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## New Tools for Understanding Epilepsy

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### 1. Introduction

Epilepsy is one of the most common serious chronic neurological disorders, which is characterized by recurrent seizures with a prevalence rate of 3-6 per 1000 worldwide (Begley et al., 2007). Epilepsy is not a form of mental illness and is not an indicator of intelligence. Epilepsy affects people of any origin or race with high and low intelligence. Some people with mental retardation may have epilepsy, but most people with epilepsy are not mentally retarded (Avoli M et.al, 2001). There are many types of epilepsies and many factors such as neurotransmitters, transporters, granule cells, voltage gated ionic currents and non-neural proliferation may act as major players for this disorder (Berkovic S.F., 1998; Zaid Afawi et al, 2010). According to the literature and our work, both ligand and voltage gated ion channels in neurons and platelets seem to work together during the onset of epilepsy (Fatima Shad K (un published data); Kocsis, J.D. and Mattson, R.H., 1996). We also found similarities in the receptor's profiles of neurons and platelets mainly for serotonin, GABA and glutamate (Fatima Shad K, 2006; Fatima Shad K and Saeed SA, 2009, 2007). Recently, there has been growing evidence that serotonergic neurotransmission modulates experimentally induced seizures and is involved in the enhanced seizure susceptibility observed in some genetically epilepsy-prone animals (Methvin Isaac, 2005). Scientists are now focusing on targets other than brain receptors for the development of anti-epileptic drugs. The brain is an extraordinarily complex organ; there are several concepts about the brain which needed to be cleared when it comes to the understanding of epilepsy (Berkovic S.F., 1998). Firstly, the brain works on electricity and continuously generates tiny electrical impulses in an orderly pattern. Secondly, loss of nerve cells may contribute to the development of epilepsy in some cases. For example, prolonged lack of oxygen may cause a selective loss of cells in the hippocampus, which may lead to epilepsy. According to one theory, epilepsy is caused by an imbalance between excitatory and inhibitory neurotransmitters. If the inhibitory neurotransmitters in the brain are not active enough, or if the excitatory ones are too active, then it's more likely to have seizures. Many of the new medicines being developed to treat epilepsy try to influence these neurotransmitters, by increasing inhibitory and reducing excitatory neurotransmitter's activities. Either way, the idea is to have less uncontrolled electrical activity in the brain, and therefore fewer seizures

(Steinlein, O. K. & Noebels, J. L., 2000). Another concept important in relation with epilepsy is that different areas of the brain control different functions. If seizures arise from a specific area of the brain, then the initial symptoms of the seizure often reflect the functions of that area. The right half of the brain controls the left side of the body, and the left half of the brain controls the right side of the body. So if a seizure starts from the right side of the brain, in the area that controls movement in the thumb, then the attack may begin with jerking of the left thumb or hand. Thus a seizure occurs when the brain's nerve cells misfire and generate a sudden, uncontrolled surge of electrical activity in the brain more precisely in the cerebral cortex. There are three to six layers of neurons found to be present in the cerebral cortex with only three distinct layers; present in the hippocampus, which is located in the medial temporal lobe. The majority of the cortex has 6 distinct cell layers and covers most of the surface of the cerebral hemispheres, the hippocampus which consists of three major regions: subiculum, hippocampus proper (Ammon's horn) and dentate gyrus has three layers in the later two regions and a transitional three to six layers in the subiculum. Given the fact that the basic mechanism of neuronal excitability is the action potential, a hyperexcitable state can result from increased excitatory synaptic neurotransmission, decreased inhibitory neurotransmission, an alteration in voltage-gated ion channels, or an alteration of intra- or extra-cellular ion concentrations in favor of membrane depolarization. A hyperexcitable state can also result when several synchronous subthreshold excitatory stimuli occur, allowing their temporal summation in the post synaptic neurons (Avoli M et.al, 2001).

Action potentials occur due to depolarization of the neuronal membrane, with membrane depolarization propagating down the axon to induce neurotransmitter release at the axon terminal. The action potential occurs in an all-or-none fashion as a result of local changes in membrane potential brought about by net positive inward ion fluxes.

As mentioned earlier hippocampus is involved in epilepsy and a special group of cells known as granule cells (GCs) are the focus of the epilepsy research. These cells are present in dentate gyrus region of hippocampus and are primary gateway of cortical inputs to CA3 region of hippocampus (Crawford I L 1973). GCs act as filters and protect temporal lobe seizures by upregulation of inward rectifier K<sup>+</sup> channels and increase leak conductance in the granule cells of dentate gyrus (Young C et.al, 2009 and Stegan M et al 2009). Repetitive firing of action potential of inter neurons exhibits diverse repetitive firing behaviour depending upon their harboring levels in the granular layer of dentate gyrus (David M.D., et al., 1997). In epileptic patients, most of granule cells display hyperexcitable, non frequency adapted cells (Dietrich D et al. 1990) which are highly susceptible for the generation of temporal lobe epilepsy. Non adapted bursting cells are characterized by their ability to generate action potential without reducing firing frequency and less after-hyperpolarization (AHP). This type of burst firing have also been recorded in inferior colliculus neurons (Tan M L et al, 2007), CA3 pyramidal neurons (Peter H et al 2008) and in spinal cord neurons (Smith M and Perrier J, 2006). The non-adapted bursting firing may depend upon calcium influx (Anthony A G and Benjamin 1984) or due to the presence of persistent sodium currents (Yunru L et al, 2004). In regular frequency adapted neurons, intracellular Ca<sup>+2</sup> increases (Knopfel T and Gahwiler B H, 1992) which activates the calcium gated potassium channels (Kca). These channels gradually decrease the positivity inside the cell and reduce the firing frequency of action potential in the later stages of Sustained Repetitive firing SRF. Buster cells (type of GCs) have low Kca channel density (Podloger M and Dirk D, 2006) due to which they exhibits the continuously same or shorter spike

duration throughout the pulse. Sodium and potassium channels are the main contributors of action potential and underlying cause of repetitive firing pattern (Joshua B C, et al 2000). In buster GCs of dentate gyrus, amplitude and firing frequency of action potential increases in response to number of pulses.  $\text{Na}^+$  and  $\text{K}^+$  voltage-gated channels change their voltage dependency for activation and inactivation in response to sustained repetitive firing (SRF). Inactivation of sodium channels is significantly modulated by growth factor (Chuan-ju L et al, 2003) and chronic compression (Zhi-Jiang H and Xu-Jun S, 2008). Similarly the potassium channel activation and inactivation are modulated by cAMP (Jian M et al, 1999), potassium channel-interacting proteins KChIPs (Frank A W et al, 2000) growth factor and tyrosine kinases (Mark B, 1997). Literature indicated that sustained repetitive firing can be good electrical tool to find out about the underlying cause of epilepsy. The present study was therefore undertaken to establish the role of underlying currents in SRF as to establish the importance of invitro models in understanding the mechanism of epilepsy. This is our recent unpublished data, exhibiting the interesting findings of the effect of repetitive stimulation on neuronal excitability and on the kinetics of voltage dependent sodium and potassium channels. We used postnatally cultured granule cells from dentate gyrus region of hippocampus from 1-5 day old rat pups to study the role of SRF in regulating voltage gated  $\text{Na}^+$  and  $\text{K}^+$  currents. These voltage gated monovalent cationic currents were recorded by patch clamping "control" and SRF-induced "epileptic cells" in whole cell configuration. We then measured the changes in the activation and inactivation of both channels before and after SRF in granule cells of dentate gyrus and observe a novel role of "window currents". Overlapping of channel activation and inactivation is referred as window current; a sustained current which is observed if a fraction of channel opens ( $\text{Popen} > 0$ ) when the probability for their inactivation is not zero. Here we report that the repetitive depolarizing stimuli alters the kinetics and gating of  $\text{Na}^+$  and  $\text{K}^+$  channels and bring the granule cell membrane to more hyper polarized potential. These electrical changes in the membrane of cultured granule cells may partially responsible for the better survival of granule cells in excitotoxic conditions. This data may put some light on the underlying homeostatic mechanism in epilepsy mainly in temporal lobe epilepsy.

## 2. Material and methods

### 2.1 Chemicals

All chemicals were purchased from Sigma (USA) and salts from Applichem (Denmark), except D-AP5, TTX, and CNQX disodium salt from Ascent Scientific (Weston-Super-Mare, United Kingdom) and KCl from Merck (Darmstadt, Germany).

