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# NLRP2 Immunoreactivity in Mouse Brain After Cortical Spreading Depolarization

**Eda DERLE[1,2], Buket DÖNMEZ DEMİR[2], Melike SEVER BAHÇEKAPILI[2], Canan ÇAKIR AKTAŞ[2], Onur Çağın GÜRLEK[2], Beyza TÜRKEN[2], Hülya KARATAŞ KURŞUN[2]**

[1]Department of Neurology, Başkent University Faculty of Medicine, Ankara, Türkiye
[2]Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara, Türkiye

## ABSTRACT

**Introduction:** The objective of this study is to elucidate the expression pattern and alterations of NLRP2 following a single cortical spreading depolarization (CSD).

**Method:** Brain tissue was obtained by cardiac perfusion at the 15th minute after CSD in optogenetically inducible Thy1-ChR2-YFP (n=3) mice. NLRP2 expression was examined in the obtained brain tissue sections by immunofluorescence staining and compared with NLRP2 expression pattern in the sections obtained from the control group (n=3) and naive mice (n=1). The cellular source of NLRP2 is demonstrated by dual staining with NeuN and S100β.

**Results:** Cellular immunoreactivity of NLRP2 was evident in the cortical and subcortical regions of sham and naive group brains. This immunoreactivity exhibited a notable reduction in CSD-induced brains. The the majority of cells expressing NLRP2 exhibited colocalization with NeuN, a neuronal marker, while a smaller subset demonstrated merging with S100β, an astrocyte marker.

**Conclusion:** This study revealed the decreased immunoreactivity of NLRP2 after single CSD in early period.

**Keywords:** Cortical spreading depolarization, inflammasome, inflammation, migraine, NLRP2

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## INTRODUCTION

The NOD-like receptor (NLR) family, a subset of pattern recognition receptors within the innate immune system, includes NLR pyrin domain-containing 2 (NLRP2). While the precise role of NLRP2 in immune responses remains incompletely understood, numerous studies indicate its involvement as an astrocytic inflammasome, contributing to inflammatory responses in various disease models such as cerebral ischemia, pain, and depression (1–3). Conversely, there are indications of its anti-inflammatory effects through the regulation of nuclear factor-κB (NF-κB) in different systems (2).

Additionally, prior research has highlighted the activation of NLRP2 by ATP through pannexin channels and purinergic receptors on astrocytes, resulting in an inflammatory cascade (1). This process has been implicated in various pathological conditions, including cerebral ischemia, pain, and depression (2,3). The dual nature of NLRP2, exerting both pro-inflammatory effects as an astrocytic inflammasome and anti-inflammatory effects through NF-κB regulation, underscores its complexity and multifaceted role in immune responses (2).

In the context of the cortical spreading depolarization model, which is accepted as the experimental model of migraine aura, where ATP-mediated astrocytic stimulation has been documented, the potential involvement of NLRP2 as a mediator in this stimulation and the subsequent inflammatory response emerges as a crucial aspect of investigation (4,5). By exploring changes in NLRP2 immunoreactivity in a single optogenetically induced cortical spreading depolarization model at a very early time point, this study aims to contribute valuable insights into the intricate mechanisms underlying NLRP2's participation in neuroinflammatory processes. Such understanding holds promise for uncovering therapeutic targets and strategies to modulate NLRP2-associated responses in neurologic disorders.

### Highlights

- **Inflammation is proposed as a key mechanism underlying the pathogenesis of migraine.**
- **NLRP2 may play a role in migraine pathogenesis.**
- **NLRP2 may also express in neuronal cells besides astrocytes.**
- **NLRP2 immunreactivity decreases very early time after CSD.**

**Correspondence Address:** Eda Derle, Başkent Üniversitesi Hastanesi Nöroloji Anabilim Dalı, E blok Bahçelievler, Ankara, Turkey • **E-mail:** edaderle@hotmail.com
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## METHOD

### Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Hacettepe University (2019/46). All animals had free access to food and water and were housed in groups under diurnal lighting conditions (12-h light–12-h dark cycle) at 22±3°C and 40–60% humidity.

Optogenetic induction of CSD stimulation is minimally invasive and minimizes procedure-associated inflammatory reactions. For optogenetic induction, we used Thy1-ChR2-YFP mice which express chanelrhodopsin-2 on cortical neurons and can be stimulated by laser light (n=3) and negative littermates as sham group (n=3), C57BL/6J (n=1) as a naive group, aged 12–16 weeks, 20–25 gr weighing. Oxygen saturation, pulse rate, and body temperature were monitored via an oximeter (The LifeSense® VET Pulse Oximeter, Nonin Medical Inc., USA).

