SLEs blocked
Ca
SKa-31 50 µM
SKa-31 150 µM
Cb
Cc

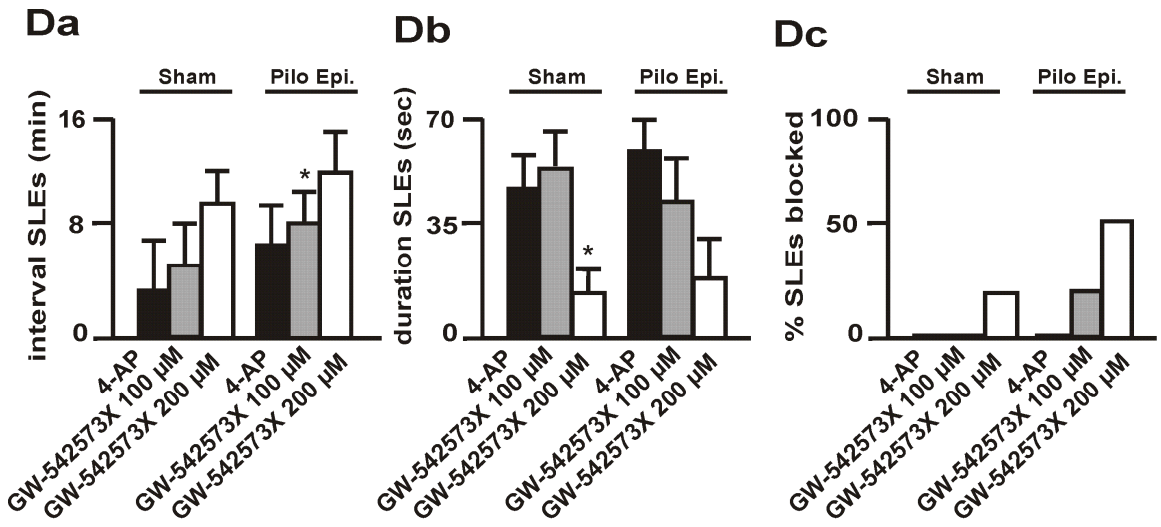


Figure 6: *Effects of SK-channel agonists on SLEs induced by 4-AP in slices from pilocarpine chronic epileptic and sham control. A) An original trace of field potential recording from entorhinal cortex of slice from normal rat's brain. Application of CyPPa (100 µM; n=8) effectively blocked SLEs in slices. Ab & Ac) SLE on extended time scale. (Ba,b,c) Effect of CyPPa as bar graphs representing the % SLEs blocked, effect on duration of SLEs and interval in between SLEs (**p<0.005) respectively. Ca,b,c) Bar graphs displayed results of SKa-31 (n=8) on the % SLEs blocked, effect on duration of SLEs (*p<0.05) and interval in between SLEs (**p<0.005), respectively. (Da,b,c) Bar graph representing the % SLEs blocked, effect on duration of SLEs and interval in between SLEs respectively by GW-542573X (n=8; (*p<0.05)). Values given as standard error of the mean (SEM).*

Effects of AEDs on SLEs induced by 4-AP in pharmacosensitive organotypic hippocampal slice cultures (OHSCs):

SLEs in slice cultures are often pharmacoresistant but in some conditions they are not (Albus et. al. 2008, Dyhrfjeld-Johnsen et. al. 2010). We therefore tested slice cultures treated with serum with respect to sensitivity to VPA (**Fig. 7**) and found that these slice cultures in contrast to slice cultures treated with artificial serum were sensitive to standard AEDs. To prove this point we used VPA at two different doses (300 µM and with 2 mM). With 300 µM, VPA blocked the 4-AP induced recurrent seizure like events in 50% slice cultures (n=6) and with 2 mM in 88% OHSCs (n=9; **Fig 7Bc**). I also used

another antiepileptic drug i.e. CBZ, at 25 μM, however it failed to block SLEs (n = 6). At 100 μM CBZ blocked recurrent SLEs in 6 out of 8 cultures (**Fig. 7Cc**). This is however a very toxic dose. Upper tolerable levels are close to 20 μM.

