**Research Article**

# Investigation of the effects of pinacidil and glibenclamide administration on HCN1, KCNT1, Kir 6.1, SUR1 gene expressions in hippocampus and cortex regions in epileptic rats

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**Abstract**
The purpose of this study was to look into the effects of pinacidil and glibenclamide on HCN1, KCNT1, Kir 6.1, and SUR1 gene expression in epileptic rats hippocampus and cortex. Male Wistar-Albino rats were used in this study. The drugs pinacidil and glibenclamide were utilized. Control, Epilepsy, Epilepsy-O, and Epilepsy-B were the five groups formed. The epileptic focus was created by intracortical administration of penicillin at a dose of 500 IU/2 µl. Hippocampus and Cortex are removed from all animals and Kir 6.1, SUR1, HCN1, and KCNT1 gene expression levels were determined by qPCR. The SPSS 21 program was used for statistics. HCN1 gene expression level is equal in the hippocampus and cortex ($p<0.05$). KCNT1, SUR1, and KIR6.1 are more expressed in the hippocampus than in the cortex ($p<0.05$). In epilepsy groups, HCN1 gene expression level was found to be higher than KCNT1, SUR1, and KIR6.1 gene expression levels ($p<0.05$). KIR6.1, SUR1, gene expression levels decreased with the application of pinacidil and glibenclamide ($p<0.05$). SUR1 and KIR6.1 gene expression levels were considerably lower in the epilepsy pinacidil group compared to the other groups. The gene expression levels in the hippocampus were found to be considerably higher than in the cortex group, according to this study. The fact that HCN1 gene expression levels are significantly greater in both the brain and the hippocampus 24 hours following the commencement of epileptic convulsions suggests that preventive medication may be possible.

**Keywords:** epilepsy, Kir 6.1, SUR1, HCN1, KCNT1, gene expressions

## 1. Introduction

Epilepsy is a neurological disorder characterized by recurring seizures that affect more than 70 million people worldwide, making it one of the most common brain diseases (1, 2).

Despite the progress in preclinical and clinical studies, the pathogenesis of epilepsy remains unclear (3). Commonly used anti-epileptic drugs relieve symptoms rather than stop the progression of epilepsy. Existing medications restore neurotransmitter equilibrium via acting on ion channels, transporters, and receptors.

Ion channels play an important role in epilepsy pathogene sis. Understanding the function of these channels is critical for both understanding the mechanism of drug-resistant epilepsy and developing new therapeutic options. HCN1, KCNT1, and $K_{ATP}$ channels, for instance, have been proven to be useful in epilepsy in various investigations.

HCN channels are classified into two types: voltage-gated K+ channels and cyclic nucleotide-gated (CNG) channels (4). When the expression profiles of all HCN isoforms in the brain were examined, it was discovered that HCN1/HCN2 is widely expressed in cortical regions while HCN2/HCN4 is largely expressed in subcortical areas (5).

In both humans and mice, HCN2 is the most predominant isoform in cortical and subcortical regions (6). Although the presence of HCN3 is the most controversial, it is assumed that it has a role in early development since its expression in different species has been observed (7). Finally, HCN4 is associated with neurons with spontaneous rhythmic activity (6). Although at least three of the four HCN isoforms are expressed in neurons (8,9) there are reports that this is primarily linked to HCN1 isoform changes (10). HCN channels have been shown to function as pacemakers, and are involved in burst action potentials due to an increase in $Ca^{++}$ in thalamocortical neurons. On the other hand, HCN channels in dendritic synapses form a shunt mechanism that restricts temporal summation while accelerating EPSP amplitude and duration (11). HCN1 and HCN2 are two isoforms related with hereditary epilepsies in humans (12). According to studies associating HCN channel expressions with epileptogenesis, dendritic HCN1 and HCN2 channels are downregulated in the pilocarpine model, but expression increases when epilepsy occurs in the chronic term (13).

Although KCNT1 channels are extensively expressed in adult central nervous system neurons, immunological labeling tests have revealed that KCNT1 is also extensively expressed

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in embryonic hippocampus and cortical mouse neurons, indicating that cells contribute to early excitability (14).

