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The role of methyl-CpG binding domain 3 in seizures and epileptogenesis

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Methyl-CpG binding domain protein 3 (Mbd3), a component of the NuRD chromatin remodeling complex, plays a role in transcriptional regulation and has been implicated in neuronal development; however, its role in epilepsy remains unclear. This study investigated the effects of Mbd3 downregulation on seizure susceptibility and behavior in rats, using adeno-associated viral vectors that code for short hairpin RNA to downregulate Mbd3 expression in the basolateral amygdala. Behavioral assessments included the open field test, elevated plus maze test, and hyperexcitability test. Seizure susceptibility was evaluated using the PTZ challenge and PTZ kindling models. A decreased Mbd3 level significantly increased latency to seizure onset in the PTZ challenge, indicating a raised seizure threshold. Rats with reduced Mbd3 expression also exhibited increased anxiety-like behavior in the open-field test. Mbd3 downregulation did not affect the progression of epileptogenesis in the PTZ kindling model. These findings suggest that Mbd3 contributes to acute seizure susceptibility and emotional behavior but not to the long-term development of epilepsy, highlighting its potential as an epigenetic modulator in seizure regulation.

Key words: epigenetic regulation, basolateral amygdala, pentylenetetrazole, seizure susceptibility, anxiety-like behavior

INTRODUCTION

Epilepsy is one of the most prevalent chronic neurological disorders, affecting approximately 50 million people globally and characterized by spontaneous and recurrent seizures due to abnormal neuronal activity in the brain (Fisher et al., 2005). Despite significant advances in pharmacotherapy, around 30% of patients exhibit drug-resistant epilepsy, underscoring the urgent need to identify novel therapeutic targets and gain a deeper understanding of the molecular underpinnings of epileptogenesis (Kwan et al., 2010; Löscher et al., 2020).

Epigenetic mechanisms, including DNA methylation and chromatin remodeling, have emerged as crucial modulators of gene expression in both physiological and pathological brain processes (Qureshi & Mehler, 2010; Kobow & Blümcke, 2014). Among the proteins responsible for interpreting epigenetic marks is methyl-CpG-binding domain protein 3 (Mbd3). Mbd3 is a non-canonical member of the MBD family that does not bind methylated DNA directly but is essential for the function of the nucleosome remodeling and deacetylase (NuRD) complex, which integrates chromatin remodeling and histone deacetylation to regulate transcription (Wade et al., 1998). Mbd3 is essential in early development, maintenance of pluripotency, and neuronal differentiation (Reynolds et al., 2012). However, its role in the mature brain, particularly under pathological conditions such as epilepsy, remains poorly understood.

Recent studies have begun to uncover the involvement of chromatin remodeling proteins in seizure-related processes (Kobow & Blümcke, 2011; Kobow et al., 2013; Debski et al., 2016; Hauser et al., 2018). Our previous work showed elevated expression of NuRD

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complex proteins, including Mbd3, in the brains of epileptic animals (Bednarczyk et al., 2016; Nizinska et al., 2023). Furthermore, we demonstrated that overexpression of Mbd3 in the basolateral amygdala (BLA) *via* adeno-associated viral (AAV) vectors significantly accelerated epileptogenesis in the PTZ-kindling model (Nizinska et al., 2023). These findings suggest a potential pro-epileptogenic role for Mbd3 and prompted us to investigate the functional consequences of its downregulation further.

This study aimed to elucidate whether reducing Mbd3 levels in the brain influences seizure threshold and epileptogenesis. Specifically, we employed AAV-shRNA constructs to knock down Mbd3 in the BLA. We assessed its effects in two experimental models: the PTZ challenge model of acute seizures and the PTZ-kindling model of epileptogenesis. In parallel, we evaluated behavioral outcomes, including anxiety-like responses, to determine whether Mbd3 also modulates behavioral phenotypes commonly associated with epilepsy. These experiments provide further insight into the role of epigenetic regulators in seizure generation, highlighting Mbd3 as a potential target for therapeutic intervention.