### Optogenetic Stimulation of Cortical Spreading Depolarization

Isoflurane was used for induction and maintenance of anesthesia during surgery and CSD induction experiments. Thy1-ChR2-YFP transgenic and littermate mice were placed in a stereotaxic frame following anesthesia and surgical procedures were performed under a stereomicroscope (Zeiss SV6). First, a skin incision was made in the midline, and bregma, frontal, and parietal bones were exposed. For electrophysiological recording, the parietal bone was thinned with a high-speed drill. During thinning, the bone was cooled with physiological saline. The thinned bone area was marked and an Ag-$AgCl_2$ pellet electrode was lowered onto the cranium. For grounding, an Ag-$AgCl_2$ disc electrode was placed between the neck muscles. Electrodes were connected to the Powerlab data acquisition system. Electrophysiological recording was started and the quality of the physiological recording was checked with the Labchart program. When the recording became stable, an optogenetic stimulation was made with a fiberoptic cable placed on the frontal bone. Laser source with a wavelength of 450 µm was adjusted to a light intensity of 4 mW, and a 0.48 numerical aperture with a diameter of 400 µm. Stimulation was applied at an intensity of 50 mJ for 10 seconds and an electrophysiological recording of the cortical spreading depolarization wave was recorded.

In the sham group, the surgical procedure was the same as the experiment group except for the laser stimulation. Furthermore, no procedure was applied on naive mouse except anesthesia. Cardiac perfusion in mice was performed at the 15th minute following the recording of CSD. Then the brain tissue was removed in all groups and kept in 4% PFA for 24 hours and then in 30% sucrose solution for 48 hours for cryoprotection. After that, 20 nm thick coronal sections were taken on a cryomicrotome (Leica CM 1100).

### Immunofluorescence Staining

Sections were placed in citrate solution (PH=6) in hot water at 80°C for 10 minutes and then allowed to cool down to room temperature. Then washed twice for 5 minutes (TBS + 0.025% triton X) and incubated for 10 minutes for permeabilization (TBS + 0.2% Triton X). The slices were incubated with blockage solution (0.3 M glycine with 10% normal goat serum) for 75 minutes, followed by the primary antibody for 48 hours (1/100 NLRP2 antibody, Proteintech 15182–1-AP). After the incubation, slices were rinsed with washing solution 3 times for 5 minutes and with secondary antibody (1/200 Goat Anti Rabbit Alexa fluoro 488 Abcam-ab150077) for 1 hour. For double staining, slices were incubated with NeuN (1/150 Milipore MAB377) and S100β (1/150 atlas antibodies MAB03629) overnight at +4°C and 1 hour at room temperature with secondary antibody (1/200 goat anti-mouse cy3). The sections were washed three times in between every step, incubated with Hoechst-33258 solution, and then covered with a coverslip.

All slices were investigated with a Leica TCS SP8 confocal laser scanning microscope (Leica, Wetzlar, Germany) and the images were obtained with a resolution of 2048 × 2048 pixels.

## RESULTS

In this study, we explored the immunoreactivity of NLRP2 15 minutes following the optogenetically induced single cortical spreading depolarization (CSD) in mouse brains. Initially, we scrutinized the expression pattern of NLRP2 in mouse brain sections through immunofluorescence staining. Notably, discernible cellular immunoreactivity of NLRP2 was evident in the cortical and subcortical regions of sham and naive group brains. Intriguingly, this immunoreactivity exhibited a notable reduction in CSD-induced brains, as illustrated in Figure 1.

Subsequently, to elucidate the cellular identity of NLRP2 expression, double immunostaining experiments were conducted. The results revealed that the majority of cells expressing NLRP2 exhibited colocalization with NeuN, a neuronal marker, while a smaller subset demonstrated merging with S100β, an astrocyte marker, as depicted in Figure 2. This dual staining approach provided valuable insights into the specific cellular populations expressing NLRP2 in response to optogenetically induced single CSD, shedding light on the potential involvement of both neurons and astrocytes in the observed immunoreactivity changes.

## DISCUSSION

Inflammation is proposed as a key mechanism underlying the pathogenesis of various neurological diseases, encompassing migraine, pain, neurodegenerative disorders, and cerebral ischemia. Inflammasomes, integral components of the innate immune system, activate proinflammatory cascades. While numerous members of the NOD-like receptor (NLR) family have been identified in different pathophysiological processes, the understanding of their roles in diseases, including NLRP2, remains insufficient (3). Especially NLRP2 is the least studied inflammasome type among others.