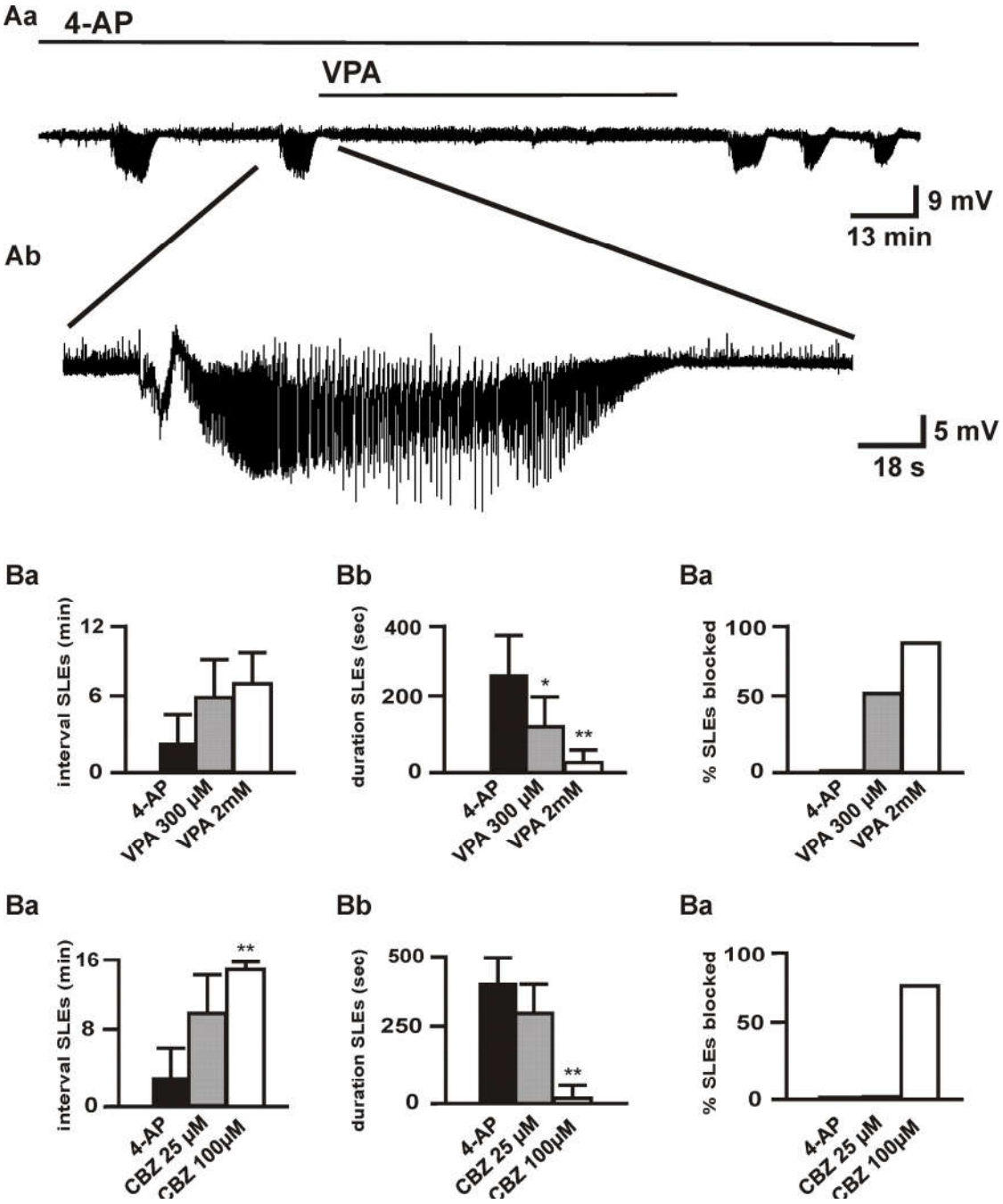


Figure 7: *Effects of valproic acid & carbamazepine on SLEs induced by 4-AP in pharmacosensitive hippocampal organotypic slice cultures (OHSCs). A) An original trace of field potential recording from entorhinal cortex in pharmacosensitive OHSCs.*

*Application of VPA (2 mM; n=9) blocked SLEs in most of pharmacosensitive OHSCs. Ab) Single SLE on extended time scale. (Ba,b,c) Effect of VPA as bar graphs representing the % SLEs blocked, effect on duration of SLEs and interval in between SLEs (*p<0.05) respectively. Ca,b,c) Bar graphs displayed results of CBZ on the % SLEs blocked (n=6), effect on duration of SLEs and interval in between SLEs, respectively (**p<0.005). Values were given standard error of the mean (SEM).*

Effects of SK-channel agonists on 4-aminopyridine induced seizure like events in pharmacosensitive organotypic hippocampal slice cultures (OHSCs):

CyPPa blocked SLEs in 100% pharmacosensitive OHSCs at 100 µM, similar to acute slices (n=6; **Fig. 8Bc**). In slice culture usually lower doses are applied by experimenter. I therefore used low doses (5 & 20 µM) to check if CyPPa still works effectively at these doses in pharmacosensitive OHSCs (see **Fig. 8**). At the lowest dose of 5 µM, CyPPa failed to block epileptiform activity (n = 6), interestingly at 20 µM CyPPa blocked SLEs in 6 out 6 pharmacosensitive OHSCs (**Fig. 8Bc**). This shows that CyPPa has a more potent effect in pharmacosensitive OHSCs than in acute slice. CyPPa increased the seizure interval from 5 ± 3 min in the control to 14 ± 2 and 16 ± 2 min at doses of 20 and 100 µM, respectively (**Fig. 8Ba**). CyPPa also reduced the duration of SLEs before the block of activity in pharmacosensitive OHSCs (20 µM). The duration was reduced by 50 % at 20 µM and in case of 100 µM by 70 % (**Fig. 8Bb**).

SKa-31 was also effective in blocking the 4-AP induced SLEs in pharmacosensitive OHSCs. Suppression of SLEs with 50 µM was achieved in 4 out of 7 pharmacosensitive OHSCs while at 150 µM in 5 out of 9 pharmacosensitive OHSCs (**Fig. 8Cc**). Low dose of SKa-31 (50 µM) did not show any effect on seizure interval whereas, at 150 µM, increment in the size of seizure interval was observed from 8 ± 3 min compared to 4 ± 2 min in control condition (**Fig. 8Ca**). Decrease in duration of SLEs was 3 ± 2 to 2 ± 1 min at 50 and 150 µM respectively, compared to 5 ± 2 in control condition (**Fig. 8Cb**).