### 2.2 Hippocampal neuronal cell culture

Animals were sacrificed for culturing post natal neurons in accordance with the guide lines of the animal ethic committee at the Panjwani Center for Molecular Medicine and Drug Research (PCMD), University of Karachi, Pakistan after getting animal ethic approval. Hippocampal dentate gyrus cells were cultured as described previously (Fatima Shad and Barry PH, 1998). Briefly, dentate gyri from both hemispheres were isolated under dissecting microscope from 1-5 days old specific pathogen free (SPF) Wister rats and plated at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> onto three 22 mm rounded cover slips previously coated with poly-L-lysine (0.05 mg/ml), and were placed in a 100 mm cell culture dishes. Cultures were

maintained at 37°C in a humidified 5% CO<sub>2</sub> incubator and were fed twice a week with DMEM (Dulbecco's Modified Eagle's Medium) plus containing 85ml. DMEM, 1ml. L glutamine, 1ml. Fungizone, 2ml. Streptomycin/Penicillin, 1ml. sodium pyruvate and 10ml. Fetal Bovine Serum. The neuronal cultures were used from the fourth day for the patch clamp experiments.

### 2.3 Patch clamp recordings

One of the author (FSK) is using patch clamp technique for last more than twenty years, and the present method was a modified version of one of her earlier papers (Fatima Shad K and Barry PH, 1992). Briefly, one polylysinated 22 mm round cover slip with cultured cells grown on it was fitted in the bath chamber (1 ml. capacity) and was mounted on the stage of an inverted microscope (Nikon TE2000-U, Tokyo, Japan). Cells were perfused continuously with Artificial Cerebrospinal Fluid (ACSF) containing (in mM): 126 NaCl, 3.5 KCl, 25 NaHCO<sub>3</sub>, 1.2, NaH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 11 glucose, and pH (7.3), and osmolality was adjusted to 325 ± 5 mOsm with sucrose. Patch pipettes were pulled from borosilicate glass capillaries using a Brown-Flaming P-97 electrode puller (Sutter Instruments, USA) with a diameter 2-3 µm. After fire polishing using micro forge, pipettes were filled with a solution containing (in mM): 145 KCl, 10 NaCl, 10 EGTA, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub> and 10 HEPES, (pH 7.2) and had a tip resistance of 4 to 5 MΩ. Osmolality was adjusted to 300±10 mOsm with sucrose. Patch clamp recordings were carried out using HEKA EPC 10 dual pre amplifiers (HEKA Instruments, Inc., New York.) placed on coarse adjustment plates of automated micromanipulators (Scientifica) at either side of the microscope. Data was recorded and analysed using PatchMaster, and temperature was maintained at 25±0.5 by TC-20 temperature controller (ALA Scientific Instruments). Data was filtered with values of 8 and 10 kHz and digitized at 10 and 20 kHz for current and voltage clamp respectively. Membrane capacitance (Cm) was recorded by using built in LockIn software after calculating the cell size and getting the current density. The offset potential was canceled before patching the cell and was re-checked after each recording for any drift. Leakage currents, determined by application of small hyperpolarizing commands were subtracted automatically from the response.

Most pharmacological experiments for the isolation of sodium and potassium currents were conducted in the presence of tetrodotoxin (TTX) and synaptic blockers, that is, D (-)-2-amino-5-phosphonopentanoic acid (D-AP5), 1, 2, 3, 4-tetrahydro-7-nitro-2,3-dioxoquinoxaline-6-carbonitrile disodium (CNQX), and picrotoxin (PTX). In K<sup>+</sup>-replacement experiments, cells were first recorded with normal intracellular solution and then with K<sup>+</sup>-free intracellular solution. In these cases K<sup>+</sup> was replaced with equimolar tetraethyl ammonium (TEA) in the ACSF and the pipette solution contained (in mM): 135 TEACl, 20 CsCl, 0.1 EGTA, 2 MgCl<sub>2</sub>, and 2 Na<sub>2</sub>ATP (pH 7.28 adjusted with TEA hydroxide). Chemicals such as CNQX, D-AP5, TEACl, and TTX were kept in H<sub>2</sub>O stocks at 20°C and PTX was kept in dimethylsulfoxide (DMSO) stocks at 20°C. Final concentrations were diluted freshly in oxygenated recording ACSF (DMSO 1:1,000) and subsequently kept in syringes of perfusion system (Octaflow, ALA Scientific, U.S.A.) under nitrogen pressurized at 1.3–1.6 bar before bath application.

Whole cell recording of GCs were obtained both in current and voltage clamped mode after checking their resting membrane potential (RMP). Only those cells having RMP more negative than -50 mV were used. Both inward and outward currents were observed in response to + 60 to - 60 mV pulses of 10 mV steps.

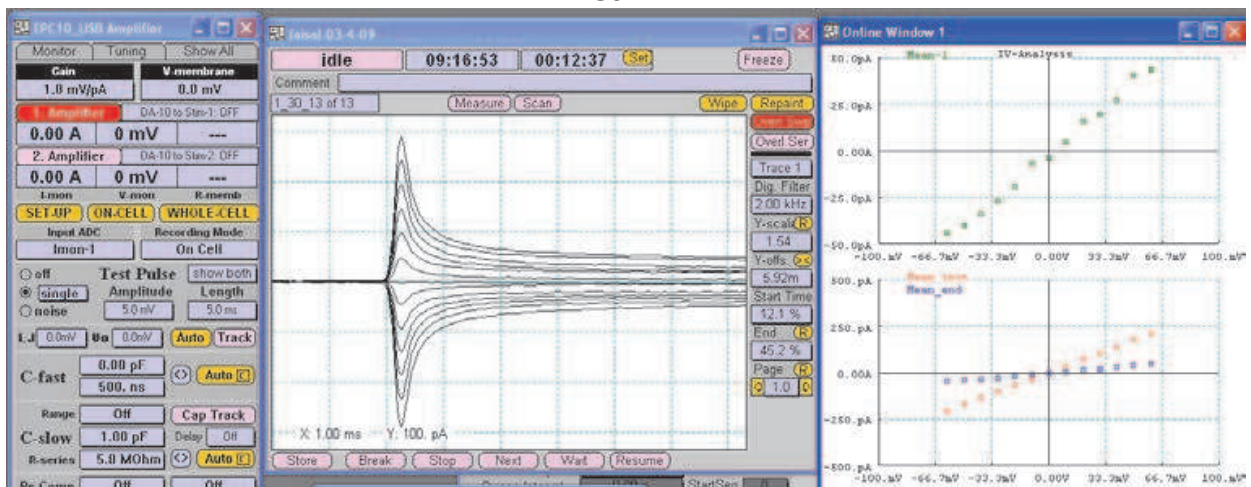


Following diagrams display some raw data to create more understanding about the process of patch clamping and how the windows of the HEKA EPC10 amplifier looks like and what are the parameters constantly regulated by the amplifier itself. Please note the gain values as well as C-fast and C-slow values (fast and slow capacitive transients) automatically adjusted by the amplifier along with liquid junction potential and series resistance.

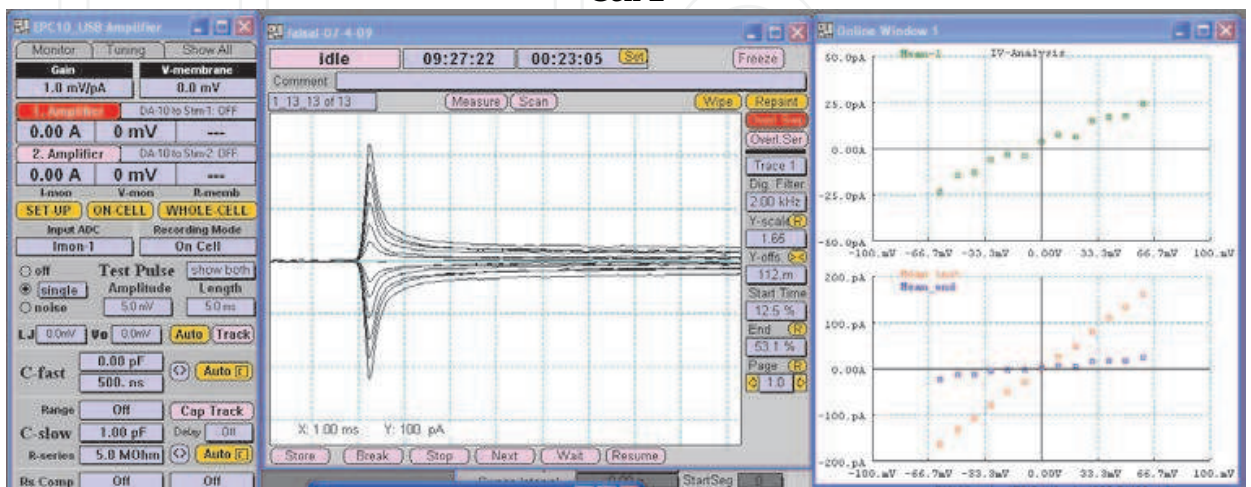
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Symmetrical Sodium solution in response to voltage steps from -60 mV to +60 mV