The objective of this study was to elucidate the expression pattern and alterations of NLRP2 following a single CSD. Surprisingly, our findings indicated basal-level expression of NLRP2 in neurons, with a discernible shift in immunoreactivity after CSD. In contrast to prior research suggesting a less abundant neuronal expression and prevalent astrocytic features, our results demonstrated marked NLRP2 expression in NeuN (+) cells, particularly in the naive and sham groups (1,6–9). Notably, there was a pronounced reduction in NLRP2 immunoreactivity following CSD, challenging the previously reported astrocytic predominance in studies.

Noteworthy studies by Zhang, Sun, Cheon, and Minkiewicz et al. underscored the astrocytic expression of NLRP2 and its association with ATP-induced pannexin and P2X7 receptor stimulation, activating inflammasome and proinflammatory cascades (1,6–9). These findings prompted us to explore the potential link between NLRP2 and CSD, considering its plausible role in the proinflammatory features observed in experimental migraine models.

Intriguingly, our study deviates from previous reports, demonstrating a marked neuronal expression pattern of NLRP2 in cortical and subcortical brain regions, contrary to the astrocytic features predominantly observed in other studies. This distinction aligns with Matsuka et al.'s identification of neuronal NLRP2 expression in dorsal root ganglia during an inflammatory pain hypersensitivity model (10). However, our study revealed a reduction in NLRP2 immunoreactivity after CSD, in contrast to Matsuka et al.'s observations of increased reactivity following ceramide-induced tissue inflammation (10). Nevertheless, , in this study, NLRP2 was

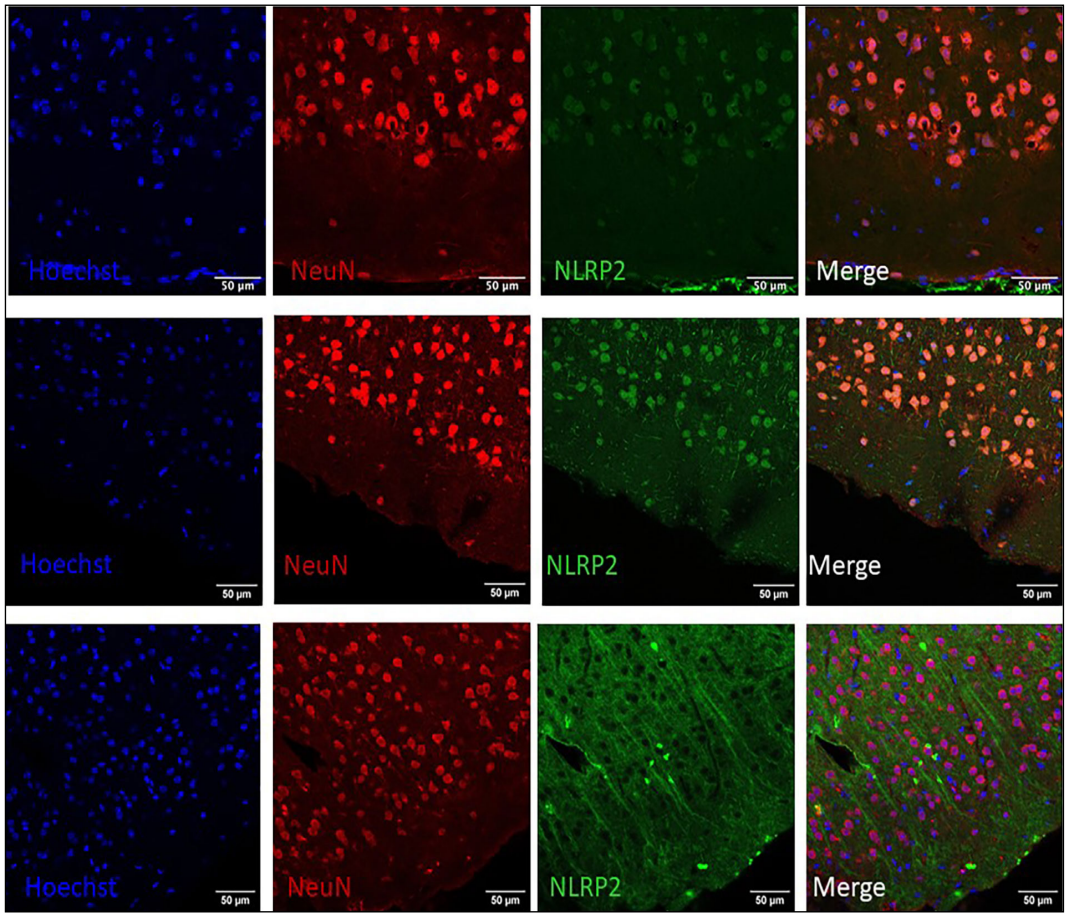


**Figure 1.** Representative images of NLRP2 immunoreactivity. Double staining with NeuN *(red)* and NLRP2 (green) was performed. Hoechst *(blue)* was used for nuclear counterstain. Naive group *(upper row)* and sham group *(mid row)* images showed cellular NLRP2 staining patterns which were diminished in the cortical spreading depolarization brain *(lower row)*. Neuronal marker (NeuN) and NLRP2 colocalizations are seen in merged images.