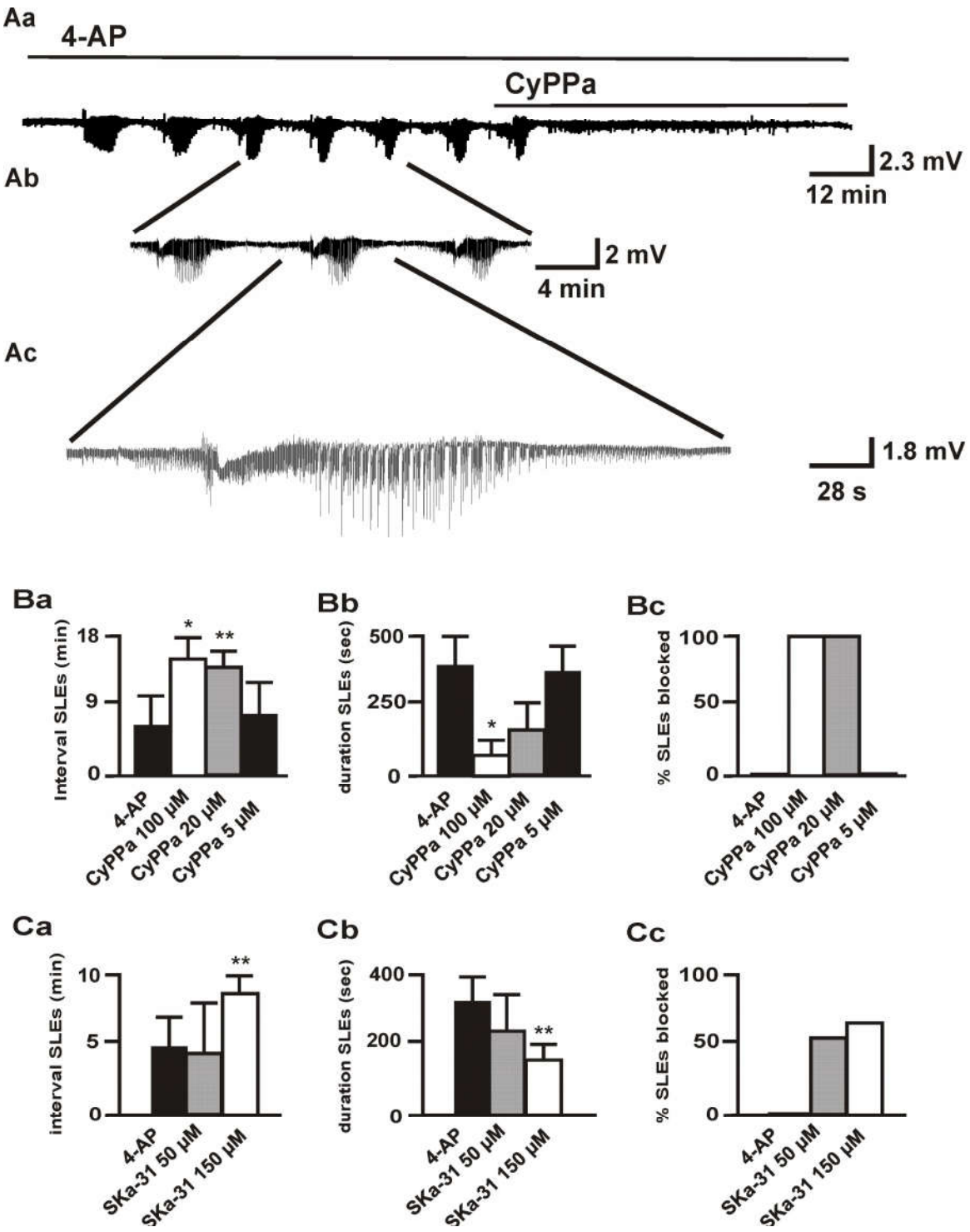


Figure 8: *Effects of SK-channel agonists on SLEs induced by 4-AP in pharmacosensitive hippocampal organotypic slice cultures (OHSCs). A) An original trace of field potential recording from entorhinal cortex in pharmacosensitive OHSCs. Application of CyPPa (100 µM; n=6) blocked SLEs in 100% of pharmacosensitive*

*OHSCs even at 20 μM dose (n=7). Ab & Ac) SLE on extended time scale. (Ba,b,c) Effect of SKa-31 as bar graphs representing the % SLEs blocked (n=9), effect on duration of SLEs and interval in between SLEs (**p<0.005) respectively. Values given as standard error of the mean (SEM).*

Effects of SK-channel agonists on 4-aminopyridine induced seizure like events in pharmacoresistant organotypic hippocampal slice cultures (OHSCs):

We had previously shown that OHSCs maintained in artificial medium were usually pharmacoresistant by the time we did our measurements (7 or more days *in vitro*). To evaluate effectiveness of SK-channel agonists in pharmacoresistance we have tested the two SK-channel agonists (CyPPa and SKa-31) that were more effective in acute slices and slices from pilocarpine treated animals. At the low dose of CyPPa, 20 μM that was able to block SLEs in pharmacosensitive OHSCs was found to be ineffective in pharmacoresistant OHSCs (n=6) CyPPa, blocked SLEs, while at 100 μM blockade of SLEs was seen in 4 out of 7 pharmacoresistant OHSCs (**Fig. 9Bc**). But with 20 μM effects on seizure interval of SLEs as well as on duration was observed (**Fig. 9Ba** & **9Bb**). CyPPa at 20 and 100 μM prolonged the time between SLEs from 3 ± 2 min to 6 ± 3 min to 12 ± 4 min, respectively (**Fig. 9Ba**). At 20 μM duration of SLEs was reduced to 340 ± 54 s, compared with control i.e. 520 ± 160 s, whereas, at 100 μM decreased in many folds on duration of SLEs was occurred 85 ± 36 s. (**Fig. 9Bb**).

Compared to acute slices, pharmacosensitive OHSCs and slices from pilocarpine treated rats, activity of SKa-31 was found less in pharmacoresistant OHSCs. At 50 μM SKa-31, suppression of SLEs was not achieved (**Fig. 9Cc**). At a concentration of 150 μM it blocked SLEs in only 2 out of 6 pharmacoresistant OHSCs. Whereas, SKa-31 enhanced the interval between SLEs to many folds compare to normal rat (control 3 ± 1 min, 5 ± 1 (50 μM) and 6 ± 2 min (150 μM), **Fig. 9Ca**). No significant change in the effect of duration was noted (**Fig. 9Cb**).