METHODS

Animal surgery

All of the animal procedures were approved by the Ethical Committee (permits no. 357/2017, 395/2017, and 838/2019) of the Warsaw Local Ethics Committee for Animal Experimentation) and conducted according to guidelines that were established by the European Council Directive 2010/63/EU and by the ARRIVE guidelines (Kilkenny et al., 2010).

Male Sprague-Dawley rats (250-270 g) from the Mossakowski Medical Research Centre, Polish Academy of Sciences (Warsaw, Poland), were used in this study. The rats were housed under controlled conditions (24°C, 50-60% humidity, 12 h light/12 h dark cycle) with food and water available *ad libitum*. The animals were housed in pairs in an enriched environment. Rats underwent EEG electrode implantation and AAV injection when they reached a body weight of 300 g.

Electrode implantations were performed according to the procedure described by Nissinen et al. (Nissinen et al., 2000), with modifications introduced by Guzik-Kornacka et al. (2011). Animals that reached a weight of 300 g underwent surgery under isoflurane anesthesia (Bartex, initial dose of 4%, and then at 1.5% to 2% in oxygen) and analgesia with 0.2 mg butorphanol (Butomidor, 10 mg/ml, Richter Pharma AG). The EEG surface electrode for seizure monitoring was implanted stereotactically over the frontal cortex (AP: 3.0; L: +2.0 mm from Bregma, #E363/20, PlasticOne). The reference and ground electrodes were implanted over the cerebellum (AP: 10.0 mm; L: ±2.0 mm from Bregma, #E363/20, PlasticOne). The electrode's ends were placed in a socket (#E363/2-TW/Spec, PlasticOne) and attached to the skull with dental acrylic (Duracryl Plus, SpofaDental).

The adeno-associated virus (AAV) coding shRNA to decrease MBD3 levels (AAV-sh(Mbd3)-GFP) was purchased from Tebu-bio (Le Perray-en-Yvelines, France). The efficacy of the vectors in reducing Mbd3 protein levels was confirmed in vitro using Western Blot in primary neuronal cell cultures. AAV stock was diluted 1:10 in sterile PBS buffer before injection into the rat's brain. AAV was injected bilaterally into the BLA. Injection site coordinates were: AP: -2.8; L: ± 4.7; DV: -7.2 (Paxinos & Watson, 2007). AAV was injected in a volume of 0.4 µl/hemisphere (0.8 µl/rat) at a 0.2 µl/minute rate using a NanoFil needle and syringe (NanoFil, WPI) under control of UltraMicroPump (#UMP3, WPI).

Behavioral tests

In the behavioral hyperexcitability test, animal behavior was assessed in four categories: approach response, touch response, loud noise, and pick-up, as previously described (Blanco et al., 2009; Nizinska et al., 2021). In the approach-response test, a pen held vertically was slowly moved toward the animal's head. Responses were scored as follows: 1 – no reaction; 2 – sniffing the pen; 3 – moving away from the pen; 4 – freezing; 5 – jumping out; and 6 – attacking the pen. In the touch-response test, the animal was gently prodded in the rump with the blunt end of the pen. Responses were scored as follows: 1 – no reaction; 2 – turning toward the touched area; 3 – moving forward, away from the touch; 4 – freezing; 5 – jerking around toward the touch; 6 – turning away from the touch; and 7 – jumping with or without vocalization. In the loud noise test, a clicking noise was generated by a timer positioned several centimeters above the animal's head. Responses were scored as follows: 1 – no reaction; 2 – jumping slightly, flinching, or flicking the ears; and 3 – jumping abruptly. In the pick-up test, the animal was picked up by grasping it around the body. Responses were scored as follows: 1 – very easy; 2 – easy with vocalization; 3 – some difficulty, with the rat rearing and facing the experimenter's hand; 4 – freezing with or without vocalization; 5 – difficult, with the rat avoiding the hand by moving away; and 6 – very diffi-

cult, with the rat behaving defensively, with or without attacking the experimenter's hand. The behavioral hyperexcitability test was repeated four times on the same day, at one-hour intervals. Median scores were used for the subsequent data analysis.