KATP channels are found in all cell types. They are found in the cell, on both the cell membrane and the inner membrane of the mitochondria. KATP channels have an important function in cell metabolism and membrane excitability (15). In cases where metabolic activity is low in the cell, $K_{ATP}$ channels are open and the membrane is hyperpolarized, which has a cell-protective effect on vascular cells and neurons (16,17).

KATP channels are generally composed of the following Kir and four SUR1 subunits, are found in the plasma membrane, and come in a variety of topologies with a wide range of Kir and SUR subunits (18).

The effects of KATP channel openers and blockers, as well as KCNT1 and HCN1 channels, on epilepsy have been studied extensively in earlier investigations (19–21). However, the effects of employing KATP channel openers pinacidil and blockers glibenclamide in epilepsy on gene expression levels of both KIR6.1, SUR1, KCNT1, and HCN1 channels have never been studied. Previous research has found that KATP channel openers lessen seizures in a variety of epilepsy models (22,23).

The purpose of this study was to look at the levels of gene expression for Pinacidil and glibenclamide KATP (Kir 6.1, SUR1), HCN1, and KCNT1 channels in the hippocampus and cortex of rats with penicillin-induced epilepsy.

## 2. Material and Methods
### 2.1. Experimental animals
Experimental animals to be used in the study were obtained from BAIBU Experimental Animals Application Research Center. All experimental animals were treated in accordance with the guiding principles established by the animal ethical committee of Bolu Abant Izzet Baysal University, and all treatments adhered to the recommendations included in the Helsinki Declaration (Registration number:2018/36/A2). The animals were kept in the Experimental Animals Application Research Center in a relative humidity of 60-70% in a 12 hours light and 12 hours dark environment, and fed ad libitum until the study started and during the study period. Male rats of the Wistar albino breed aged 2-4 months were used. Four groups were created: Control (C), Epilepsy, Epilepsy-O, Epilepsy-B. A total of 36 animals were used, with 9 animals in each group. Rats were anesthetized with 1.2 g/kg (IP) urethane. The epileptic focus was created by intracortical administration of penicillin at a dose of 500 IU/2 μl. Drugs were applied 30 min after penicillin administration. Hippocampus and cortex tissues were removed from animals 24 hours after penicillin administration. After that Kir6.1, SUR1, HCN1, KCNT1 gene expression levels are detected in the hippocampus and cortex.

### 2.2. Surgical operation
All rats were anesthetized with 1.2 g/kg urethane (IP) (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) and placed in a stereotaxic device. Left cerebral cortex 2 mm posterior to bregma and 3 mm lateral to sagittal skull bone is removed, then dura matter is removed. To create epileptic focus 500IU with a Hamilton microinjector (701N, Hamilton Co., Reno, NV, USA) to a depth of 1.2 mm, 2 μl of penicillin G was injected.

### 2.3. Drug administration
In this study, $K_{ATP}$ channel-opener Pinacidil (1 mg/kg), and $K_{ATP}$ channel-blocker glibenclamide (5 mg/kg) were given intraperitoneally (i.p). All drugs were applied 30 min after penicillin administration.

### 2.4. QPCR method
To detect changes in gene expression levels, the total gene was isolated, cDNA synthesis was performed, and quantitative real-time PCR (qRT-PCR) experiments were performed.

**RNA isolation:** For RNA isolation from tissue samples, 1 ml of Trizole solution was added to a 50 mg tissue sample and homogenized. The tubes were incubated at room temperature for 5 minutes, then 200 μl chloroform was added, and manually shaken quickly for 15 seconds. The tubes were kept at room temperature for 3 minutes, centrifuged at 12,000 g, and 4 °C for 15 minutes. The transparent colored upper phase was taken into a new tube and 500 μl of 100% isopropanol was added. After incubation at room temperature for 10 minutes, the tubes were centrifuged for 10 minutes at 12,000 g and 4 °C At this stage, the RNA in the sample formed a white precipitate at the bottom of the tube. The liquid in the tube was removed, taking care not to touch this precipitate, and the RNA precipitate was washed with 1 ml of 75% ethanol and centrifuged at 7500 g and 4 °C for 5 minutes. The resulting RNA was dissolved with 20-50 μl of DEPC-ddH2O and its concentration was measured.