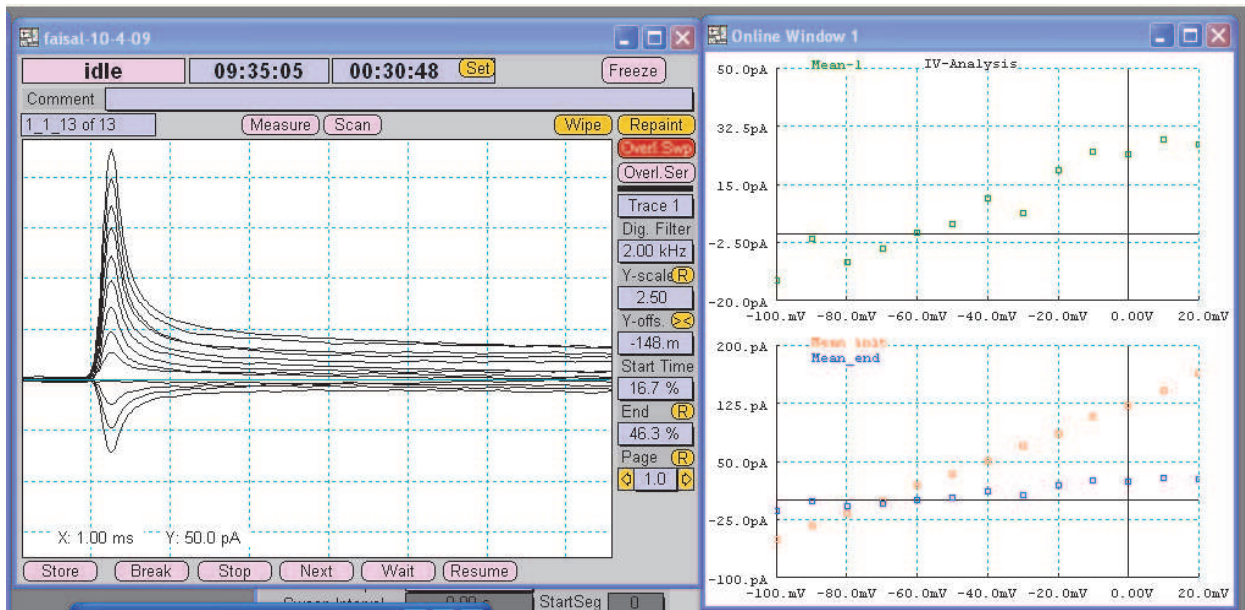
Cell 1



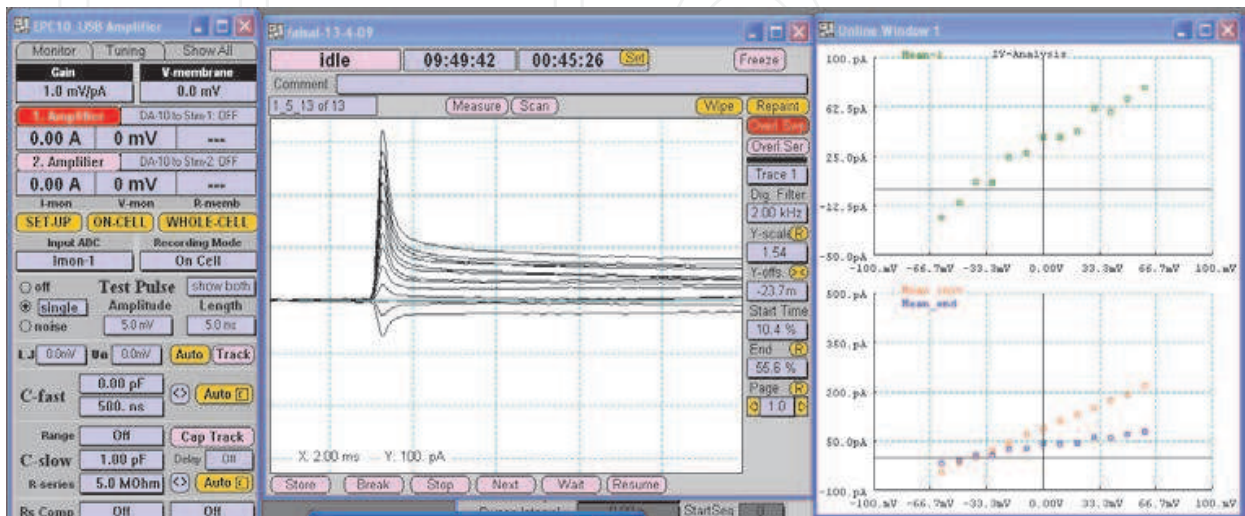
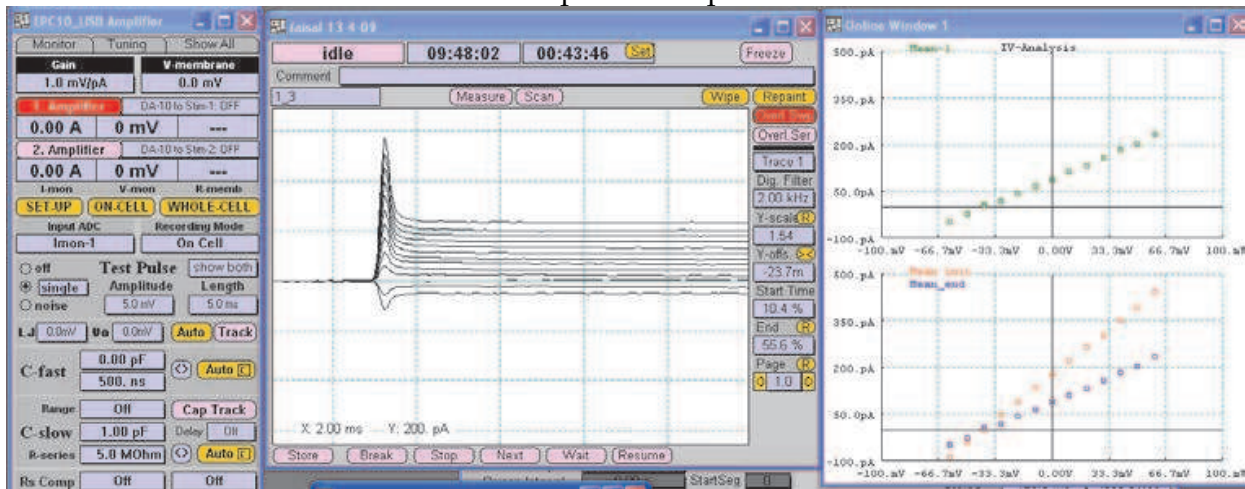
Cell 2