The open field test was conducted in a square, dark gray box with dimensions of 1 × 1 m and a wall height of 35 cm. The arena was made by the Laboratory of Animal Models at the Nencki Institute of Experimental Biology. The rat's movements were monitored for 20 minutes. The latency to enter the inner area of the arena, the latency to enter the central area, and the speed were monitored during the test.

The elevated plus maze consisted of two open arms (50 cm long, 14 cm wide) and two closed arms (wall height 29 cm) and was 50 cm above the floor. The rat was placed in the central area of the maze and monitored for 15 minutes. The number of entries into the closed and open arms, as well as the speed, were monitored during the test.

The open-field and elevated plus-maze tests were recorded using WinTV software (Hauppauge, NY, USA). Video files were analyzed using EthoVision 8.5 software (Noldus, Leesburg, VA, USA), and the data were exported to Microsoft Excel. The order of animals within trials was randomized.

PTZ challenge

Pentylenetetrazole (PTZ) dissolved in saline was applied intraperitoneally at a convulsive dose (50 mg/kg body weight) (Davoudi et al., 2013; Dhamne et al., 2015). Control animals received an intraperitoneal injection of saline. Rats were monitored with video-EEG (Panasonic WV-CP480; Comet EEG system, Grass Technologies, USA) for 60 minutes after PTZ injection. EEG recordings were analyzed manually to detect the start of electrographic seizures using TWin EEG software (v.4.5.3.23, Grass Technologies, USA). Video was used to detect behavioral generalized tonic-clonic seizures. Animals for mRNA analysis were sacrificed at 1, 4, 8, 24, and 48 h after the seizure initiation; animals for protein analysis were sacrificed at 4, 8, 24, and 48 h after the seizure initiation. The mortality rate during the PTZ challenge was 0%.

PTZ kindling

During PTZ kindling, rats received intraperitoneal injections of PTZ at a dose of 35 mg/kg, 3 times per week (Racine, 1972; Samokhina & Samokhin, 2018). This dose of PTZ in our hand is routinely used for kindling. In the present cohort, some animals had low-grade seizures already during the first session. Behavioral seizures were recorded for 30 minutes using a video camera (Panasonic WV-CP480) and scored according to the Racine scale (Racine, 1972). The criterion for full kindling was defined as the induction of seizure scores 4-5 according to the Racine scale during three consecutive sessions. The experiment was conducted until all animals met the accepted criterion of the kindling model. For ethical reasons, each animal reaching the criterion (3 consecutive sessions with tonic-clonic convulsions) was withdrawn from the experiment and did not participate in subsequent sessions. The mortality rate was 10% (n=1 per group).

Immunofluorescence and image analysis

Rats were anesthetized with isoflurane followed by an intraperitoneal injection of 2 ml/kg pentobarbital (Morbital, Biowet, 133.3 mg/ml). Perfusion was performed with 200 ml of saline, followed by 200 ml of 4% paraformaldehyde in PBS (pH 7.4). The brains were post-fixed in a 4% PFA solution for 4 hours and then cryoprotected in 30% sucrose in 0.02 M KPB (pH 7.4) solution at 4°C. The brains were frozen on dry ice and stored at -80°C, cryosectioned to 30 µm sections in a 1-in-5 series in the coronal plane using a cryostat (Leica CM1860). Sections were stored in the Tissue Collection Solution (30% ethylene glycol, 25% glycerol, 0.05 M PB buffer) at -20°C.

Sections were stained with primary monoclonal antibody (chicken anti-Neun, ABN91 or mouse anti-GFAP, #MAB3402, Millipore, 1:1000) followed by anti-chicken Alexa Fluor 568 (A11041 ThermoFisher Scientific, 1:2000) or horse anti-mouse Texas Red™ (TI-2000, Vector Laboratories, 1:2000), respectively. Sections counterstained with Hoechst (#62249, ThermoFisher 1:1000), mounted, and coverslipped in Vectashield® Mounting Medium (#H-1000, Vector Laboratories).

The sections were photographed using a Nikon Eclipse 80 microscope and a Lumen 200 fluorescent lamp (Prior Scientific), with a Nikon 10/0.30 DIC L/N1 objective. Images covering the virus expression were superimposed to visualize the co-localization of GFP with NeuN and Hoechst staining using ImagePro Plus 5.0.