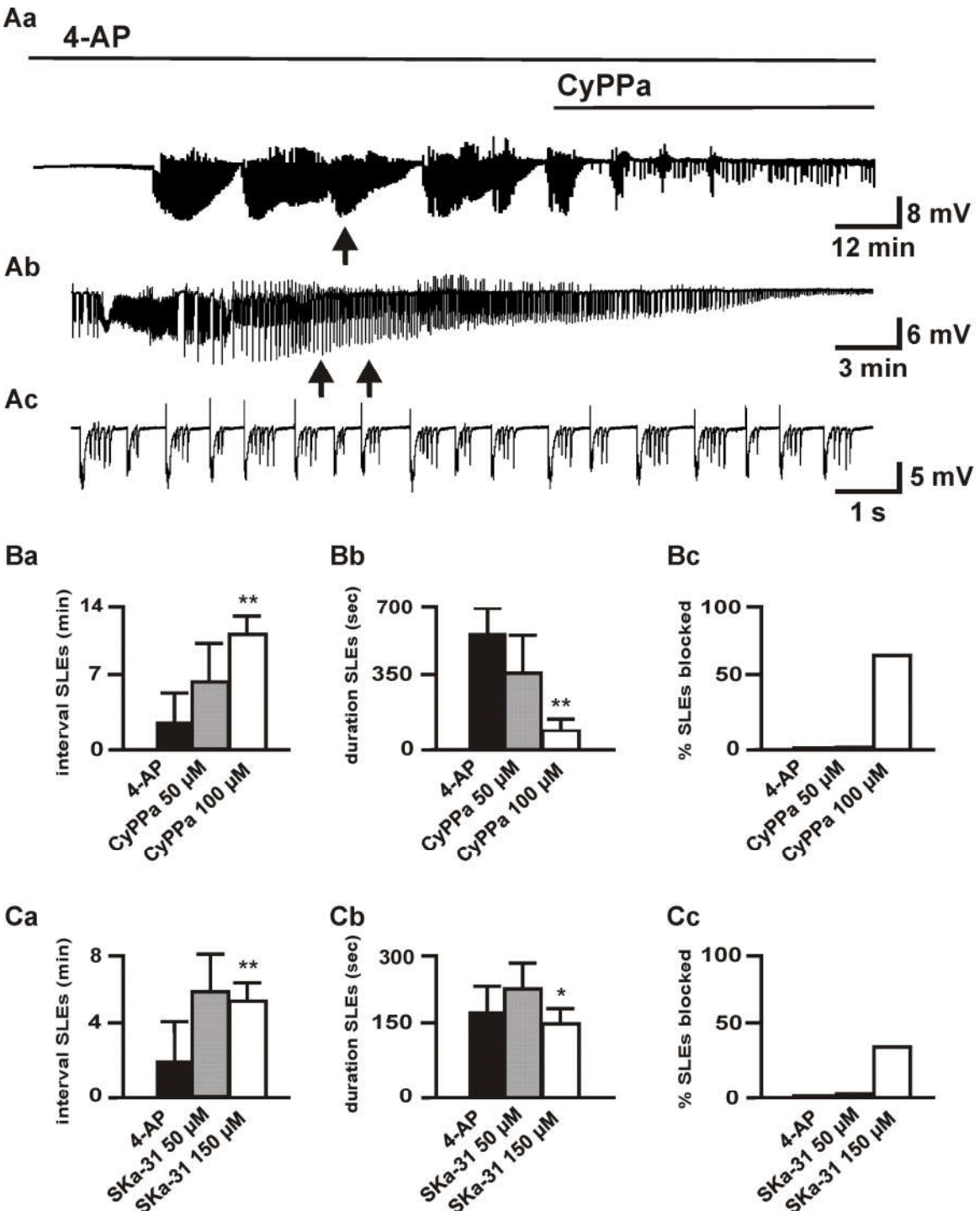


Figure 9: *Effects of SK-channel agonists on SLEs induced by 4-AP in pharmacoresistant hippocampal organotypic slice cultures (OHSCs). A) An original trace of field potential recording from entorhinal cortex in pharmacoresistant OHSCs. Application of CyPPa (100 µM; n=7) blocked SLEs in 66% of pharmacoresistant*

*OHSCs even at 20 μM dose. Ab & Ac) SLE on extended time scale. (Ba,b,c) Effect of SKa-31 as bar graphs representing the % SLEs blocked (n=6), effect on duration of SLEs (*p<0.05) and interval in between SLEs (**p<0.005) respectively. Values given as standard error of the mean (SEM).*

Effects of SK-channel agonists on 4-aminopyridine or high potassium and bicuculline induced SLEs in human neocortical tissue from pharmacoresistant epileptic patients:

Among the problems linked with new AEDs drug development for pharmacoresistant epilepsies is the unavailability of a suitable model. It is very important that the testing model should provide information of reliable efficacy that addresses the superiority in patients who are non-responsive to current AEDs (Dudek et. al. 2002). Further in this regard, precise dose finding regimens should be established during early development phase, followed by toxicology studies. It is a tedious and lengthy process, but could quickly and conveniently be done by using human specimens obtained from pharmacoresistant epileptic patients. Interestingly, I have found effective SLEs suppression both by CyPPa (100 μM) and SKa-31 (150 μM) in the human neocortex surgically resected from patients. In case of CyPPa a total of 16 slices obtained from 8 patients were used. 4-AP (100 μM) was able to induce SLEs that were relatively shorter in duration in 7 slices from temporal neocortex slices, compared to OHSCs. CyPPa blocked 4-AP evoked SLEs in 100% slices (n=7; **Fig. 10Bc**). I have used another protocol in those slices which did not respond to 4-AP. SLEs were induced by combining elevation of extracellular potassium concentration (8 mM) with application of bicuculline methiodide at a concentration of 50 μM in total of 9 slices, CyPPa blocked SLEs in 66% i.e. 9 out of 6 slices (**Fig. 10Bc**). CyPPa also increased the seizure interval by three folds compared to control from 4 ± 1 min to 13 ± 3 min (**Fig 10Ba**). In the same way, duration of SLEs decreased to half from 45 ± 14 s to 21 ± 8 s (**Fig 10Bb**).

SKa-31 showed potential on human tissue similar to CyPPa. From 5 different patients a total of 11 slices were included in the study. Out of them, 4 slices were administered with 4-AP (100 µM). SLEs were successfully suppressed in all 4 slices by SKa-31 at 150 µM (**Fig 10Cc**). While in seven slices 8 mM K^+ combined with the application of bicuculline methiodide were used for induction of SLEs, SKa-31 blocked SLEs in 4 of out of 7 slices (**Fig. 10Cc**). Significant reduction in the SLEs' interval were achieved by SKa-31 from 210 ± 85 s to 85 ± 40 s (**Fig. 10Ca**). Furthermore, the duration of epileptiform events was shortened from 70 ± 40 s to 21 ± 9 s as indicated in **Fig. 10Cb.**

Property of GW-542573X in human tissue to block SLEs was not promising. A total of 12 slices were used to test GW-542573X. At the concentration of 200 µM it only blocked SLEs induced by elevated potassium combined with bicuculline (50 µM) in 2 out 8 slices used in the study. However, in case of 4-AP it failed to block SLEs induced by 4-AP (100 µM) in all four slices. (**Fig. 10Dc**). GW-542573X did not show any effect on the interval of SLEs, on the other hand, duration of SLEs were reduced from 49 ± 12 s to 36 ± 8 s (**Fig. 10Db**). Whereas, GW-542573X (200 µM).