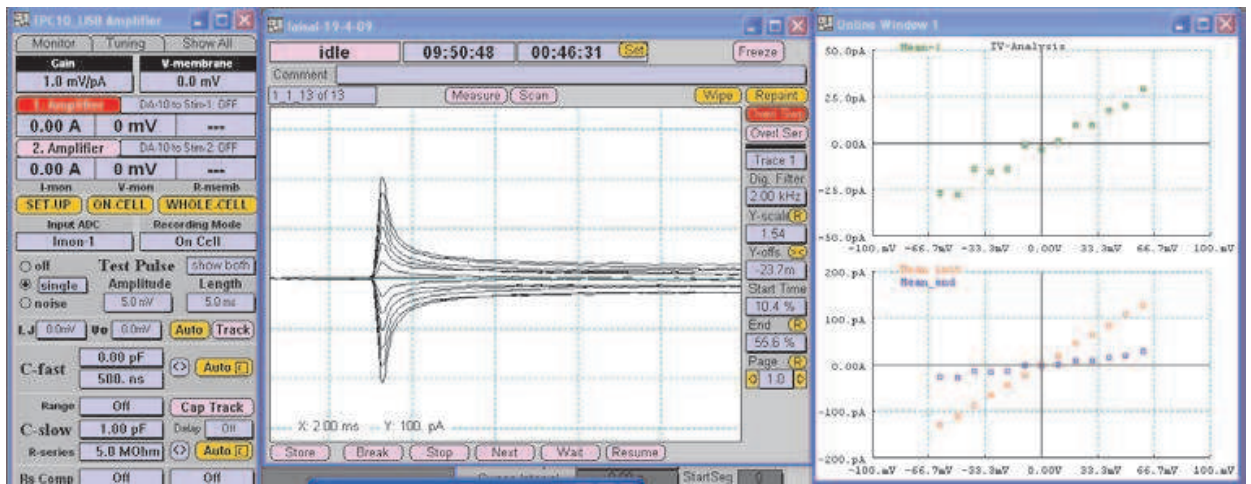
Low sodium



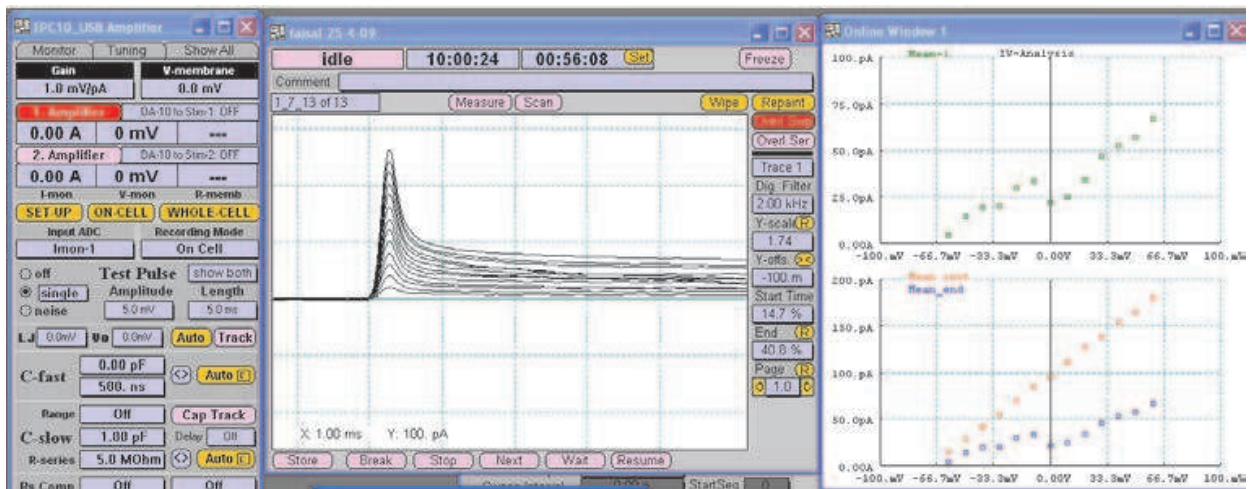
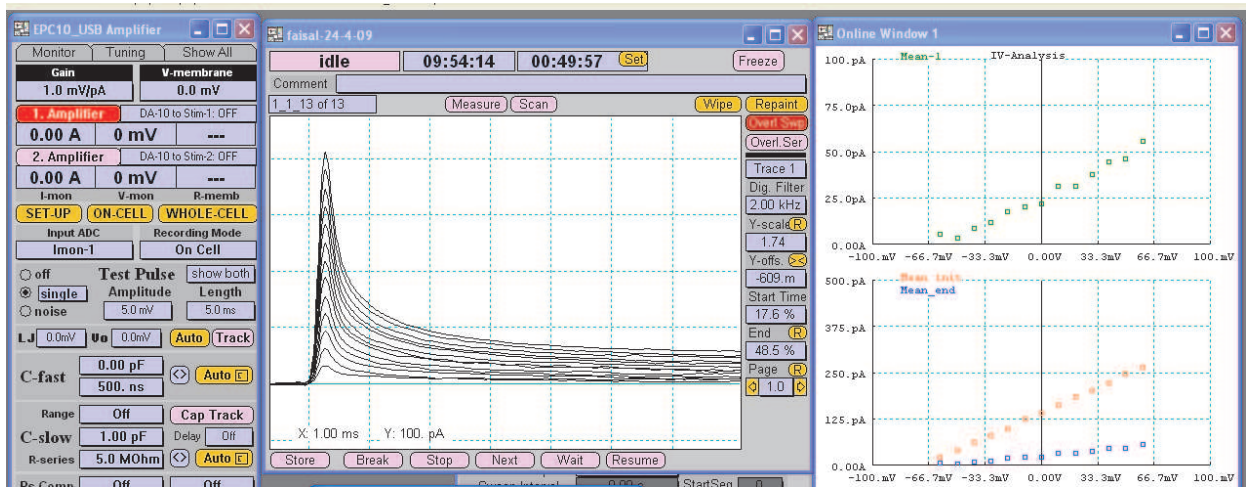
Another patch clamped cell







Absence of inward currents





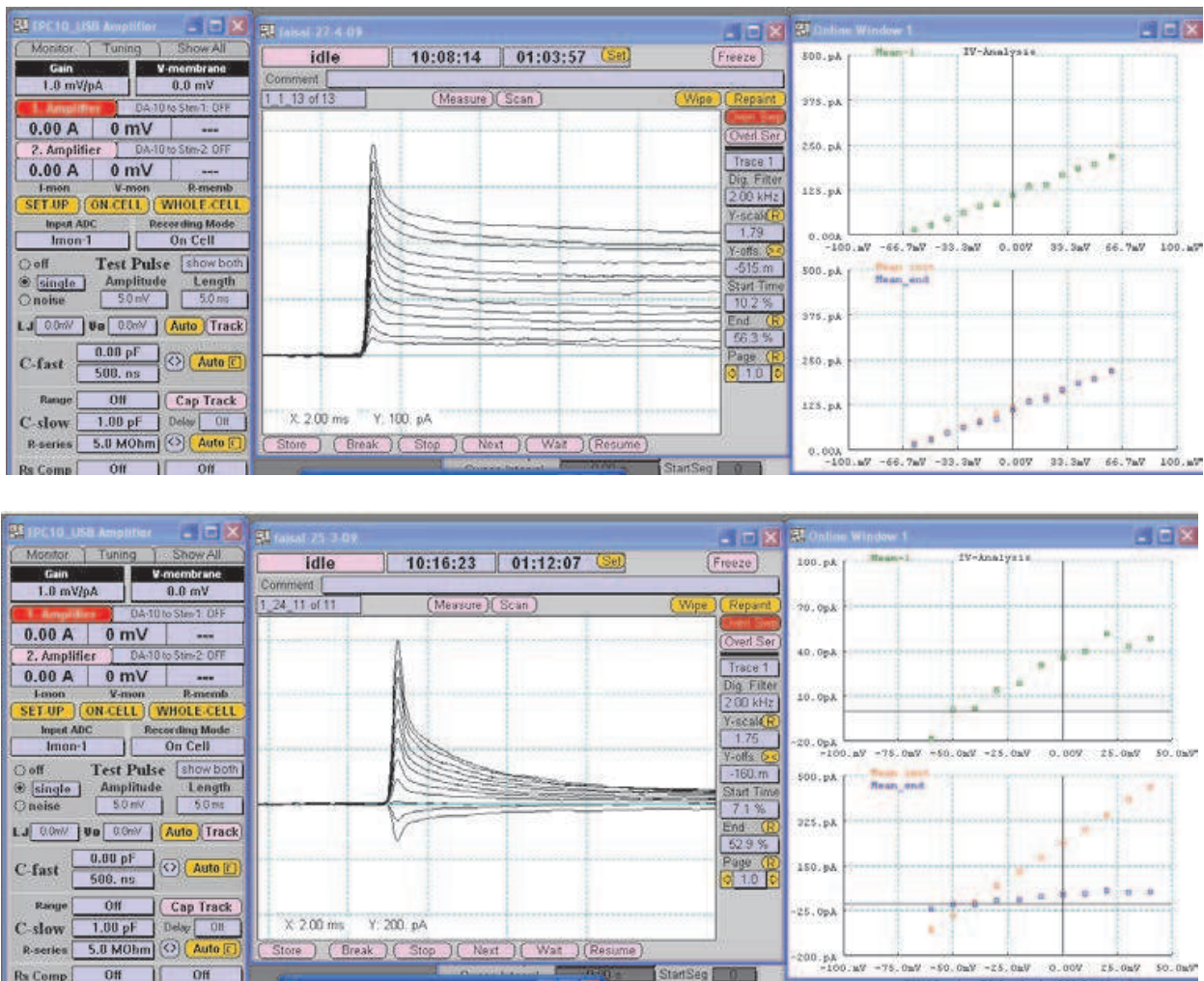
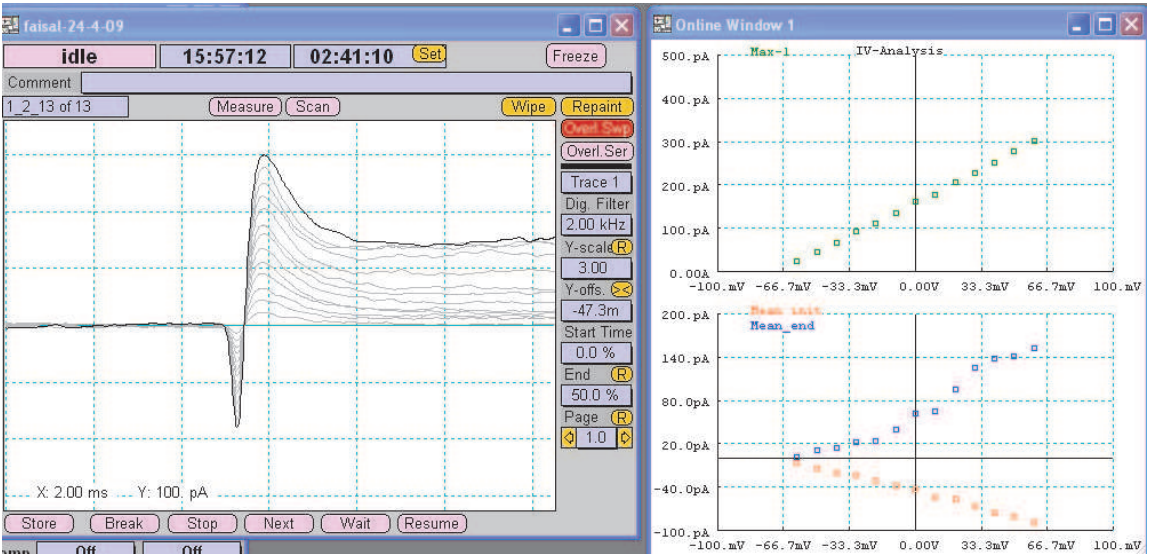


