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Cell injury and receptor expression in the epileptic human amygdala

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ABSTRACT

Neuropathological findings in the amygdala obtained from patients with mesial temporal lobe epilepsy (MTLE) indicate varying degrees of histopathological alterations, such as neuronal loss and gliosis. The mechanisms underlying cellular damage in the amygdala of patients with MTLE have not been fully elucidated. In the present study, we assess cellular damage, determine the receptor expression of major inhibitory and excitatory neurotransmitters, and evaluate the correlation between the expression of various receptors and cell damage in the basolateral complex and the centromedial areas in the amygdala specimens resected during brain surgery on 30 patients with medically intractable MTLE. Our data reveal an increased rate of cell damage and apoptosis as well as decreased expression levels of several GABAergic receptor subunits ($GABA_AR\alpha1$, $GABA_AR\beta3$, and GABABR1) and GAD_{65} in the amygdalae obtained during epilepsy surgery compared to autopsy specimens. Analyses of the expression of glutamate excitatory receptor subunits (NR1, NR2B, mGluR1α, GluR1, and GluR2) reveal no significant differences between the epileptic amygdalae and autopsy control tissues. Furthermore, the increased occurrence of apoptotic cells in the amygdala is negatively correlated with the reduced expression of the studied GABAergic receptor subunits and GAD_{65} but is not correlated with the expression of excitatory receptors. The present data point to the importance of GABAergic neurotransmission in seizure-induced cell injury in the amygdala of patients with MTLE and suggest several GABA receptor subunits as potential druggable target structures to control epilepsy and its comorbid disorders, such as anxiety.

1. Introduction

Mesial temporal lobe epilepsy (MTLE), the most common type of focal epilepsy, is associated with structural and functional abnormalities in mesial structures of the temporal lobe, particularly in the hippocampus and amygdala. Hippocampal damage and its role in the symptomatology of MTLE have been well studied and our understanding of the molecular and cellular mechanisms underlying human epileptogenesis is largely based on data obtained from this brain structure. However, little attention has been paid to the changes occurring in the human amygdala during the course of epileptogenesis (Kullmann, 2011; Yilmazer-Hanke et al., 2016).

Functional integration of the temporal lobe is implemented by extensive interconnection and multiple pathways mediating direct and indirect communications among different structures of this brain region and beyond (Lopes da Silva et al., 1990). Animal studies have suggested that seizure activities originating in the amygdala or the hippocampus may cause damage to other structures of the temporal lobe or to the surrounding cortex (Mascott et al., 1994; Supcun et al., 2012). Shrinkage of the amygdala has been suggested to follow the reduction

Abbreviations: BLC, the basolateral complex; CM, the centromedial nucleus; MFI, mean fluorescence intensities; MTLE, mesial temporal lobe epilepsy; NGS, normal goat serum; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling

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Table 1
Clinical data of patients.