**cDNA Synthesis:** For each sample, 1 μg of RNA, 2 μl of oligo dT, and DEPC-ddH2O were mixed with a final volume of 8 μl and incubated for 5 minutes at 70 °C. After 10 μl of 2X reaction buffer and 2 μl of reverse transcriptase enzyme were added, the samples were incubated for 1 hour at 42 ˚C and 5 minutes at 80 ˚C. The cDNA samples were stored at -20˚C.

**Quantitative Real-Time PCR (qRT-PCR):** Primers that bind with high specificity to the target gene regions to be tested for RT-PCR experiments were designed. The oligo design was carried out using the Amplify program, and its properties such as melting temperatures (Tm) and primary-dimer formation were studied using the same program. To ensure that the selected primers do not bind to other unwanted regions (unspecific) in the genome, the primers were selected from the exon-intron junction regions. However, the specificity of the primers was confirmed by the in-silico PCR method using the UC Genome Browser. To investigate the level of gene expression, 1 μl of cDNA, 1 μl of primer mixture (10 μM, forward+reverse), 10 μl of 2X SYBR Green, and 8 μl of ddH2O were added to each qRT-PCR reaction. The following program was used for the reaction: 95˚C for 5 min, [95˚C for 15 sec, 60˚C for 30 sec, 72˚C for 30 sec] x40, 72˚C for 5 min

**Analysis of the qRT-PCR results:** Normalization with a housekeeping gene such as GAPDH was performed to prevent differences between samples and possible pipetting errors during the detection of gene expression levels. The analysis was performed using the ddCt method by the following equation (Table 1).

$$ddCt = Ct \text{ (target gene)} - Ct \text{ (housekeeping gene)}$$
$$\text{Target gene expression} = 2^{\wedge}(- ddCt)1$$

**Table 1.** Primers list

| Primers name | Primers | Tm (°C) |
|---|---|---|
| HCN1-F | GGATCCCAATTTCGTGACGG | 59 |
| HCN1-R | AGAGCCGTCTGTCAACTTCA | 57 |
| KCNT1-F | ATACTTCCAGCCCAGCCTTT | 57 |
| KCNT1-R | AGATGAAGGCAGTGGAAGCT | 57 |
| SUR1-F | TGGGGAACGGGGCATCAACT | 61 |
| SUR1-R | TGGCTCTGGGGCTTTTCTC | 59 |
| KIR6.1-F | GAGTGAACTGTCGCACCAGA | 59 |
| KIR6.1-R | GGATCACCAGAACTCAGCAA | 57 |
| GAPDH-F | ACCACCATGGAGAAGGCTGG | 61 |
| GAPDH-R | CTCAGTGATGCCCAGGATGC | 61 |

## 2.5. Statistical analysis

The difference between groups was analyzed with SPSS v.21 ANOVA and the Post-Hoc LSD test. A p-value of $<0.05$ was considered significant.

## 3. Results

HCN1 gene expression levels in the hippocampal area are significantly higher in the Epilepsy group than in the other groups $p < 0.05$. The level of HCN1 gene expression in the cortex is significantly higher than in the control group $p < 0.05$ (Fig. 1).
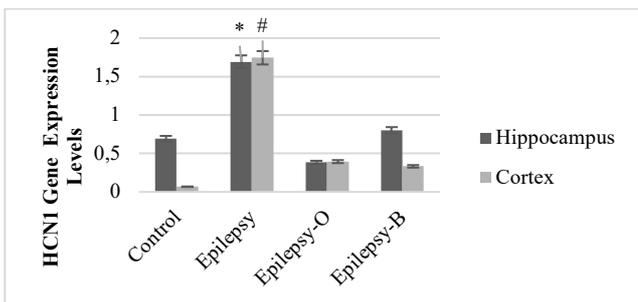