To analyze the efficacy of the in vivo transfection, the numbers of GFP+ cells, NeuN+ neurons, and double-stained cells were assessed.

For the measurements of virus spread in the brain tissue, the volume within which the GFP^+ appeared was calculated. To this end, the area containing GFP^+

was measured in every fifth section (30 µm). The volume was calculated by multiplying these areas by the distance between the sections and summing the results. Fourteen to sixteen sections covering the whole extent of virus expression were used for calculations.

To evaluate the transfection efficacy in neuronal cultures, 24-29 non-overlapping images were captured along the axis of the culture round coverslip, using different filters for GFP, AlexaFluor 568/Texas Red, and Hoechst, with a Nikon 10x/0.30 DIC L/N1 objective. The numbers of GFP^+ cells, NeuN+ neurons, and double-stained $NeuN^+/GFP^+$ neurons were assessed. The percentage of NeuN+ neurons containing GFP was calculated.

Figures were prepared using Adobe Photoshop 7.0 and CorelDRAW 12. Brightness and contrast were adjusted to regain the sections' natural appearance.

Statistical analysis

The non-parametric Mann-Whitney test was used to investigate the differences in the latency of electrographic seizures in PTZ challenge and PTZ kindling results. The Student's t-test was used to analyze the behavioral hyperexcitability test, open-field test, elevated plus maze test, MTT test, and PTZ challenge. The Chi-square test was used to determine the number of animals that developed tonic-clonic behavioral seizures in the PTZ challenge. A one-way ANOVA for repeated measures was performed to examine the development of kindling. Statistical significance was calculated using GraphPad Prism software (version 5.0, GraphPad Software) and determined for *p<0.05, **p<0.01, and ***p<0.001. Results are presented as a mean ± standard error (SEM).

RESULTS

AAV-viral constructs efficiency *in vitro* and *in vivo*

Expression of the AAV transgene used in the study was evaluated *in vivo*. Immunofluorescence staining revealed the cellular localization of viral expression using antibodies directed against neuronal and astrocyte markers. AAV for Mbd3 downregulation (sh(MBD3)), as well as control AAV (sh(scrambled)) expression, was primarily observed in neurons (Fig. 1A, C) and only incidentally in astrocytes (Fig. 1B, D). Product of sh(MBD3) spread in the volume of 228.6±40.6 µm3 and was detected in 70.34±8.4% of neurons, while the product of control AAV (sh(scrambled)) was observed in 71.8±6.6% of neurons.

Changes in Mbd3 expression levels in the amygdala selectively influence animal behavior

To test the influence of downregulation of Mbd3 in the amygdala on behavior, we injected an AAV that reduces Mbd3 expression (sh(MBD3), n=20), or control AAVs (sh(scrambled), n=19), into the basolateral amygdala (BLA). After the recovery period, the following behavioral tests were conducted: the behavioral hyperexcitability test (day 14 of the experiment), the open field test (day 17 of the experiment), and the elevated plus maze test (day 18 of the experiment).

The behavioral hyperexcitability and elevated cross-maze tests showed no differences between groups. Interestingly, in the open field test, animals with reduced Mbd3 expression (sh(MBD3)) spend more time in the outer zones of the arena (sh(scrambled): 264.1±19.1 *vs.* sh(MBD3): 338.2±29.3 [s]; p=0.043) while less time in the inner arena zone compared to control animals (sh(scrambled): 927.2±19.0 *vs.* sh(MBD3): 854.4±28.7 [s]; p=0.043) (Fig. 2A, B).

Downregulation of Mbd3 prolongs the latency time to tonic-clonic seizure in a model of acute seizure induced by intraperitoneal injection of PTZ

Since Mbd3 is suspected of having proepileptic function, we hypothesized that a decrease in Mbd3 protein level would affect the seizure threshold. To verify this, we performed the PTZ challenge test in animals with a shMbd3-evoked decrease in Mbd3 levels.