No	Gender	Age (year)	Seizure duration (year)	Seizure frequency	Drugs	Seizure free after surgery	Psychiatric disorders	Lesion site	MRI
1	M	30	18	weekly	VPA, CBZ	yes	yes	R	Sclerosis
2	F	50	30	monthly	BHP, PHT, LTG, LOR	yes	yes	L	Sclerosis
3	M	18	9	monthly	TPM, LTG, VPA	yes	no	L	Sclerosis
4	M	31	19	weekly	CBZ, VPA, SER	yes	yes	L	Sclerosis
5	M	50	28	daily	CBZ, TPM, PRM, FLUX	yes	yes	L	Sclerosis
6	M	34	31	weekly	CBZ	yes	no	L	Sclerosis
7	M	46	33	daily	CBZ, PHT, CLB	yes	yes	L	Sclerosis
8	F	38	29	monthly	CBZ, LTG	yes	no	L	Sclerosis
9	M	28	20	weekly	CBZ, TPM, VPA	yes	no	R	Sclerosis
10	M	18	4	weekly	CBZ, TPM	yes	no	L	Sclerosis
11	F	17	12	weekly	VPA, CBZ	no	yes	L	Dysplasia
12	M	23	10	daily	VPA, CBZ, LEV	yes	no	L	Sclerosis
13	M	35	32	monthly	CBZ, VPA, LOR	yes	yes	R	Sclerosis
14	M	46	20	monthly	LEV, CBZ, PHT	no	no	L	Sclerosis
15	F	46	39	monthly	LEV, CBZ, LOR	yes	yes	R	Sclerosis
16	F	39	35	monthly	CBZ, LTG, LEV	yes	no	R	Sclerosis
17	M	35	8	weekly	CBZ, LEV	yes	no	R	Sclerosis
18	M	42	19	weekly	OCBZ, LEV	yes	no	L	Sclerosis
19	M	30	28	weekly	CBZ, VPA	yes	no	R	sclerosis
20	M	25	10	monthly	VPA, CLZ	yes	no	R	Tumor
21	F	27	16	weekly	LTG, CBZ, LEV	yes	no	L	Sclerosis
22	M	26	23	weekly	CBZ, LTG, VPA	yes	no	L	Sclerosis/ dysplasia
23	M	14	12	weekly	LEV, VPA, PRM	yes	no	L	Sclerosis
24	M	35	25	monthly	CBZ, CLZ	yes	no	L	Sclerosis
25	M	47	30	weekly	CBZ, PHT, CLB	yes	no	R	Sclerosis
26	M	30	26	daily	CBZ, TPM, VPA, LEV	yes	yes	L	Sclerosis
27	F	30	14	weekly	CBZ, LTG, FLUX	yes	yes	R	Sclerosis
28	F	21	14	weekly	CBZ, TPM	yes	no	L	Sclerosis
29	F	33	31	weekly	PHB, CBZ, FLUX	no	yes	L	Sclerosis
30	F	27	11	weekly	CBZ, CLZ, PRM, LTG	yes	no	L	Sclerosis

CBZ, carbamazepine; CLB, clobazam; F, female; LAM, FLUX; Fluoxetine, lamotrigine; LEV, Lorazepam; LOR, levetiracetam; LTG, lamotrigine; M, male; MRI, magnetic resonance imaging; OCBZ, oxcarbazepine; PHB, phenobarbital; PHT, phenytoin; PRM, primidone; TPM, Sertraline; SER, topiramate; Valproate, VPA. Seizure duration (self-reported); Seizure frequency (Daily ≥1 per 24 h, Weekly ≥1 per 7 days, Monthly ≥1 per 30 days).

of the volume of the hippocampus in MLTE (Bernasconi et al., 2005). More pronounced amygdaloid atrophy has been observed in epileptic patients who more commonly had prolonged febrile convulsions and secondarily generalized seizures (Cendes et al., 1994). Patchy neuronal loss and gliosis in the human amygdala, especially in the ventral portion of the lateral nucleus, have been observed in most investigations of both adults and children with MLTE or status epilepticus since the 1950s (Pitkänen et al., 1998). Animal studies have revealed that both neurons and interneurons of different nuclei of the amygdala are susceptible to seizure induced damage (Tuunanen et al., 1996; Pitkänen et al., 1998). Damage of the excitatory spiny projection neurons and GABAergic interneurons in the medial and ventral regions of the lateral amygdala associated with patchy fibrillary gliosis may play a role in epileptogenesis and comorbid symptoms of medically intractable MTLE patients (Yilmazer-Hanke et al., 2016). Indeed, improved long-term outcomes have been observed in most of the patients who underwent epilepsy surgery when both the amygdala and the hippocampus were resected (Schramm, 2008).