**Fig. 1.** HCN1 Gene expression levels in the hippocampus and cortex *$p<0.05$ compare with all groups in the hippocampus. # $p<0.05$ compared with all groups in the cortex

The control group's KCNT1 gene expression level in the hippocampal region was significantly higher than the other groups *$p<0.05$. The level of KCNT1 gene expression in the cortical region was significantly higher in the epilepsy group than in the other groups #$p<0.05$ (Fig. 2).
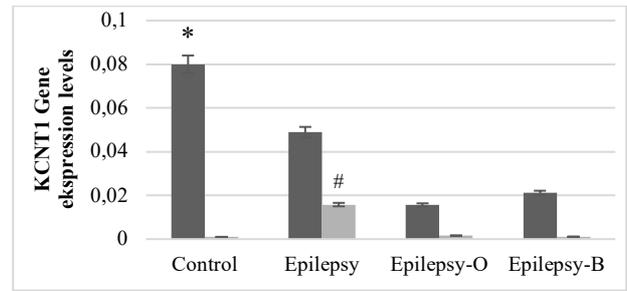


**Fig. 2.** KCNT1 Gene expression levels in the hippocampus and cortex *$p<0.05$ compare with all groups in the hippocampus. # $p<0.05$ compared with all groups in the cortex

The expression level of the Kir.6.1 gene expression levels in the Hippocampus region was significantly higher in the Epilepsy-B group than in the Epilepsy and Epilepsy-O groups. *$p<0.05$ Kir.6.1 gene expression levels in the cortical region were significantly lower in the Epilepsy group than in the Epilepsy-O and Epilepsy-B groups. #$p<0.05$ (Fig. 3).
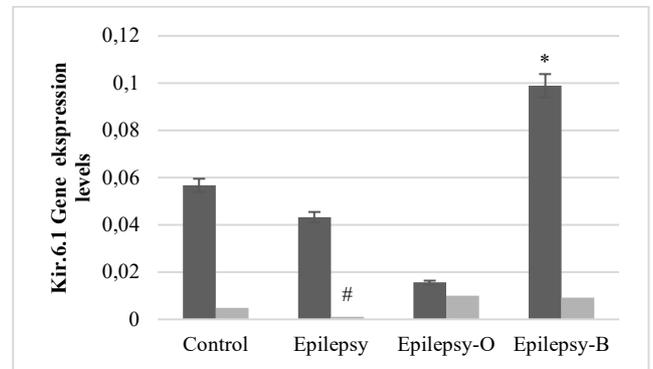


**Fig. 3.** Kir.6.1 Gene expression levels in the hippocampus and cortex *$p<0.05$ compare with all groups in the hippocampus. # $p<0.05$ compared with all groups in the cortex

When compared to the other groups, SUR1 gene expression was significantly lower in the Epilepsy-O group in the hippocampal region (Fig. 4).
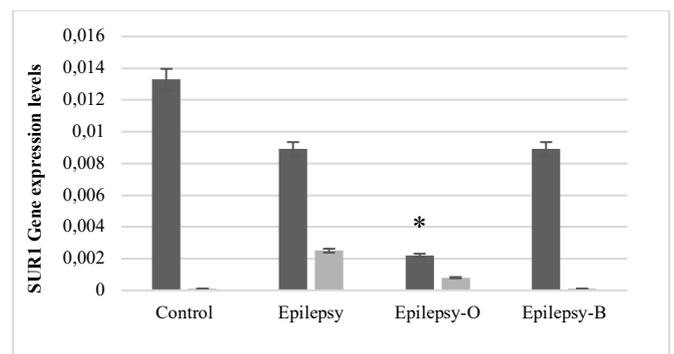


**Fig. 4.** SUR.1 gene expression levels in the hippocampus and cortex *$p<0.05$ compare with all groups in the hippocampus. # $p<0.05$ compared with all groups in the cortex

When gene expression levels are compared according to regions, there is no significant difference in HCN gene expression level. HCN gene expression level was expressed in both cortex and hippocampus. KCNT1, Kir.6.1, SUR1 gene expression levels are significantly higher in the Hippocampus region than in the Cortex region (Table 2).