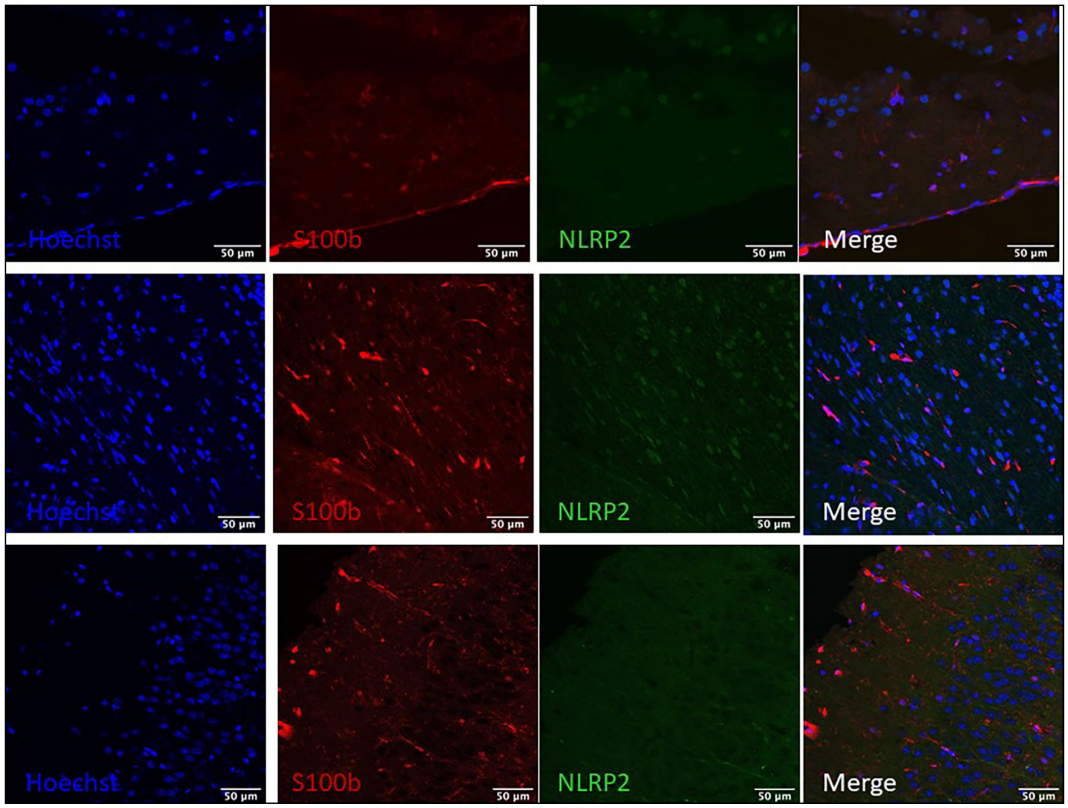


**Figure 2.** Representative images of NLRP2 immunoreactivity. Double staining with S100b *(red)* and NLRP2 *(green)* was performed. Hoechst *(blue)* was used for nuclear counterstain. NLRP2 immunopositivity was barely seen in S100b positive astrocytes *(merged images)*.

detected two days after ceramide induction. In our study, we detected NLRP2 expression at a very early time point after CSD induction (10). Considering the timing of response patterns elicited by stimulation, further investigations may unravel the expression and activation intervals of NLRP2. Pharmacologic or genetic manipulation of NLRP2 presents a promising avenue for understanding its role as a potential limiting factor in inflammation, possibly through NF-κB regulation. Future research exploring the temporal dynamics and manipulations of NLRP2 could offer valuable insights into its involvement in the inflammatory responses triggered by CSD.

**Ethics Committee Approval:** This animal study was approved by Hacettepe University, Animal Experiments Ethics Committee (Approval No: 2019/46)

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept- ED, HK; Design- ED, HK; Supervision- ED, HK; Resource- ED, HK; Materials- ED, HK; Data Collection and/or Processing- ED, HK, BDD, MSB, CÇA, OCG, BT; Analysis and/or Interpretation- ED, HK; Literature Search- ED; Writing- ED, HK; Critical Reviews- ED, HK.

**Conflict of Interest:** The authors declared that there is no conflict of interest.

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