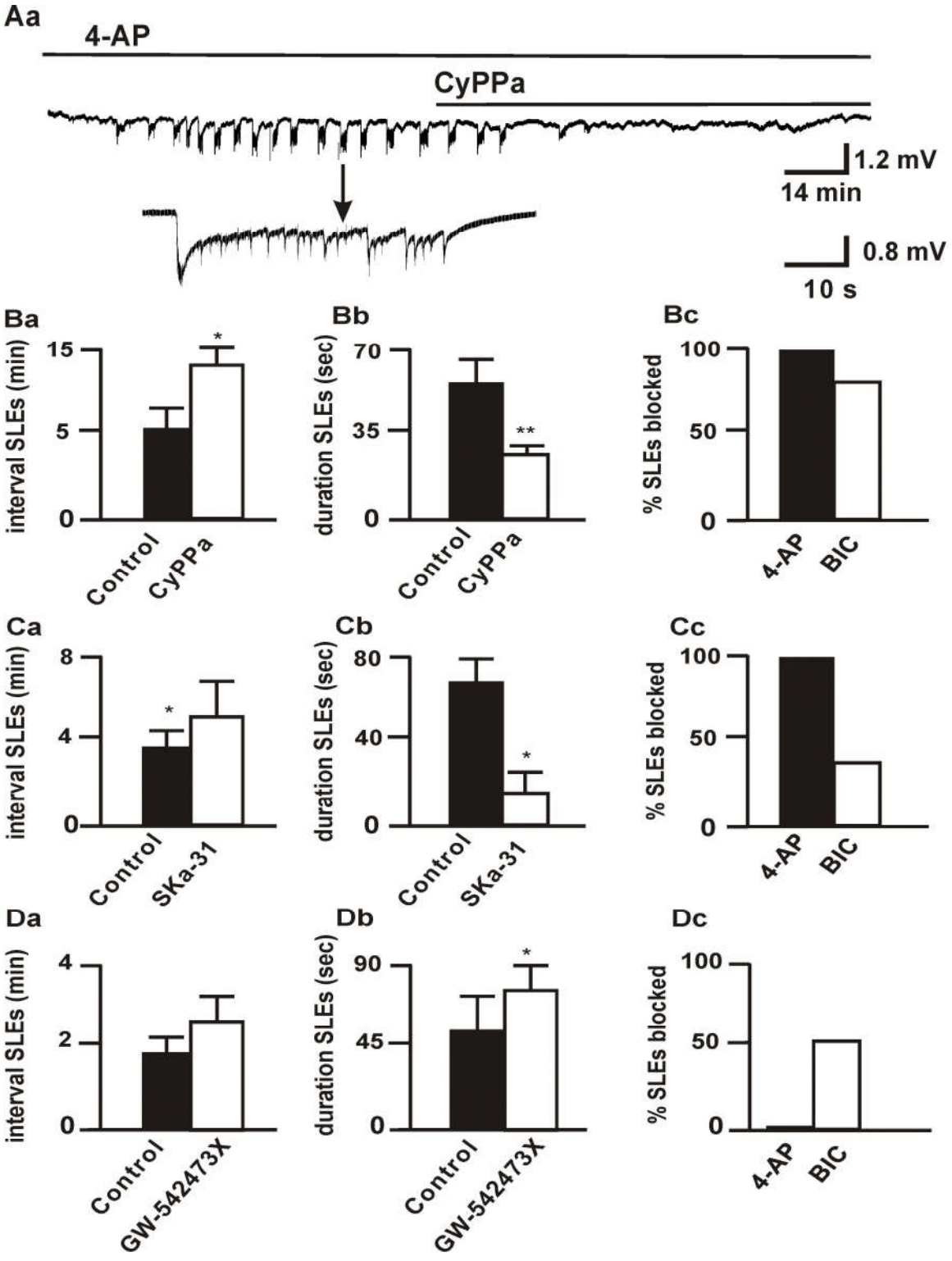


Figure 10: *Effects of SK-channel agonists on SLEs induced by 4-AP or combined application of 8 mM K^+ and bicuculline methoiodide (50 μM). A) An original trace of field potential recording from layer VI/V of neocortex from pharmacoresistant epileptic*

*patient. Application of CyPPa (100 µM) blocked SLEs in 100 % of slices induced by 4-AP (n=7). While 83% suppression of SLEs in presence of 8 mM K^+ and bicuculline methoiodide (50 µM; n=9). (Ba,b,c) Effect of CyPPa as bar graphs representing the % SLEs blocked, effect on duration of SLEs (**p<0.005) and interval in between SLEs (*p<0.05) respectively. Ca,b,c) Bar graphs displayed results of SKa-31 on the % SLEs blocked (n=4 with 4-AP, n=7 with bicuculline), effect on duration of SLEs (*p<0.05) and interval in between SLEs, respectively. (Da,b,c) Bar graph representing the % SLEs blocked (n=4 with each of 4-AP and bicuculline.) effect on duration of SLEs (*p<0.05) and interval in between SLEs respectively by GW-542573 X. Values given as standard error of the mean (SEM).*

Effect of SK-channel agonists on SLEs induced by Mg^{2+} free aCSF:

Among all the three SK-channel agonists used in this study, only CyPPa was found to be effective in the blockade of SLEs induced by Mg^{2+} free aCSF. In acute slice preparations, CyPPa only blocked SLEs at 100 µM (n=7), whilst at low dose of 50 µM no suppression of SLEs was achieved (n=6). Compared to this, at low dose of 50 µM the blockade of SLEs was observed in 2 out 6 studied OHSCs (both pharmacosensitive and pharmacoresistant). However, at 100 µM, CyPPa blocked SLEs in 100% pharmacosensitive OHSCs. But in pharmacoresistant OHSCs, CyPPa at 100 µM SLEs were blocked only in one out of six studied OHSCs. Contrary to this, both SKa-31 and GW-542473X failed to block SLEs in acute slice preparations and also in OHSCs (n=8, n=6) respectively.

Effect of SK-channel agonists on stimulation induced evoked potentials in hippocampus:

SK1 channel agonist, GW 543574X at 200 µM, did not show any powerful effect on paired-pulse index, only small change was observed in index of CA3 regions (n=6; **Fig. 11 Aa and Ab**). However, CyPPa (100 µM) resulted significant increased in the paired-pulse index. In CA3, CyPPa increased paired pulse index from 1.9 ± 0.2 to 2.2 ± 0.3 (n=7; **Fig. 11 Ba and Bb),** whereas, in CA1 it increased the index value from 1.3 ± 0.2

to 1.6 ± 0.2. Similarly, SKa-31 (150 µM) also significantly increased from 1.8 ± 0.3 to 2.6 ± 0.4 in CA3 region. Increased in index by SKa-31 in CA1 was from 1.4 ± 0.1 to 1.5 ± 0.1 (n=7; **Fig. 11 Ca and Cb**).