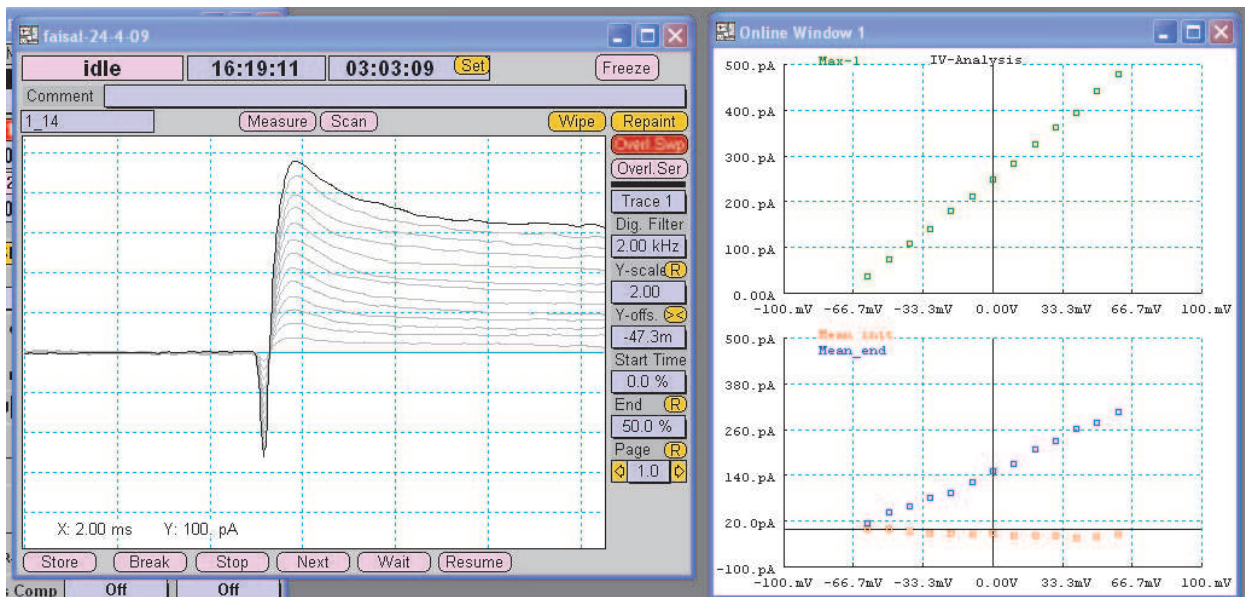
Fig. 1. In the above figures (A-J) presenting raw data (directly from the computer) simply exhibiting that how different cells behave differently and how changes in ionic concentration changes the inward and outward deflection of currents. Literature indicated a variety of approaches for in vitro modeling of epilepsy mainly temporal lobe epilepsy. One can design an epileptic model by using chemical (absence of Mg) or electrical (SRF) tool. These artificial epileptic models are capable of mimicking the silent features of this pathology and can be tested for novel therapeutic agents but with great caution during their interpretation.

We thought it will be beneficial for non expert readers to see how a cell can be patched and how the values of transient and steady state currents look like in response to voltage pulses. One can use the following values to draw a current voltage graph as well as the following equation can be used for practice to see which ion is more permeable for the non selective cation or anion channel.  $E_{rev}$  = is the voltage at which the concentration of that particular ion is equal in ECF and ICF and after this voltage the ion will change its direction,  $z$  is the valency and  $[A]_o$  are the  $[A]_i$  are the concentration out side and inside.  $P_A$  and  $P_B$  are the permeability of ion A and ion B  
Sodium currents (Number of Sodium channels, permeability of Na ions per unit change in voltage and reversal potential of the sodium or any other ion can be calculated using the following equation)

$$E_{rev} = RT/zF \ln P_A [A]_o / P_B [A]_i$$



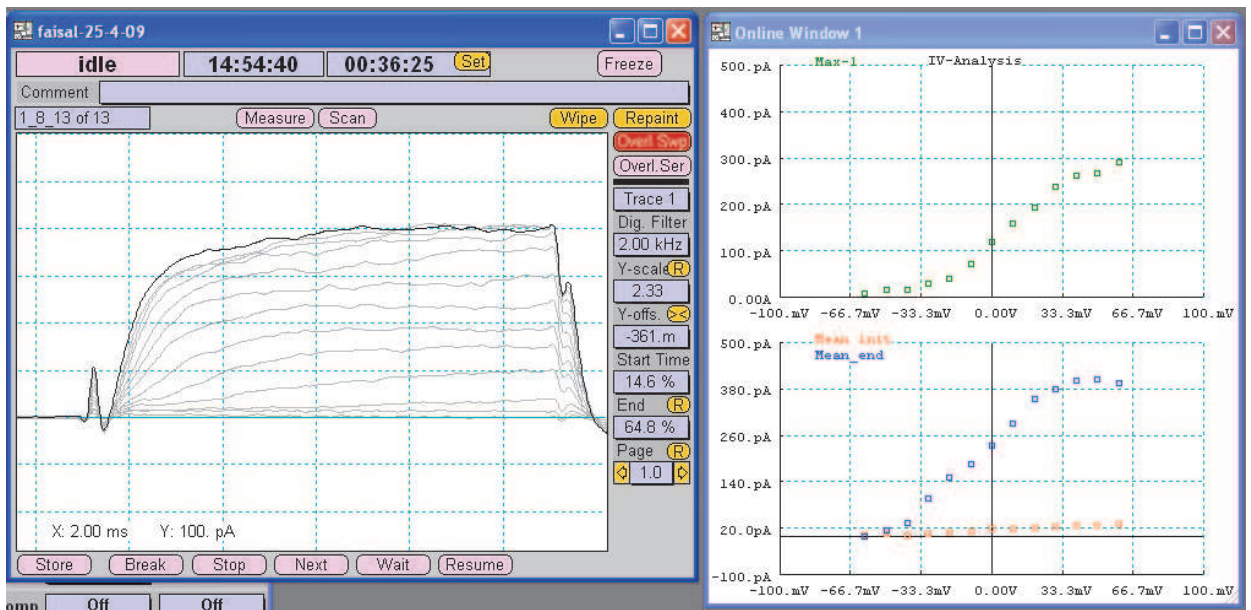
#	Holding Potential (mV)	peak (o) I (pA)	inward I (pA)	Persist (o) I (pA)	Time to peak (i) μ sec.
1	-60	22.567	-6.9942	2.0444	265.21
2	-50	45.241	-13.975	11.293	276.52
3	-40	65.281	-20.338	13.925	275.34
4	-30	91.400	-23.784	22.002	273.24
5	-20	110.64	-31.609	23.278	272.97
6	-10	135.58	-38.035	39.726	275.55
7	0	161.08	-42.521	61.990	274.19
8	10	176.76	-53.981	65.602	275.32
9	20	205.64	-57.132	95.058	278.12
10	30	227.72	-66.613	125.31	275.80
11	40	250.62	-76.349	137.64	277.57
12	50	278.18	-81.710	140.65	277.32
13	60	300.42	-88.700	152.24	276.56



#	Holding Potential (mV)	peak (o) I (pA)	Inward I (pA)	Persist (o) I (pA)	Time to peak (i) sec.
1	-60	37.013	-1.3052	15.280	500.00
2	-50	72.857	-1.6571	41.372	500.00
3	-40	107.55	-7.0724	59.973	500.00
4	-30	140.62	-11.647	80.917	500.00
5	-20	179.49	-9.6198	93.364	500.00
6	-10	211.62	-14.327	122.26	500.00
7	0	249.49	-12.186	149.24	500.00
8	10	282.44	-15.985	170.72	500.00
9	20	324.09	-17.815	207.99	500.00
10	30	363.06	-16.406	230.13	500.00
11	40	394.45	-23.742	261.85	500.00
12	50	442.30	-20.464	277.07	500.00
13	60	478.56	-13.076	306.75	500.00