**Table 2.** Gene expression levels in the hippocampus and cortex

| | Region | Mean | SEM | Min | Max | F | P |
|---|---|---|---|---|---|---|---|
| HCN1 | Hippocampus | 0.893 | 0.1766 | 0.01 | 4.68 | 0.258 | 0.486 |
| | Cortex | 0.635 | 0.29078 | 0 | 1.08 | | 0.450 |
| KCNT1 | Hippocampus | 0.041 | 0.00615 | 0 | 0,13 | 22.513 | 0.001* |
| | Cortex | 0.004 | 0.00296 | 0 | 0,13 | | 0.001* |
| Kir.6.1 | Hippocampus | 0.054 | 0.00931 | 0.01 | 0.25 | 36.01 | 0.001* |
| | Cortex | 0.006 | 0.00114 | 0 | 0,03 | | 0.001* |
| SUR1 | Hippocampus | 0.008 | 0.00109 | 0 | 0.02 | 20.693 | 0.001* |
| | Cortex | 0.0008 | 0.0005 | 0 | 0,02 | | 0.001* |

## 4. Discussion

Epilepsy is a complicated condition with numerous risk factors. In most cases, a genetic component is regarded in the absence of a recognized cause of epilepsy, and thanks to developments in genomic technology, the complex genetic structure of epilepsy, the mechanisms of its generation, and their link with mutations have begun to be proven. Comorbidities are becoming more widely acknowledged as major etiological and prognostic indicators. Anti-epileptic medications decrease seizures in more than two-thirds of persons with epilepsy but have no long-term effect on prognosis. In terms of quality of life, morbidity, and risk, epilepsy is a significant burden (2). Epilepsy is characterized by recurrent spontaneous seizures (24). It is an illness that affects a large number of people and costs a lot of money in terms of social and medical care (25). It is more common in children (26,27), and it is typically associated with a disorder that causes impairment (28,29). Recently, many studies aimed at revealing the mechanism of epilepsy have focused on ion channels and related pathways. Excessive depolarization of cells is involved in the known mechanism of epilepsy, and in this case, aiming to hyperpolarize the cells is among the strategies of anti-epileptic drugs. There are ion channels that help the hyperpolarization of the cell. In these channels, HCN1, KCNT1, and $K_{ATP}$ channels have a role in the hyperpolarization of cells.

In neurons, HCN channels have two functions. HCN channels are partially open at rest in many cells and contribute to the correction of membrane potential. Second, HCN channels inherently provide negative feedback due to their ability to endure both membrane hyperpolarization and depolarization. HCN channels open when the membrane is hyperpolarized, and the depolarizer generates a current that flows directly into the cell. The HCN channels become inactive when the membrane is depolarized, making hyperpolarization easier. As a result, HCN channels can actively attenuate both inhibitory and excitatory inputs arriving at the cell membrane, assisting in the stabilization of the membrane potential and playing a key role in controlling the excitability and electrical response of cells (30).

In addition to the information in all of these investigations, the epilepsy group's HCN1 gene expression level was shown to be considerably greater in both the hippocampus and the cortex. HCN1 gene expression increased, demonstrating its importance in epilepsy. The KCNT1 channel subunit is engaged in gradual hyperpolarization following a single action potential or the recurrent firing of action potentials (31). It is thought that in individuals with KCNT1 mutations, increased potassium current in inhibitory interneurons generates an imbalance between neuronal excitation and inhibition, resulting in protracted hyperpolarization and seizures (32).

In either situation, it is predicted that the KATP channels will open and protect the neuron by blocking activation (33). KATP channels have been found in numerous kinds of neurons inside the central nervous system, where they may play a protective function during anoxia or hypoglycemia (33,34).