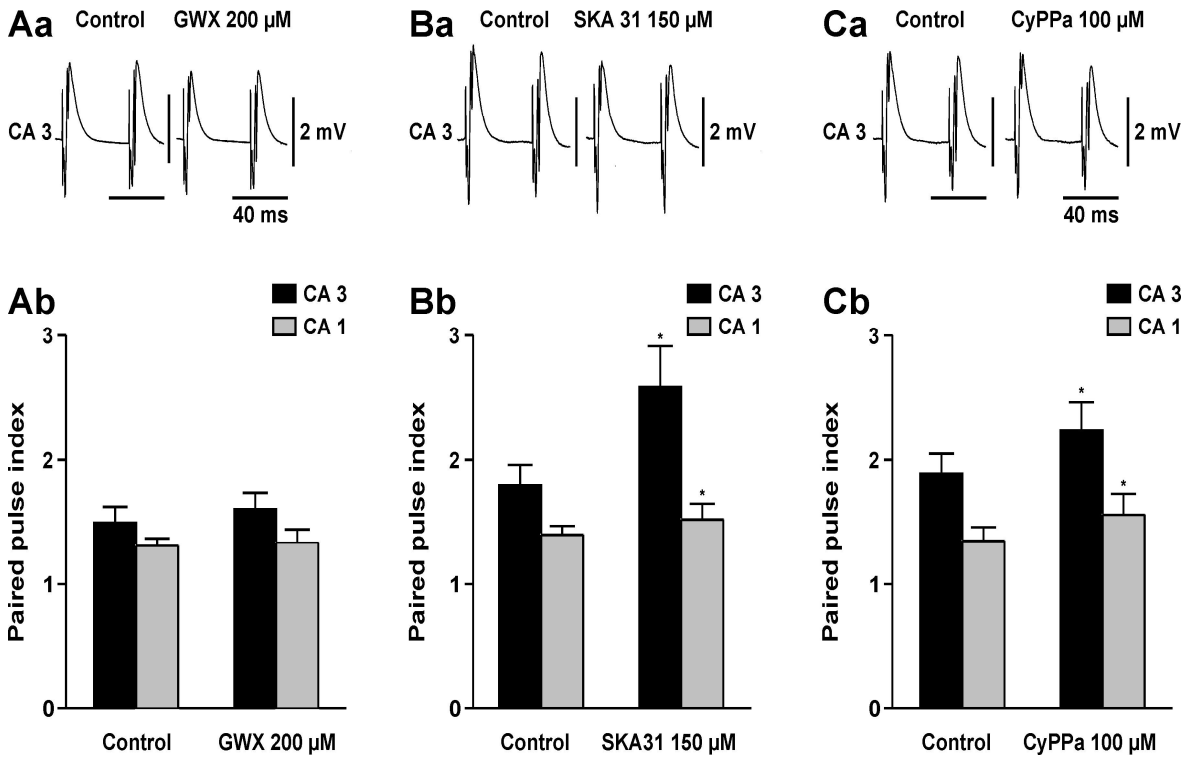


***Figure 11**: Effect of SK-channel agonists on stimulation induced evoked potentials in acute slices. Aa, Ba and Ca showing original trace recording from CA3 region and effects of GW 543574X (200 µM), SKa-31 (150 µM) and CyPPa (100 µM) respectively. Ba, Bb and Bc demonstrating comparative paired pulse indices of CA1 and CA3 regions from control values of following SK-channel agonists GW 543574X (200 µM; n=6), SKa-31 (150 µM; n=7; *p < 0.05) and CyPPa (100 µM; n=7; *p < 0.05) respectively.*

Discussion:

In my Ph.D thesis I have addressed the role of SK-channel agonists in different in vitro epilepsy models. The main purpose of choosing epilepsy was due to current medications showing pharmacoresistance in one third of all patients. Moreover, side effects related to AEDs restrict a patient from performing many daily life activities such as operating cranes, or to engage in sensitive operations that need normal cognition etc. Patients with generalized seizures are very much prone to injuries due to sudden collapsing during seizures. There are many head injuries reported in children also due to collapsing on hard surfaces. In many epilepsies which have associated convulsions, neurons fire at much higher rate than in normal animals. Therefore targeting a drug that can reduce neuronal firing would be of interest. Such a drug that controls neuronal excitation or firing rate and possesses anticonvulsant activity in a pharmacoresistant model would be a potential drug candidate. In principle SK-channels are involved in regulation of neuronal excitability by contributing to medium after-hyperpolarisations following an action potential and by contributing to afterhyperpolarisations following a train of action potentials (Leuranguer et. al. 2008, Pedarzani et. al. 2005; Pedarzani et. al. 2001; Storm 1990). Activation of these channels showed anticonvulsant activity in animal modesl of epilepsy. Different SK-channel activators were studied before: namely 1-EBIO, Des-EBIO and NS-309 in various models of epilepsy such as PTZ, MES test, pilocarpine treated chronic epilepsy rat models. All these SK-channel agonists were found effective in blocking epileptiform activities (Oliveira et. al. 2010; Kobayashi et. al. 2008, Anderson et. al. 2006; Garduno et. al. 2005; Lappin et. al. 2005). According to literature search, no data was found on the effect of SK-channel agonists in kindling model of epileptogenesis. Similarly, so far no data was reported to test SK-channel agonists on human tissue from pharmacoresistant patients. These were the strong supporting clues for me to test the anticonvulsant potential of SK-channel agonists with regard to model of pharmacoresistance and especially on human tissue specimen from patients who are clinically non-responders to AEDs.

In my study I have used neuronal tissue from different regions and also at different postnatal age. This is important since expression of SK-channels is dependent on age. SK1 and SK2 are highly expressed in young neurons. In the hippocampus SK2 and SK3 channel expression is early restricted to the ER and only after P15 expression in

spines and dendrites is noted (Ballesteros-Merino et. al. 2012). Interestingly Ca^{2+} sources for activation of these channels may differ (Wang et. al. 2014). Expression of SK-channels may also differ with respect to pathology.