#	Holding Potential (mV)	peak (o) I (pA)	inward I (pA)	Persist (o) I (pA)	Time to peak (i) sec.
1	-60	7.5033	1.7111	-84.261	249.24
2	-50	15.568	5.0703	13.233	258.98
3	-40	16.076	1.9596	34.109	253.96
4	-30	27.778	5.7529	96.613	252.26
5	-20	39.568	9.0225	149.87	253.74
6	-10	71.728	9.6337	184.22	250.65
7	0	119.43	16.273	231.95	249.77
8	10	158.85	18.046	290.64	248.82
9	20	193.04	21.480	353.00	248.69
10	30	239.25	25.096	379.17	252.02
11	40	261.33	27.338	403.03	251.91
12	50	267.82	26.792	404.47	252.70
13	60	290.25	31.005	395.27	249.12

Fig. 2. In the above table and figures bipolar cells were patch clamped and currents were measured in response to the voltage pulses. Despite of the fact that both cells used were similar and pulse protocol applied was exactly the same but the responses were quite different for both transient and sustained currents, indicating the existence of heterogeneity of the current population in each cell. This again warns us for the presence of significant differences in the same type of cells experiencing the similar electrical stimulus.

Basically these diagrams and tables are given for those who are interested in practicing for making current voltage (I/V ) curves for the understanding of the phenomenon of normal impulse as well as the mechanism of epileptic train.

We hope by now its clear that sodium and potassium are the major cations involved in the process of action potential and a high frequency series of action potentials are knows as epileptic episodes. Both Na and K gated currents go through the process of activation and inactivation. These currents when recorded before and after the application of SRF gives two

very different kinetic profiles and this information will be helpful in detecting the cell membrane status its protein channel and their integrity. To analyze the voltage dependence of channel activation, the conductance (G) was calculated by using the equation:

$$G = I / (V_m - V_{rev})$$

Where I is the peak current,  $V_m$  is the test pulse voltage and  $V_{rev}$  is the reversal potential of under study ion. Conductance was plotted against  $V_m$  and fit to a Boltzmann distribution equation:

$$G = G_{max} / (1 + \exp (V_{1/2} - V_m) / k)$$

Where  $G_{max}$  is the maximum conductance,  $V_{1/2}$  is the potential at which activation is half maximal, and k is the slope of the curve.

For the estimation of channel inactivation, the inactivation parameters were fitted to Boltzmann distribution equation:

$$I / I_{max} = 1 / (1 + \exp (V_{1/2} - V_{pre}) / k)$$

Where  $I_{max}$  is the maximum current after the most hyperpolarized prepulse, the  $V_{pre}$  is the prepulse potential,  $V_{1/2}$  is the potential at which inactivation is half-maximal.

2.4 Statistics

Significance of differences were tested by Student’s *t*-test with *P* < 0.05 considered to be significant.

3. Results

Dentate gyrus granule cells of hippocampus were plated at a concentration of 1×10<sup>5</sup> cells/cm<sup>2</sup> per plate (containing three 22 mm round cover slips). These cells were identified mostly to be granule cells (\*Fig.3B right panel). Only shiny healthy intact cells were used for patch clamp experiments. All granule cells of dentate gyrus have similar passive membrane properties (Table. 1) and are of very homogenized morphological properties. (\*Though Fig 3 was suppose to be Fig 1 of the result section)

	Pre SRF	Post SRF
Input Resistance (MΩ)	263.1±25.2	192.6±30.6
Resting Membrane Potential, RMP (mV)	-63 ±5.3	-62 ±4.7
Threshold for action potential (mV)	-49±2.6	-42±6.6
Spike Amplitude (mV)	90±10.4	72±12.2
No of Spikes in the burst	15±2	11±3
After hyperpolarization, Amplitude (mV)	1.1±0.2	1.8±0.3

Table 1. Membrane properties of granule cells of mammalian dentate gyrus

After patch clamping the cells for the measurement of their passive membrane properties and their morphological features. We have decided to use them for our in vitro model of epilepsy. For that purpose we have used an electrical tool called Sustained repetitive firing (SRF), a protocol of depolarizing pulses of 0.5 nA amplitude with 500 ms duration and of 0.3 Hz frequency as to mimic epileptic burst in the cultured granule cells. We have used HEKA amplifier pulse protocol program to generate SRF and introduced it into the clamped cell through the recording electrode. In response to SRF pulse, patched cell exhibit regular and continues series of action potentials mimicking an epileptic episode as been observed earlier (DeLorenzo RJ et al, 2000). The duration and frequency of SRF pulses were remains constant for all experiments. Those cells which were patched in whole cell configuration (with out any SRF) were considered as “control cells”. The patch clamped cultured neuronal cells were also tested to confirm that the SRF induced hyperexcitability effect is activity and not time dependent. For testing this phenomenon, the cell was patch clamped in whole cell configuration without any stimulus for a duration of approximately eight seconds (equivqlent to the time of three depolarized pulses) then the SRF pulses were injected (Fig. 3).

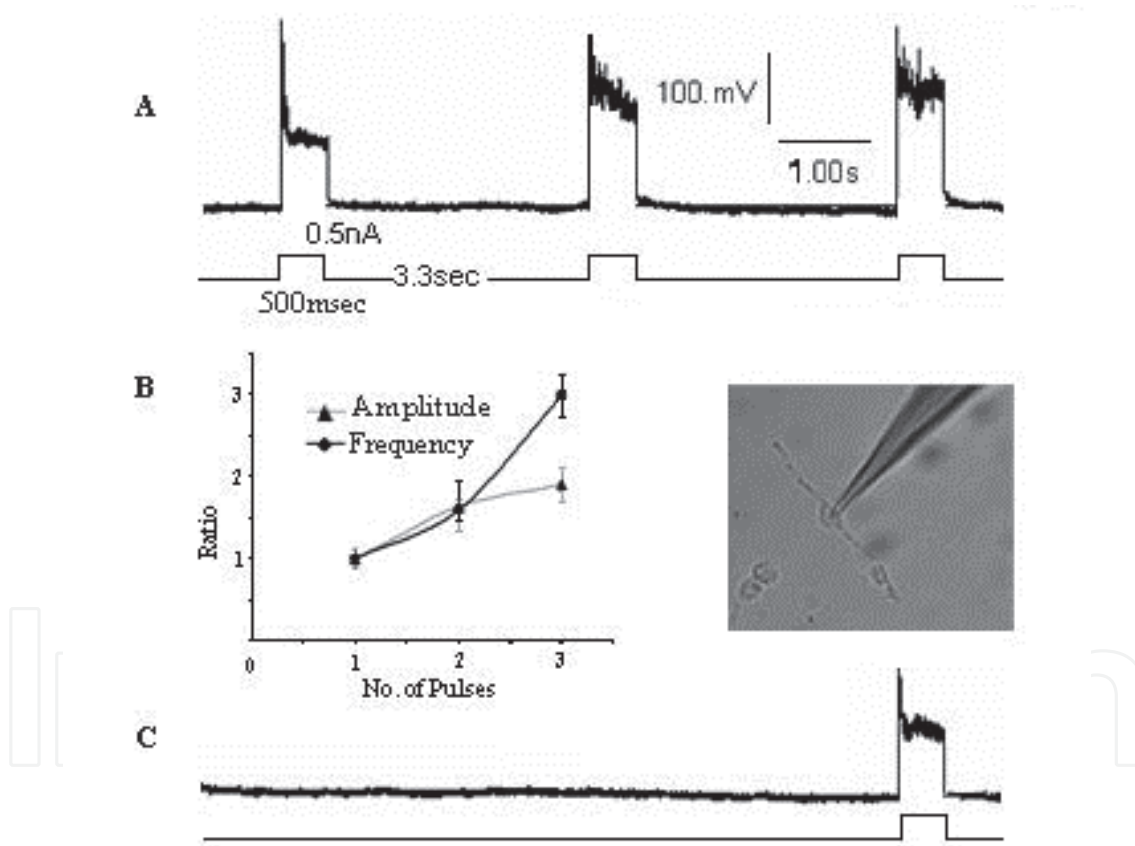


Fig. 3. Effect of SRF on frequency and amplitudes of action potential in granule cells Spike amplitude and firing frequency of action potentials were increased as a result of SRF depolarizing pulses of 0.3 Hz. **A:** In response to sustained repetitive pulses of 0.5 nA, a train of action potential was observed in a patch clamped granule cell in the current clamp mode of whole cell configuration. **B:** exhibits the relationship between the number of pulses with spike amplitude and frequency, inset showing a patch clamped granule cell. **C:** demonstrates the voltage response of SRF after a delay of 8 sec confirming that the increase in amplitude and frequency of action potential is activity not time dependent.