Kir6.1 was found to be only mildly expressed in the neurons of the rat central nervous system, in contrast to the widely distributed Kir6.2 component. Although positivity was relatively low in many brain areas, considerable immunoreactivity was found in the Striatum, Dorsomedial, and paraventricular hypothalamic nuclei, zona incerta, and substantia nigra pars compacta. Surprisingly, the dispersed Kir6.1-immunopositive neurons in the striatum were previously identified as tonically active large cholinergic interneurons using double-label immunofluorescence (35). It should be explained if Kir6.1 contributes to the KATP channels that protect neurons during energy depletion in all of these neurons (36), maybe synergizing with Kir6.2, or whether they perform additional functions that have not yet been determined.In the pinacidil group, HCN1 gene expression was lower than in the control and epilepsy groups. Under normal conditions, it can be thought that the role of HCN1 channels is more. HCN1 gene expression level decreased in groups using pinacidil and glibenclamide. HCN1 gene expression may be reduced by pinacidil and glibenclamide.

The cytoplasmic membrane and the inner membrane of the

mitochondria are both home to K$_{ATP}$ channels in the cell. They work depending on ATP. Under normal conditions, the K$_{ATP}$ channels are closed, but when the amount of intracellular ATP decreases, these channels open and allow the cell to hyperpolarize. KATP and HCN1 channels may have some sort of link. In this study, the K$_{ATP}$ opener Pinacidil and the blocker Glibenclamide were used. The level of HCN1 gene expression was found to be low when Pinacidil and Glibenclamide were administered compared to those in the Epilepsy groups. Opening or blocking K$_{ATP}$ channels may be effective in decreasing HCN1 gene expression levels. Under normal conditions, when the amount of ATP in the cell decreases during excessive activation in the cell, the ATP channels open and the cell becomes hyperpolarized.

The seizures during epilepsy occur due to the depolarization of cells, and seizures can affect the entire brain with the propagation of this depolarization wave. Thus, hyperpolarization of cells is a protective strategy. Ion channels located in the cell membrane contribute to hyperpolarization. Among these channels, the K$_{ATP}$, KCNT1, and HCN1 ion channels contribute to the hyperpolarization of the cell.

We investigated how much three of these channels contribute separately in epilepsy, how they act, do they have an effect on HCN1 and KCNT1 gene expression by opening or blocking K$_{ATP}$ channels during an epileptic seizure. As a consequence, it's been discovered that giving pinacidil and glibenclamide to people with epilepsy lowers the amount of HCN1 gene expression.

Excessive and synchronous depolarization occurs in epilepsy, resulting in seizures. The realization of hyperpolarization in cells, on the other hand, is critical, and ion channels play the most vital role. The effects of pinacidil and glibenclamide on the gene expression levels of HCN1, KCNT1, and KATP channels in epilepsy were investigated in this study. As a result, HCN1 gene expression is highest in the hippocampus and cortex of epilepsy patients. Although the gene expression levels of KCNT1 and K$_{ATP}$ channels were not significant during epilepsy, both K$_{ATP}$ channel openers and blockers decreased KCNT1 and HCN1 gene expression levels. But the expression levels of Kir6.1 and Sur1 gene reduced with openers, Kir6.1 rose with blockers, while Sur1 remained unaffected.

The fact that there is a drug-resistant group in epilepsy indicates the need to conduct studies that can pave the way for developing new treatments against epileptic seizures and help to accurately understand the pathogenesis of the disease.

The fact that HCN1 gene expression levels are significantly higher in both the cortex and the hippocampus 24 hours after the onset of epileptic seizures provides promise for preventative therapy.

**Conflict of interest**
The authors declared no conflict of interest.

**Ethical Committee Approval**

All experimental animals have been treated based on the guiding principles approved by the animal ethical committee of Bolu Abant Izzet Baysal University as well as all the treatments comply with recommendations provided on the Declaration of Helsinki (Registration number:2018/36/A2).

**Authors' contributions**
Concept: Ü.K, H.S., Design: Ü.K, H.S., Data Collection or Processing: Ü.K, H.S., Analysis or Interpretation: Ü.K, H.S., Literature Search: Ü.K, H.S., Writing: Ü.K, H.S.

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