During the PTZ challenge, 100% (n=10) of the control animals developed generalized tonic-clonic seizures. In comparison, only 70% (n=7) of the animals with reduced Mbd3 expression developed generalized tonic-clonic seizures, and 30% (n=3) of the animals with reduced Mbd3 expression did not. This difference, however, was not significant (p=0.075, Chi-square test). Interestingly, a statistically significant increase in latency time to electrographic seizures was observed in animals with reduced Mbd3 expression compared to control animals (sh(scrambled) PTZ: 79.5±11.7 *vs.* sh(MBD3) PTZ: 261.3±70.8 [s]; p=0.021; n=10) (Fig. 2C).

Mbd3 downregulation does not influence epileptogenesis in the PTZ kindling model

No differences were observed between the group with reduced Mbd3 expression (sh(MBD3)) and control animals in any of the studied kindling parameters: the number of sessions to reach the criterion

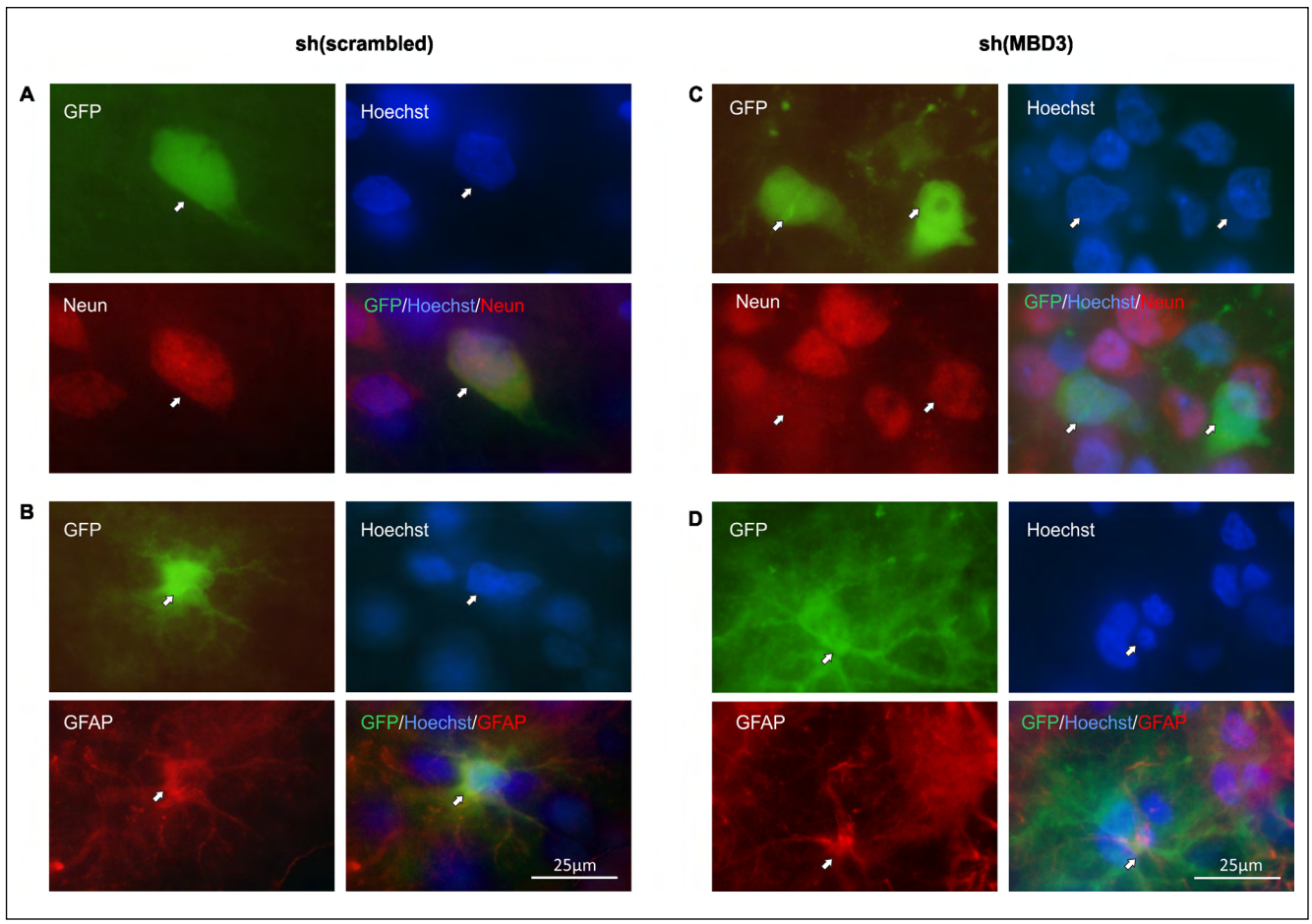

Fig. 1. Cellular localization of control AAV (sh(scrambled)) and AAV for Mbd3 downregulation (sh(Mbd3)) in the rat brain. Representative images of the BLA region of the rat brain infused with AAV viruses with the sequence for Mbd3 