Most of the investigations in human epilepsy have focused on both excitatory and inhibitory receptor alterations in the sclerotic hippocampus. These studies provided detailed information on receptor expression and putative alterations in subunit composition of different receptors in these brain structures (Das et al., 2012; Cendes et al., 2014). Changes in the subunit compositions of inhibitory and/or excitatory receptors may result in a reduction of seizure threshold and contribute to brain hyperexcitability and seizure generation in epileptic patients (Sánchez Fernández and Loddenkemper, 2014). In spite of these intense investigations in the hippocampus and neocortex, data focusing on the expression of inhibitory and excitatory receptors in the amygdala of epileptic patients are scarce. An increased expression of NR1 mRNA in the amygdala of 27 patients with MTLE has been shown (de Moura et al., 2012). A significantly higher number of binding sites of glutamate AMPA and kainate subreceptors, metabotropic glutamate type 2/3 receptors, muscarinic type 2 and adrenoceptor α1 as well as lower muscarinergic type 3 and serotonergic type 1A receptor densities in the lateral amygdala of another 26 MTLE patients have been reported (Graebenitz et al., 2011).

We performed our investigations on amygdala tissues that were obtained during brain surgery on patients with medically refractory MTLE. Our experimental plans were: (*i*) to assess cellular damage and apoptosis in different regions of the amygdala; (*ii*) to determine the receptor expression of major inhibitory and excitatory neurotransmitters in the basolateral complex (BLC) and the centromedial nucleus (CM) of the amygdala; and (*iii*) to evaluate the correlation between the expression of various receptors and cell damage in the epileptic amygdala specimens. The findings in surgical specimens of patients with MTLE were systematically compared to data from the amygdaloid tissues obtained from autopsy controls.

2. Materials and methods

The research protocol was approved by the local ethics committee of Shefa Neuroscience Research Center, Tehran, Iran and informed consent was obtained from all patients. Human tissue obtained was a portion of the tissue which is excised for treatment of medically intractable focal epilepsy originating in the temporal lobe. All patients underwent pre-surgical evaluation and removal of the temporal lobe was indicated to achieve seizure control in these patients. The predominant seizure types were focal impaired awareness seizures. As defined by the International League Against Epilepsy, the patients had drug-resistant epilepsy which were resistant to adequate trials of at least two tolerated, appropriately chosen, and used antiepileptic drug