After calculating the activation and inactivation kinetics by using the formulas mentioned earlier, we have found that the value of half maximum activation  $V_{1/2}$  for  $\text{Na}^+$  was  $-25.8 \pm 3.2$  mV before and increased to  $-29 \pm 4.1$  mV after SRF. Whereas for  $\text{K}_{\text{sus}}$  (sustained K currents) values were  $-30 \pm 4.3$  mV before and was changed to  $-33 \pm 5.1$  mV after SRF (Fig. 4A) but transient potassium activation  $V_{1/2} = -30.4 \pm 2.8$  mV significantly get changed to  $-41 \pm 5.2$  mV after SRF. The transient potassium current ( $\text{K}_t$ ) was not decreased in the presence of 10 mM tetraethylammonium (TEA) in the extra cellular solution but 4AP in the bath reduces this current up to 75%.

Voltage dependent of sodium current before and after SRF

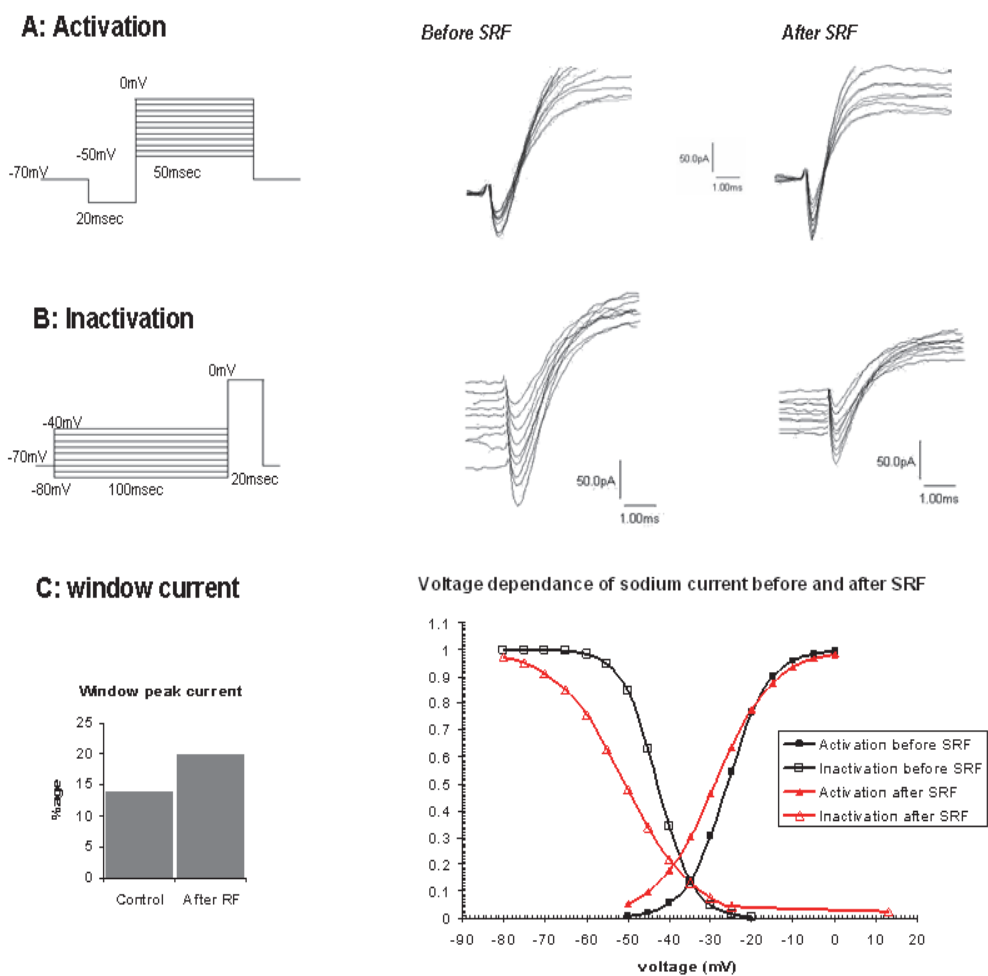


Fig. 4. **A:** Left hand panel exhibits activation pulse protocol (from -50mv to 0mV) and right hand graph shows  $\text{Na}^+$  activation current response before and after SRF **B:** Steady state inactivation pulse protocol on the left before and after SRF current recordings on the right. **C:** Left panel shows voltage dependency of  $\text{Na}^+$  activation and inactivation currents. Right panel shows histogram of changes in window currents before and after SRF, drawn from the activation-inactivation curves. Peak window current was observed at -35mV which was shifted to -39mV after SRF.  $V_{1/2}$  for activation before SRF was calculated to -25.8mV and for inactivation -42.7mV which were also shifted to -29mV and -50.6mV respectively after SRF.

Similarly  $V_{1/2}$  mV values of Na<sup>+</sup> inactivation =  $-42.7 \pm 6.6$ ;  $K_t$  =  $-68.5 \pm 5.6$ ; and  $K_{sus}$  =  $-58 \pm 5.6$  are markedly shifted towards -ve potential, Na<sup>+</sup> =  $-50.6 \pm 7.4$ ;  $K_t$  =  $-75.6 \pm 8.2$ ;  $K_{sus}$  =  $-63 \pm 9.4$ . These changes in voltage dependency of activation and inactivation currents results in an increase in sodium and decrease in potassium window currents. (Table 2)

	Activation		Inactivation				
	$V_{1/2}$ (act)	$K_{(activation)}$	$V_{1/2}$ (inactivation)	$K_{(inactivation)}$	Window Peak-Volt	Window range	Window Peak
	(mV)		(mV)		(mV)	(mV)	%
Sodium Current							
Before SRF	$-25.8 \pm 3.2(12)$	$5 \pm 0.5$	$-42.7 \pm 6.6(8)$	$4.2 \pm 1.1$	-35	-45 to -25	14
After SRF	$-29 \pm 4.1(11)$	$7.2 \pm 0.6$	$-50.6 \pm 7.4(6)$	$8.3 \pm 0.9$	-39	-54 to -20	20
Potassium Transient							
Before SRF	$-30.4 \pm 2.8(10)$	$6.6 \pm 0.8$	$-68.5 \pm 5.6(9)$	$7 \pm 1.2$	-51	-60 to -40	10
After SRF	$-41 \pm 5.2(10)$	$5 \pm 1$	$-75.6 \pm 8.2(9)$	$8 \pm 1.3$	-55	-60 to -45	8
Potassium Sustained							
Before SRF	$-30 \pm 4.3(10)$	$8.9 \pm 1.1$	$-58 \pm 5.6(9)$	$11 \pm 1.5$	-35	-60 to -10	20
After SRF	$-33 \pm 5.1(9)$	$8 \pm 0.6$	$-63 \pm 9.4(8)$	$9.2 \pm 1.3$	-39	-60 to -20	15

Table 2. Voltage dependence of activation and inactivation before and after SRF in mammalian granule cells.

$V_{1/2(act)}$ : is the value of the voltage at which membrane reaches 50% of the maximum activation state  
 $V_{1/2(inact)}$ : Is the value of the voltage at which membrane reaches 50% of the maximum inactivation state  
 $K( act )$ : slope of the activation curve  
 $K( inact )$ : slope of the inactivation curve  
Window peak volt: Voltage at which window current reaches its peak.  
SRF: Sustained Repetitive Firing  
The number in the parenthesis indicate the number of experiments  
 $P < 0.01$  indicate significant differences compared with control group  
These cells show non adapting firing frequency in response to superathreshold (0.5nA) current pulses (Fig.5A).