On the other hand, due to wide expression of these channels throughout, they might show serious side effects. They play an important role in blood pressure regulation therefore the KCNQ1 channel, which is expressed in the brain as well as in the heart may contribute to prolong QT interval resulting SUDEP (Goldman et. al. 2009). Further activation of SK-channel might result in disturbed atrial contractility in the heart as SK currents are involved in regulation of atrial contractility. Increase in SK-channel current may cause antiarrythmic or proarrythmic effects (Chang & Chen 2015; Diness et. al. 2015; Gui et. al. 2013). During pregnancy both SK2 and SK3 channels are highly expressed. They tend to take part in myometrium contractility to facilitate child birth under estrogen and also to regulate blood flow in the uterus during the pregnancy period. They are expressed in endothelial cell as well as smooth muscle of uterus (Ronghui et. al. 2013; Veerareddy et. al. 2002; Xiao et. al. 2001).

In addition, involvement of SK-channel in cognitive decline should not be overlooked; studies on knockout mice revealed that absence of SK-channel deficient performed poor then wild type in cognitive models (Jacobsen et. al. 2009). Blockers of SK-channel are found useful in reversing decline in cognitive function (Chen et. al. 2014). It is therefore important to explore the negative impact of SK-channel activator on network oscillations. If the network oscillations that have different frequencies and present in various physiological state such as during sleep, state of alertness or awakening remain under normal frequency range. These oscillations are involved in various cognitive and behavioral function of the body (Buzsáki 2006). Gamma oscillations that range from 20-80 Hz are found in different brain regions including the hippocampus (Murthy et. al. 1992). Besides contributing to various functions it also involved in the regulation of synchronized action potential (Buzsáki 2006). Therefore, I have tested the most effective SK-channel agonist found in my experimental project, i.e. in kainite induced gamma oscillations. CyPPa at 20 μM suppressed gamma oscillation (data not shown). Gamma band frequency arises when there is excitation or tonic like activity (Traub et. al. 1996)

We know that SK-channels are expressed in human as well as in rat cortex with regional moderate down regulation of SK2 channels in models of chronic epilepsy (Oliveira et. al. 2010). During initial 10 days of post status-epilepticus, reduction in expression of both SK2 and SK3 channels were observed. However, SK1 channel was not altered during initial phase after status epilepticus (Oliveira et. al. 2010). Data from epilepsy prone rats indicated that SK1 and SK3 expression is greatly reduced in these animals (N'Gouemo et. al. 2009). In the pilocarpine model of chronic temporal lobe epilepsy down regulation of SK2 and SK3 channels persisted into the chronic phase (Oliveira et. al. 2010, Schulz et. al. 2012). Down regulation of Ca^{2+} dependent after hyperpolarisation in part due to expression changes in SK-channels was also noted in the kindling model of epilepsy (Behr et. al. 2000; Gloveli et. al. 2003). Apamin sensitive K^+ dependent K conductances were also noted in granule cells obtained from human epileptic tissue (Beck et. al. 1997). Agonists of SK 3 agonists may serve as an effective target in various indications such as substance that usually results in enhanced signalling. SK3 thereby interferes with NMDA ionophore and blocks synaptic currents and overcomes effects of abuse.

Among the three agonists used in the study, CyPPa was found to be most effective in blocking the SLEs induced by 4-AP. As found, both CyPPa and SKa-31, effectively blocked SLEs in all acute slices at 100 & 150 µM, whereas, GW-542573X was not found to be that effective in blocking SLEs, rather it prolonged interval in between SLEs. Similarly, at lower dose range, CyPPa and SKa-31 demonstrated significant effects on reduction in duration and prolongation in interval of SLEs.

Like acute slices both CyPPa and SKa-31 at upper dose range suppressed 4-AP induced SLEs in slices from in chronic epileptic rat and sham operated animals. In contrast, the result on acute slices where activity of GW-542573X was not showing promising results in regard to blockade of SLEs. GW-542573X exhibited better SLEs suppression in pilocarpine treated rat slices. However in sham operated animal effects were similar to acute slices from young animals. All three SK-channel agonists significantly reduced duration of SLEs. They also increased the interval in between SLEs. These findings indicate that in the case of the chronic model of epilepsy that all three SK-channel agonists affecting i.e. SK1, SK2 and SK3 channels possessed anticonvulsant potential in blocking 4-AP induced SLEs.

I have used 4-aminopyridine in a concentration of 100 µM which mostly affect Kv 1.4 and Kv 1.5 as well as Kv 3 channels. These channels are mostly expressed presynaptically thereby controlling Ca^{2+} influx and transmitter release. This treatment induced reliable SLEs in the entorhinal cortex of rats and in juvenile tissue studied in organotypic slice cultures. In tissue from epileptic rats the capability to induce SLEs is often reduced presumably due to activity dependent editing of Kv 1 channel (Streit et. al. 2011). There was decrease in expression Kv 7.2 and Kv 7.3 in pilocarpine epileptic rats which are targeted by retigabine (a Kv 7 opener) (Maslarova et. al. 2013). Also in the human neocortex, the capability to induce SLEs is variable as it seems to fail in temporal neocortex of patients with mesial temporal lobe epilepsy. However, it works in patient material with developmental alterations (Avoli et. al. 1991). I therefore used another protocol to induce SLEs in temporal neocortex by combining the elevation of potassium concentration with the application of bicuculline methiodide (50 µM). Elevation of potassium concentration leads to depolarization of neurons and glia, reduction of voltage dependent K^+ currents, changes in Cl^- equilibrium potential due to interference with Cl^- transport and also to enhanced transmitter release. Such treatment in hippocampus is sufficient to induce SLEs but not in the temporal neocortex. We therefore employed additional block of GABA currents by bicuculline.

Slice cultures provide a platform to study various diseases in vitro including epilepsy. Organotypic slice cultures can express spontaneous SLEs but also respond to 4-AP. The frequency of SLEs was not found as much in preparation without DG compared to slice culture with intact DG that showed high number of SLEs (Gloveli et. al. 1995). The possible interpretation could be due to pathways of hippocampus that connects DG to CA1 or DG to CA3 and then in turn to CA1 (Gloveli et. al. 1995). CyPPa was superior over SKa-31 in blocking SLEs in both types of slice culture used in this project. The effect of CyPPa in pharmacosensitive OHSCs was comparable to acute slice and pilocarpine treated rats' slice. In pharmacoresistant OHSCs, CyPPa blocked SLEs in half of studied culture. SKa-31 blocked SLEs in 50% of pharmacosensitive OHSCs but it was found less effective in pharmacoresistant OHSCs. In addition, both compounds significantly altered other parameters i.e. duration and interval in between SLEs. Dependent on preparation the induced SLEs are either pharmacoresistant or pharmacosensitive responding to clinically relevant concentrations of CBZ and other

AEDs. It is important to know the thickness of the slice preparation as it plays an important role during the recording of the activity, however age of the animal is another factor that may cause variation in results, for instance the frequency of SLEs was found higher in younger animals as opposed in adult animals (Gloveli et. al. 1995).