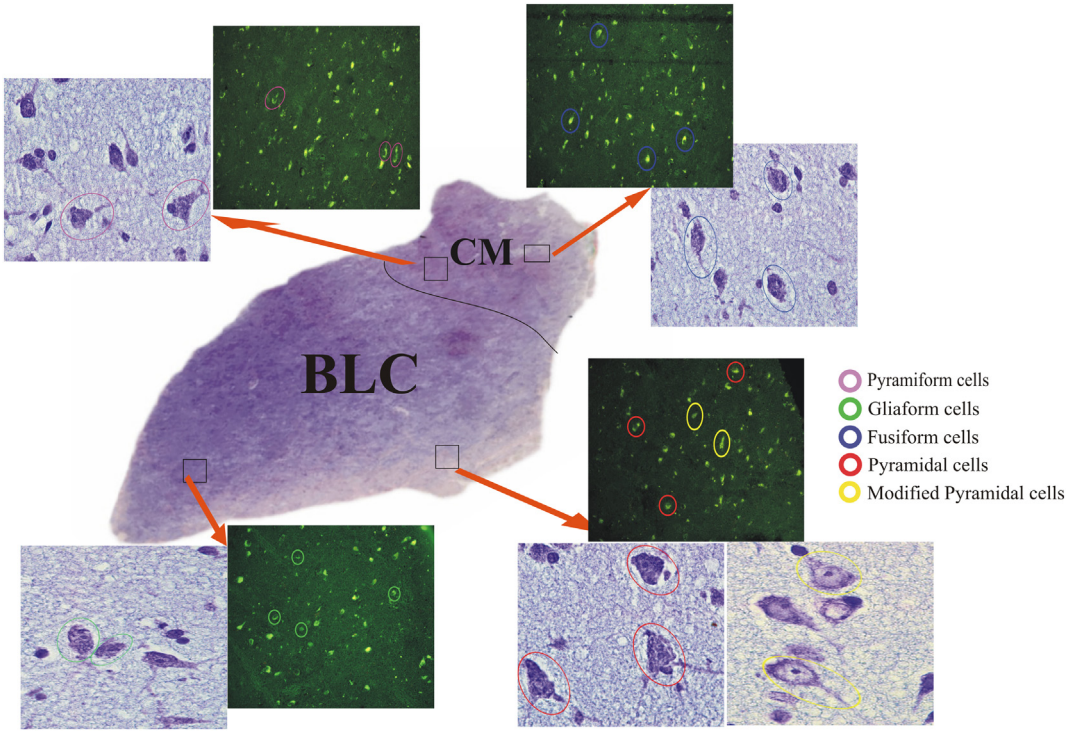

Fig. 1. Identification of different regions of the resected human amygdala obtained during epilepsy surgery. Characteristic neurons (pyramidal, modified pyramidal and gliaform) are identified in the basolateral complex (BLC; pyramidal, modified pyramidal and gliaform) and the centromedial nuclei (CM; pyramiform and ovoid or fusiform types that have been also known as medium spiny neurons). Magnification of Nissl stains is 100× and magnification of immonoreactive cells is 20×.

schedules (Kwan et al., 2010). The amygdalae were obtained from 30 patients (mean: 32.4 ± 10.2 years, median: 30.5, 25.7–39.7) with MTLE during epilepsy surgery in Khatam Hospital, Tehran, Iran between 2011 and 2016. The brain surgery consisted of temporal lobectomy including surgical resection of the amygdala, the parahippocampal gyrus and the hippocampal formation. Detailed patient characteristics, including age, gender, seizure duration and frequency, medication, and magnetic resonance imaging results, are given in Table 1. As control tissue, the amygdalae were obtained during autopsies performed on bodies from the body donor program of the Legal Medicine Organization, Tehran, Iran. The control subjects (mean: 44.5 ± 11.3 years; median: 45 years, 34–51.5) had no known history of psychiatric and/or neurological disorders and causes of death were cardiac arrest (n = 6), cardiorespiratory insufficiency (n = 3), multiple organ failure (n = 2), and abdominal trauma (n = 2). Autopsy delay varied between 2 and 12 h. There was no significant difference in age, gender, and hemisphere between patients and control subjects.

2.1. Morphological evaluation

The brain tissue was surgically removed *en bloc* and cut into a 5 × 6 mm dimension for every block within 1–2 min after surgical resection and then immerged in 4% paraformaldehyde solution for 1 week before they were processed for histological studies. Tissue sections were selected by random systemic sampling from each specimen and stained with cresyl violet. BLC and CM areas were studied under a light microscope (BX51, Olympus, Japan) connected to a digital camera. Microphotographs were taken with 40× and 100× objective lenses (Olympus, Japan). Every 10th section was used for the identification of cells and the next section was processed for immunohistochemistry studies.

Depending on the functional connectivity and the cellular architecture, two main clusters in the amygdala are recognized; a superficial BLC and a deeper CM nucleus (McDonald, 1998; Mosher et al., 2010). The BLC in the human brain exhibits three major cell types; pyramidal neurons, modified pyramidal neurons, and gliaform neurons (Tosevski et al., 2002). CM areas contain two main cell types; fusiform or ovoid-shaped neurons and pyramiform neurons (Schiess et al., 1999; Sah et al., 2003). To identify discrete populations of BLC and CM neurons in the amygdala within the specimens, the characteristic features of the neurons in each area were evaluated by both light- and immunofluorescent microscopes (Fig. 1).

2.2. Histopathological assessment

Five successive uniform random sections were selected from each resected specimen and were stained with cresyl violet (Nissl). Different areas of the amygdala tissue, including the BLC and CM nucleus, were studied under a light microscope (BX51, Olympus, Japan) coupled to a digital camera (BX51, Olympus, Japan) and images were taken under an objective lens (40×). The magnification was calculated using an objective micrometer. Several micrographs were taken to calculate the number of dark cells in different sub-regions of the amygdala in control and epileptic tissues (Jafarian et al., 2010 and Jafarian et al., 2015).