downregulation sh(MBD3) (right panels, green), or a control sequence sh(scrambled) (left panels, green). Sections were immunostained using an anti-Neun antibody (neuronal marker, red) (A, C) or an anti-GFAP antibody (astrocyte marker, red) (B, D), and Hoechst (cell nucleus marker, blue) (A-D). Arrows indicate neurons (A, C) or astrocytes (B, D). Expression of transgenes from the AAV constructs under the control of the U6 promoter was primarily observed in neurons (A, C) and a very few astrocytes (B, D); scale: 25 µm.

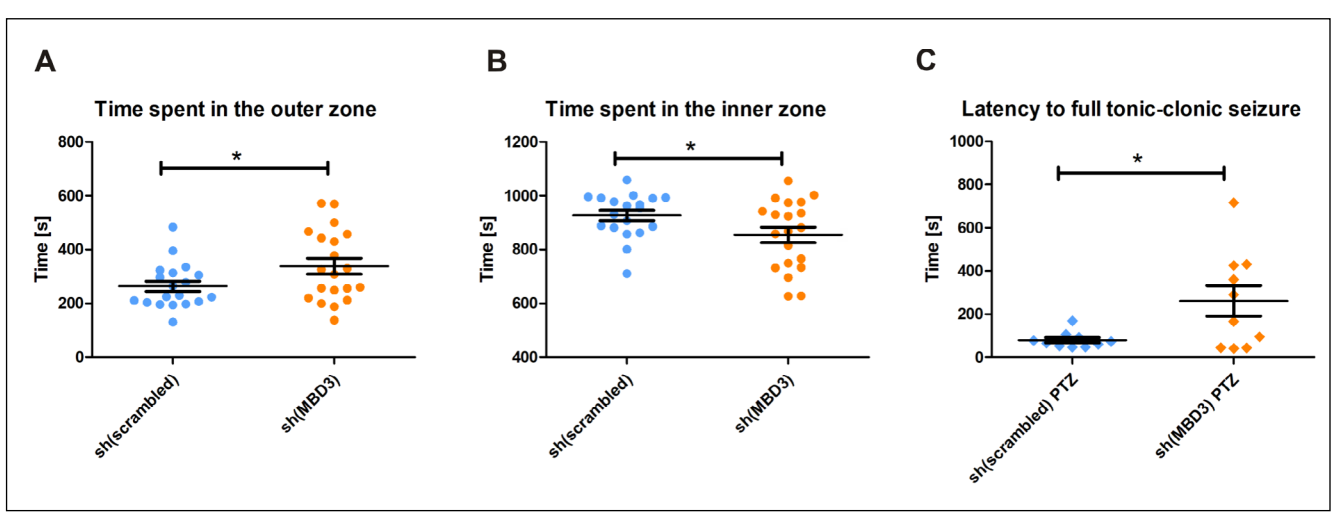

Fig. 2. Open field test performance and PTZ-induced acute seizures upon Mbd3 downregulation. (A) time spent in the outer zone; (B) time spent in the inner zone; (C) latency time to the electrographic seizure evoked by PTZ injection (mean ± SEM, Mann-Whitney test, n=10, *p<0.05).

(Fig. 3A), the number of sessions from the first 4/5 grade seizure to the session of reaching the criterion or death (Fig. 3B), and number of session with stage 4/5 seizures (Fig. 3C). Mortality in this experiment was 10% (n=1) in the group of animals with reduced Mbd3 expression and 10% (n=1) in the group of control (sh(scrambled)) animals.