In the other preparations the induced SLEs vary in pharmacological sensitivity. In rats' entorhinal cortex the 4-AP induced SLEs responds well to the most clinically employed standard AEDs including carbamazepine (Brückner & Heinemann 2000). By contrast SLEs induced in human tissue are usually not blocked by clinically employed anticonvulsants if the patient is pharmacoresistant. In my study irrespective of the tissue and seizure model I found that the induced SLEs could be blocked by agonists of SK-channel. Out of three agonists I have used in this study SK 2 and SK 3 agonists i.e. SKa-31 and CyPPa were superior to the SK1 channel agonist. This applies particularly to human tissues and slices from pilocarpine treated epileptic rats and to some extent also to SLEs in slice cultures. The employed drug concentrations in this study were rather high. However, it should be considered that diffusional equilibration in slices under interface conditions is usually a slow process. Diffusion of the applied drug may take more than an hour depending on molecular size, distribution between aqueous and lipid phases, uptake and metabolism. Usually such long application times are avoided. It is therefore of interest that relatively, low concentrations of SK-channel agonists were useful in pilocarpine treated chronic epileptic rats and in slice cultures particularly.

There are many AEDs that were found ineffective in zero magnesium model. Interestingly, CyPPa was effective in this model. It blocked SLEs not only in acute slices preparation but also OHSCs even at a low dose of 20 µM. This effect of CyPPa in zero magnesium model adds another edge to its anticonvulsant potential in pharmacoresistant types of epilepsies.

To segregate amongst presynaptic and postsynaptic effects in synaptic transmission, pair-pulse protocol is adapted (Zucker & Regehr, 2002). To see the behavior of SK-channel agonists on normal neuronal function, evoked potentials were studied in the hippocampus. It provides useful information about readily releasable pool (RRP) of

neurotransmitter, higher release decreases the pool ratio (Betz 1970). Paired-pulse was given in CA1 schaffer collateral via stimulation electrode. Paired pulse stimulation protocol can be used to study both excitatory as well as inhibitory synapse (Kraushaar and Jonas, 2000; Zucker, 1989). Recording electrodes were placed in CA1 and CA3. It is calculated by dividing amplitude of second pulse with first.

Analysis of SK-channel agonists on evoked potential showed that CyPPa and SKa-31 increased paired-pulse index in CA3, while no change was present in paired-pulse index of CA1 region. However, SK1 agonist i.e. GW-542573X did not cause any significant alteration in paired-pulse index of both CA1 and CA3. It is known that a decrease in pair paired facilitation is proof for presynaptic component. It may be due to the likelihood of the increased chance of transmitter release. However increment in transmitter quantum, presumably because of an expanded number of postsynaptic receptors (Kleschevnikov et. al. 1997). Contrary to this a study reported that PPR has no effect on release probability particularly in schaffer collateral (Manita et. al. 2007). Increase of amplitudes by CyPPa and SKa-31 might indicate the role of SK-channels in controlling excitability in hippocampal neurons. Increase in excitability was observed due to loss of SK-channels as found in epileptic states (Oliveira et. al. 2010).

The most interesting finding in my study is that SLEs induced in human tissue from patients with pharmacoresistant seizures can be blocked. CyPPa in both protocols effectively blocked SLEs i.e. induced by 4-AP and high K^+ combined with bicuculline. While GW-542573X failed to block 4-AP induced SLEs, but was effective in suppression SLEs induced by high K^+ combined with bicuculline. Our lab previously reported that carbamazepine was found ineffective in blocking SLEs in human tissue (Jandova et. al. 2006). This finding is of importance as it shows pharmacological efficacy in human tissue proving that human tissue can be employed for detection of beneficial effects for seizure control even before the toxicology studies are done which are required before an agent can be tested in phase 1 and 2 clinical studies.

I have shown that CyPPa and SKa31 are particularly effective in controlling SLEs events in almost all used preparations and epilepsy models. In some models SKa-31 was also similar in results with that of CyPPa. But CyPPa remained in a leading

position compared to two SK-channel agonists used in this study. It suggests that SK2 and SK3 channels are a promising target for seizure control.

Conclusively, the findings suggest that activation of SK-channels is potentially a way by which seizure control may be achieved even in the condition of pharmacoresistance. There is need before translating SK-channel agonist in clinical trails to investigate side effects related to SK-channel agonist. Transgenic animals would be a better choice as a vital paradigm that can be utilized to investigate this question.

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Affidavit

I, Muhammad Liaquat, Raza certify under penalty of perjury by my own signature that I have submitted the thesis on the topic ‘Effects of small conductance calcium activated potassium channel agonists on seizure like events in *in vitro* slices and slice cultures' I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of any eventual publications

Muhammad Liaquat Raza had the following share in the following publications:

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

Curriculum vitae

Not disclose due to privacy

List of publications

- **Mishto M, *Raza ML*, DeBiase D, Vasuri F, Martucci M, Keller C, Bellavista E, Buchholz TJ, Kloetzel PM, Vezzani A, Pession A, Heinemann U. (2015) Immunoproteasome β5i subunit is a key contributor to ictogenesis in a rat model of chronic epilepsy. Brain, Behaviour & Immunity, 49:188-96.**

- ***Raza ML*. (2011) Alternative Treatment Approaches in Epilepsy: Mini-review. Clinical, Experimental & Medical Journal, 4:189-197.**

- ***Raza ML*, Zeeshan M, Ahmad M, Shaheen F, Simjee SU. (2010) Anticonvulsant activity of DNS II fraction in the acute seizure models. *J Ethnopharmacol* 128(3):600-605.**

- ***Raza ML*, Zeeshan M, Ahmad M, Shaheen F, Simjee SU. (2007) Actions of DNS II in epilepsy models. Neuron Glia Biology. 3:S33-S79.**

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