2.3. Neuronal apoptosis assay

After deparaffinization of sections, three sections from each specimen were selected to detect DNA fragmentation. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) was performed by using an in situ Cell Death Detection Kit® (Roche, Mannheim, Germany), as previously represented (Jahanbazi Jahan-Abad et al., 2018). Briefly, sections were deparaffinized and rehydrated at 60 °C, followed by xylene wash, and rehydration with diluted alcohol. After being washed with 10 mMTris–HCl (pH = 7.6), sections were incubated in methanol containing 0.3% H_2O_2 for 10 min to quench endogenous peroxidase activity. These sections were then treated with proteinase K (Roche, 20 µg/ml in Tris buffer) at 37 °C for 30 min. The sections were incubated in TUNEL reaction mixture (450 µl of label solution and 50 µl of enzyme solution) at 37 °C for 60 min and

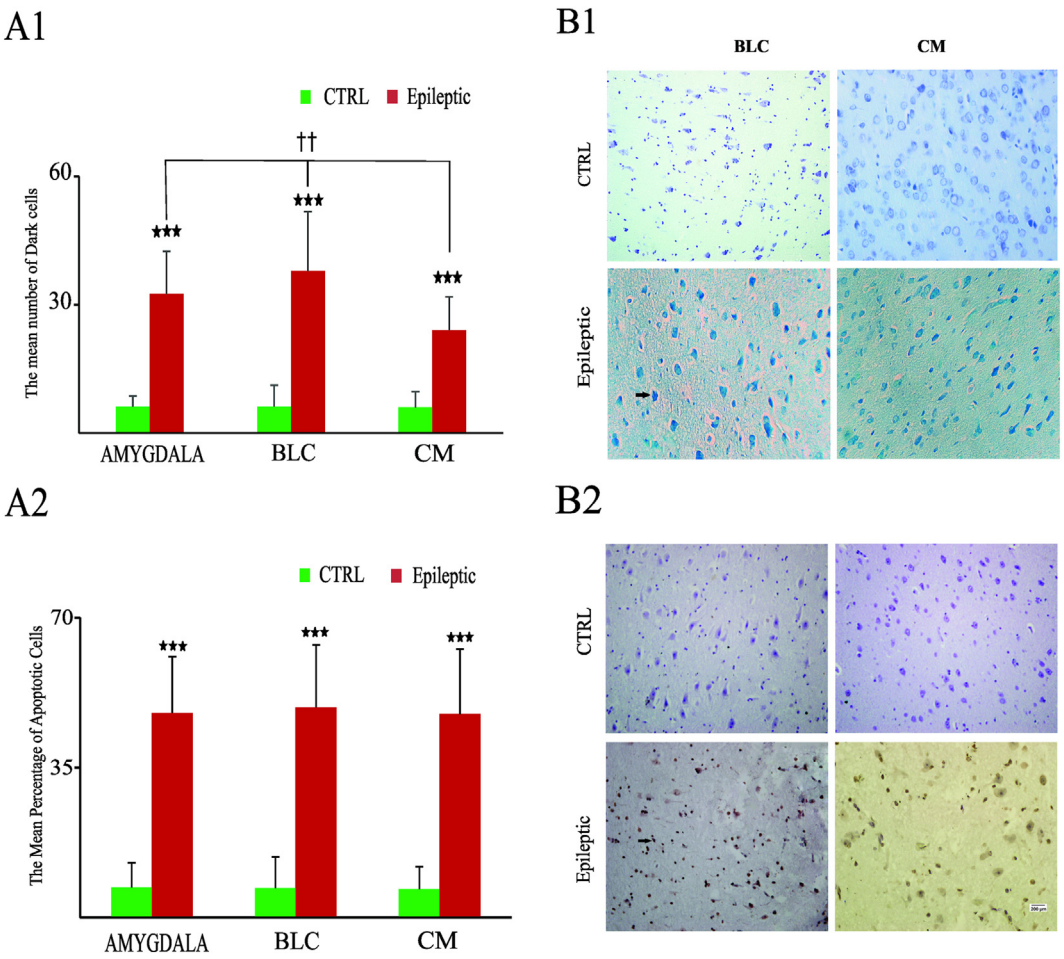

Fig. 2. Evaluation of dark cells and TUNEL-positive cells in the resected human amygdala obtained during epilepsy surgery and in control autopsy tissues. The mean percentage of dark cells and TUNEL-positive cells were analyzed in the epileptic amygdala and compared to the control tissue. A1-A2: The mean percentage of dark cells and TUNEL-positive cells in the basolateral complex (BLC) and the centromedial nuclei (CM) of the amygdala in the epileptic and autopsy brain specimens. The mean percentage of dark cells was significantly higher in the epileptic amygdala compared to the control tissues in BLC and CM, In addition, a significant lower percentage of dark cells was observed in CM compared to BLC and whole amygdala in epileptic tissues (A1). B1-B2: Photomicrographs of dark cells and TUNEL-positive cells (40× objective lens) in the BLC and CM areas of the epileptic and control amygdala tissues. The arrows denote dark cell (B1) and apoptotic cell (B2). *** $P \leq .001$ and $_{\#}P < .002$.

Table 2
Clinical data of autopsy control patients.

No	Gender	Age (year)	Autopsy delay (hour)	Etiology
1	M	57	6	Cardiorespiratory insufficiency
2	M	30	10	Cardiorespiratory insufficiency