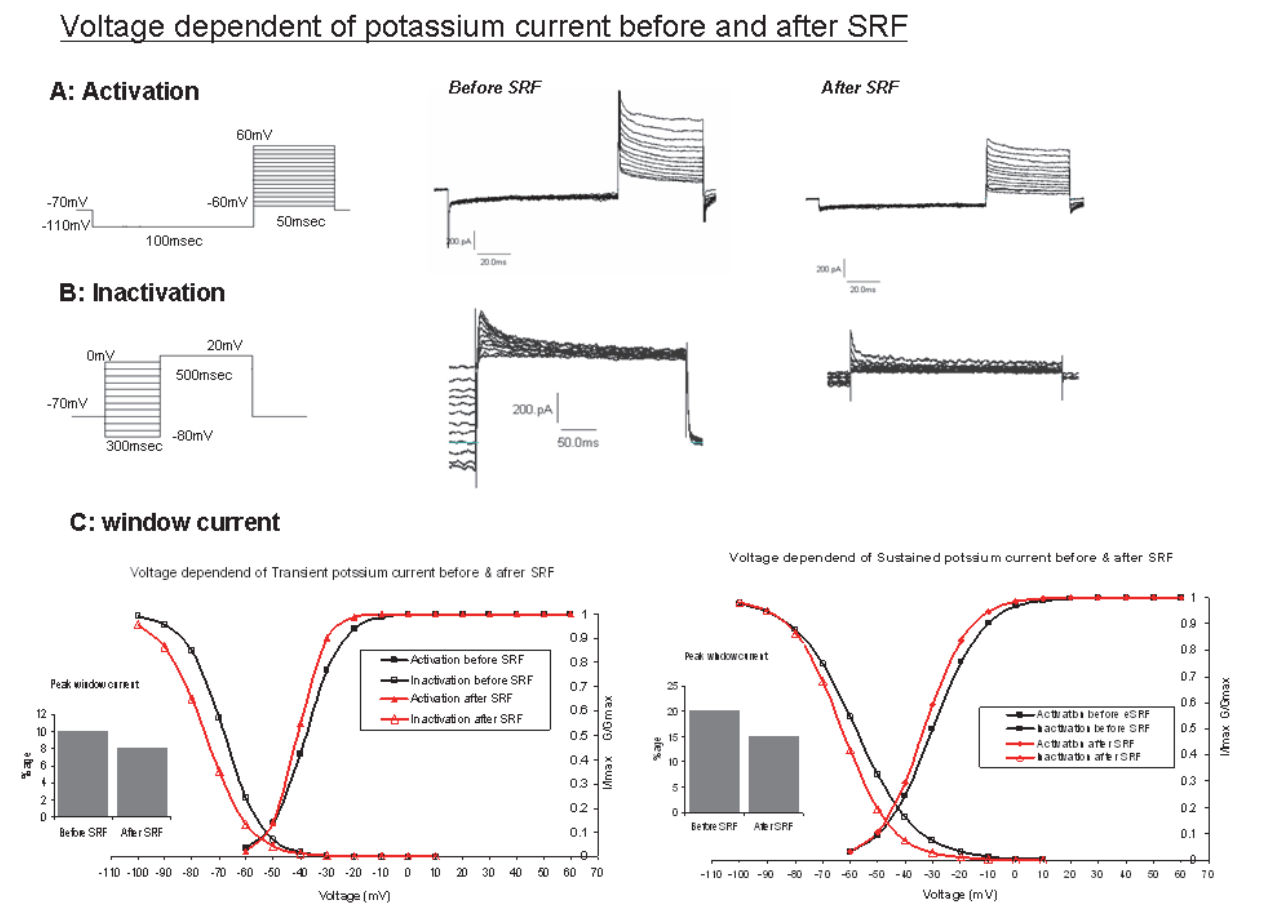


Fig. 5. This figure exhibits both sustained and transient potassium currents **A:** Activation pulse protocol is on the left, control current recording in the middle and after SRF in the right panel **B:** Exhibits (from left to right) inactivation pulse, control and after SRF current responses, for potassium channels. **C:** Voltage dependence of activation and inactivation of transient and sustained potassium currents. Histogram (in the inset) shows percentage of window currents before and after SRF. Amplitude of window current for transient potassium was at -51mV which was shifted to -55mV and sustained peak window current was shifted from -35mV to -39mV after SRF indicating voltage dependency of both type of potassium currents.



For measuring the activity dependent increase in amplitude and firing frequency, we applied three consecutive current pulses at 0.3 Hz (inter pulse duration of 3.3 sec). We observed that the amplitude and frequency of spikes in the next pulse are greater than previous one (Fig. 5C). Amplitude and frequency increased with the ratio of 1.0:1.6:1.9 and 1.0:1.6:3.0 for three pulses respectively. Ratio of amplitude and frequency at the second pulse (after 4 sec) is almost equal but during third pulse (after 8 sec), the ratio for frequency after SRF get increase significantly (Fig. 5B). Amplification of amplitude and number of spikes were not found to be present when only one pulse was given for the same duration of the three pulses (approximately 8 sec) indicating that these changes in spikes were not time but activities dependent (Fig. 5D).

#### 4. Discussion

The present study investigated the electrophysiological profile of granule cells and role of voltage gated  $\text{Na}^+$  and  $\text{K}^+$  channels in response to sustained repetitive firing. Amplitude and firing frequency of action potential for cultured granule cells were increased with increasing number of SRF pulses. We observed the voltage dependency of activation and inactivation of both  $\text{Na}^+$  and  $\text{K}^+$  currents after repetitive firing indicating both ionic currents have some underlying role in the modulation of action potential train in response to SRF. We have observed that both  $\text{Na}^+$  and  $\text{K}^+$  inactivation traces become less leaky after the exposure of SRF. We also observed that  $\text{Na}^+$  channel inactivation and  $\text{K}^+$  current activation has been shifted to more negative values indicating the reduce excitabilities in these cells after sustained repetitive firing. Similar observation was also been made by (Christina C et al 2009 and Stegan M et al 2009) and ascribed that granule cells become leaky with Decreasing severity of temporal lobe epilepsy. Thus similar to our conclusion both authors suggested that seizures triggers expression of different neuroprotective reactions, including upregulation of  $\text{K}^+$  channel density.

Literature also indicated that the levels of  $\text{Ca}^{+2}$  may gradually get increased intracellularly in response to SRF. Increased intracellular  $\text{Ca}^{+2}$  levels then may triggers the calcium gated potassium channels inducing after-hyperpolarization (AHP) in normal frequency adapting cells. Because of the low density of calcium gated potassium channels in granule cells of dentate gyrus, less adaptation of firing frequency may observed in some cases. Decrease levels of extracellular calcium due to repetitive firing results in sodium persistent current ( $I_{\text{NaP}}$ ) (Hailing S et al 2001).  $I_{\text{NaP}}$  is capable of amplifying a neuron's response to synaptic input and enhancing its repetitive firing capability (Carl E S 2007). These granule cells are thought to play an important role in electrical signaling, in neuronal synchronization, and rapid information transmission (Adam K et al 2002) and their number found to be increased in the epileptic hippocampus. Similarly, increase intracellular calcium levels results into many changes in the cell such as increased cytosolic cAMP, which inturn significantly increases persistent sodium currents (Bibiane F et al, 1997) as well as inducing prolonged neuronal plasticity (Nikitin ES et al 2006). Literature indicated that sodium inactivation gates are involved in the repetitive firing response and the impairment of the inactivation results in an increase in whole-cell persistent current (Kristopher M.K. et al, 2006). PKA phosphorylate the inactivation gates of sodium channels which in turn prolong their activation state and enhances repetitive firing.  $\beta 1$  subunit of the  $\text{Na}^+$  channels seems to be involved in the modification of gating properties (Earl PD et al 1994). Similarly  $\beta 4$  subunit of potassium channel observed to be participating in reducing dentate gyrus excitability and

resultant protection against temporal lobe seizures (Robert B et al 2005). When  $\beta 4$  subunits of potassium channel get phosphorylated they alter the channel conductance and decrease the efflux of potassium and hence resultant increased positivity inside the cell. Our findings of shifting of window currents of granule cells to hyperpolarized voltages may mimic in vivo condition during epileptic seizures. Our results also reveals a complex dynamics behind repetitive spike discharge and suggests that a persistent  $\text{Na}^+$  current plays an important role in action potential initiation and in the regulation of dentate gyrus granule cells transmission. The resting leak conductance was found to be doubled in epileptic granule cells and roughly 70–80% of this difference was sensitive to  $\text{K}^+$  replacement. SRF exposed “epileptic granule cells” had strongly enlarged inwardly rectifying currents with a low micro molar  $\text{Ba}^{2+}$  sensitivity. Further investigations needed to be done in this regard. Beside SRF in conjunction with cultured granule cells, we have other tools in hand as to explore underlying mechanism of epilepsy and its seizure type. Our initial experiments on platelets from epileptic patients suggested that peripheral benzodiazepine receptors of platelets could be a novel and effective therapeutic target.

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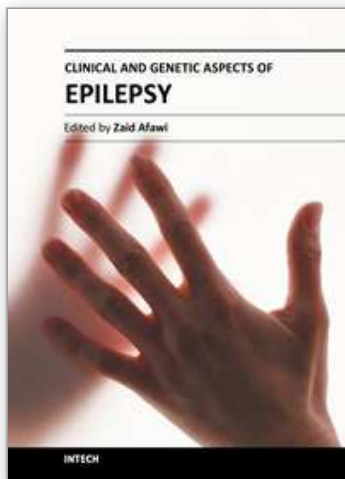
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