DISCUSSION

In the present study, we demonstrated that Mbd3 downregulation in the basolateral amygdala (BLA) affects anxiety-related behavior and significantly delays the onset of acute seizures induced by PTZ, without altering the progression of epileptogenesis in the PTZ kindling model. These findings suggest a modulatory role for Mbd3 in seizure susceptibility and emotional processing, possibly mediated through epigenetic regulation of gene expression.

The observed anxiogenic effect, indicated by reduced time spent in the center of the arena in the open field test, highlights a previously underappreciated role of Mbd3 in regulating emotional behavior. This aligns with the well-established function of the BLA in processing anxiety and fear responses (Duvarci & Pare, 2014; Janak & Tye, 2015; Boulasiki et al., 2023). The NuRD complex, of which Mbd3 is a central component, is known to modulate gene expression profiles involved in synaptic plasticity and neurodevelopmental processes, all of which can contribute to anxiety phenotypes when dysregulated (D'Souza et al., 2021; Boulasiki et al., 2023). Mbd3 downregulation did not affect performance in the elevated plus maze or hyperexcit-

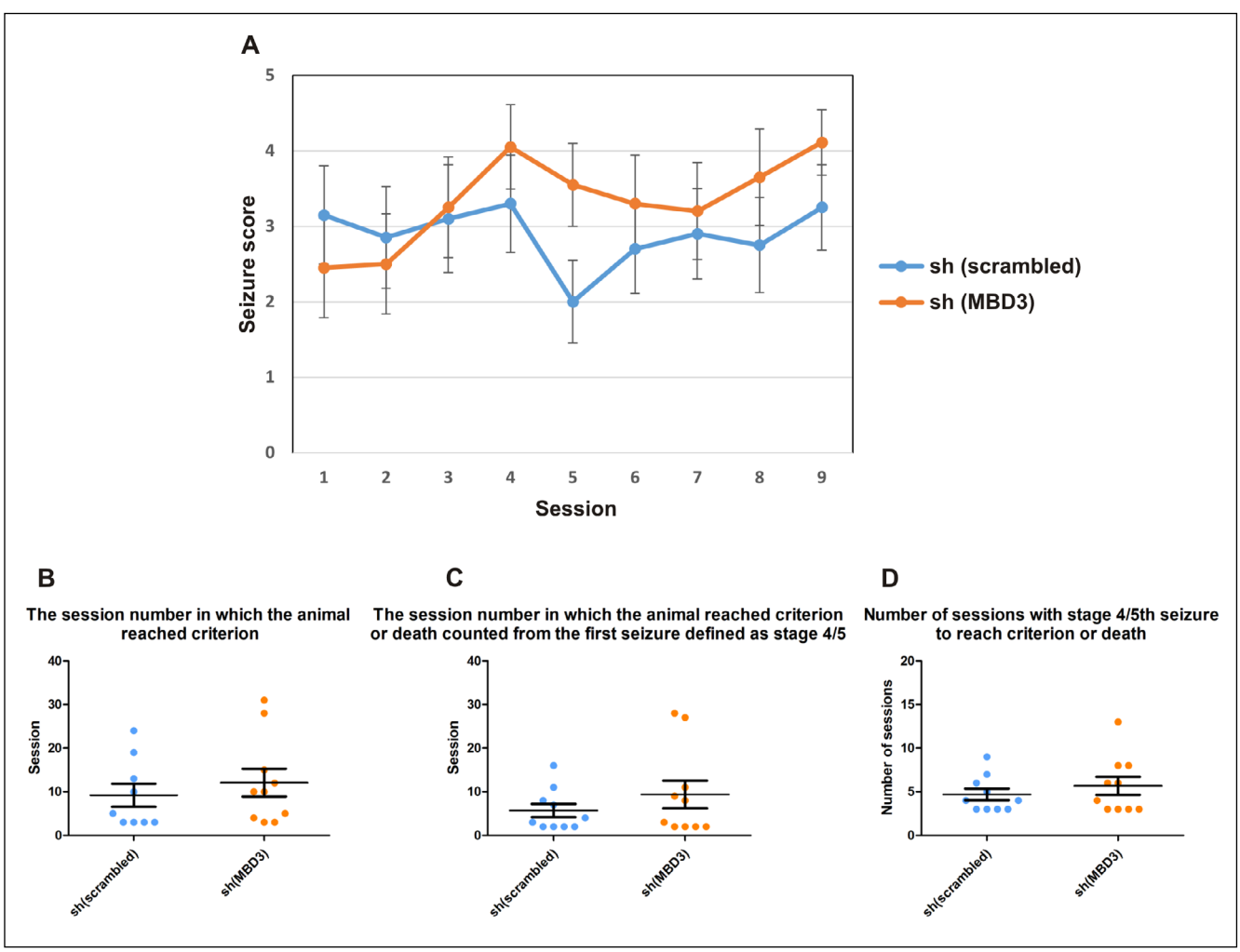

Fig. 3. Effect of Mbd3 downregulation on PTZ kindling. (A) Seizure development in control animals (sh(scrambled)) and animals with Mbd3 downregulation (sh(Mbd3)) during first nine kindling sessions (mean seizure score ± SEM, ANOVA for repeated measures, *p<0,05, **p<0,01); (B) The session number in which animals reached criterion; (C) The session number in which animal reached the criterion or death counted from the first score 4/5 seizure; (D) number of sessions with a score 4-5 until the criterion or death (mean ± SEM, Mann Whitney test, n=10, *p<0.05).

ability tests. This suggests that the anxiety-related behavioral changes are task-specific and possibly reflect altered risk assessment or exploratory behavior rather than general arousal or motor activity.

Our results also support the role of Mbd3 in modulating seizure susceptibility. Rats with reduced Mbd3 expression exhibited a significant increase in latency to electrographic seizure onset following PTZ administration. This suggests that Mbd3 facilitates seizure initiation, potentially by modulating neuronal excitability through epigenetic repression of genes involved in inhibitory neurotransmission or ion channel regulation. This finding is consistent with prior work implicating chromatin remodeling complexes in seizure thresholds and epileptogenesis (Kobow et al., 2009; Henshall & Kobow, 2015; Kobow & Blümcke, 2018). For example, dysfunction of MeCP2 and other methyl-binding proteins has been linked to increased seizure susceptibility in human and animal models (Samaco et al., 2009; Zhang et al., 2010).

However, the protective effect of Mbd3 downregulation did not extend to the PTZ kindling model of chronic epilepsy. No differences were observed in kindling acquisition, severity, or progression. This dissociation suggests that Mbd3 may play a more prominent role in acute seizure thresholds rather than the long-term plasticity changes necessary for epileptogenesis. The kindling process involves cumulative changes in gene expression, network remodeling, and synaptic reorganization. It is possible that compensatory mechanisms or redundancy in epigenetic pathways can buffer the effects of Mbd3 loss in chronic models.

Interestingly, previous findings from our group demonstrated that overexpression of Mbd3 accelerates epileptogenesis, pointing to a dose-sensitive, bidirectional effect of Mbd3 in seizure regulation (Nizinska et al., 2023). This duality highlights the crucial role of tightly regulated NuRD complex activity in maintaining homeostasis within neural circuits. It also raises the possibility that therapeutic targeting of Mbd3 must consider the direction and magnitude of intervention.

From a translational perspective, Mbd3 and the NuRD complex represent intriguing targets for therapeutic modulation in epilepsy. Given their involvement in epigenetic regulation, future work could explore pharmacological or gene-editing approaches to selectively modulate Mbd3 activity in specific brain regions. Furthermore, it remains to be determined whether Mbd3-mediated changes in seizure threshold are specific to chemically induced seizures or whether similar effects are seen in other models of epilepsy, such as kainic acid or traumatic brain injury models.

CONCLUSIONS

Our findings indicate that Mbd3 downregulation in the amygdala increases seizure threshold and anxiety-like behavior but does not affect epileptogenesis. These results suggest Mbd3 as a potential modulator of acute seizure susceptibility and emotional regulation, warranting further investigation as a therapeutic target in epilepsy.

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