3	M	30	11	Multiple organ failure
4	F	50	5	Cardiac arrest
5	M	53	8	Cardiac arrest
6	M	68	11	Multiple organ failure
7	M	47	2	Cardiac arrest
8	M	38	7	Cardiac arrest
9	M	48	9	Abdominal trauma
10	M	45	5	Abdominal trauma
11	M	30	7	Cardiorespiratory insufficiency
12	M	39	4	Cardiac arrest
13	M	44	12	Cardiac arrest

then in horse-radish peroxidase solution (Santa Cruz, Heidelberg, Germany) for 30 min. The color reaction was developed by applying 3–3′-diaminobenzidine (DAB, Roche; 0.5 µl DAB and 1.5 µl peroxide buffer) for 5–10 min, and was counterstained with hematoxylin. A set of sections was incubated in the absence of TUNEL as a negative control. Images were acquired at 40× magnification.

2.4. Immunocytochemistry assay

Paraffin embedded sections with 8 mm thickness were mounted on glass slides. Slides were then cleared and rehydrated through serial application of xylol and washed with phosphate buffered saline (PBS; pH 7.4) three times. The sections were treated with 1% trypsin for 30 min and then the slides were incubated in 10% normal goat serum (NGS) and 0.2% TritonX-100 in PBS for 1 h at room temperature. The slides were next washed with PBS three times and incubated overnight at 4 °C with commercial primary antibodies (Santa Cruz, Germany) in a solution containing 1% NGS in 0.3% Triton X-100 and 0.1 M PBS at pH 7.4. The sections were thereafter rinsed three times with PBS (10 min per rinse) and incubated with FITC antibodies (Jackson Immune Research Labs, USA) diluted at 1:650 in PBS with 0.3% Triton X-100 and 5% NGS at 22 °C for 1 h. After several PBS washes, the slides were counterstained with povidone/iodine (1:800 *v*/v) solution. Finally, the slides were cover slipped with 90% glycerol mounting buffer and data was acquired using a fluorescence microscope (BX51WI; Olympus, Tokyo, Japan). Negative control staining was performed without the primary antibodies. Intensity of primary antibody immunoreactivity in different brain regions was quantified by measuring the relative optical densities. Images were acquired at low magnification (20×) and the background values were measured in

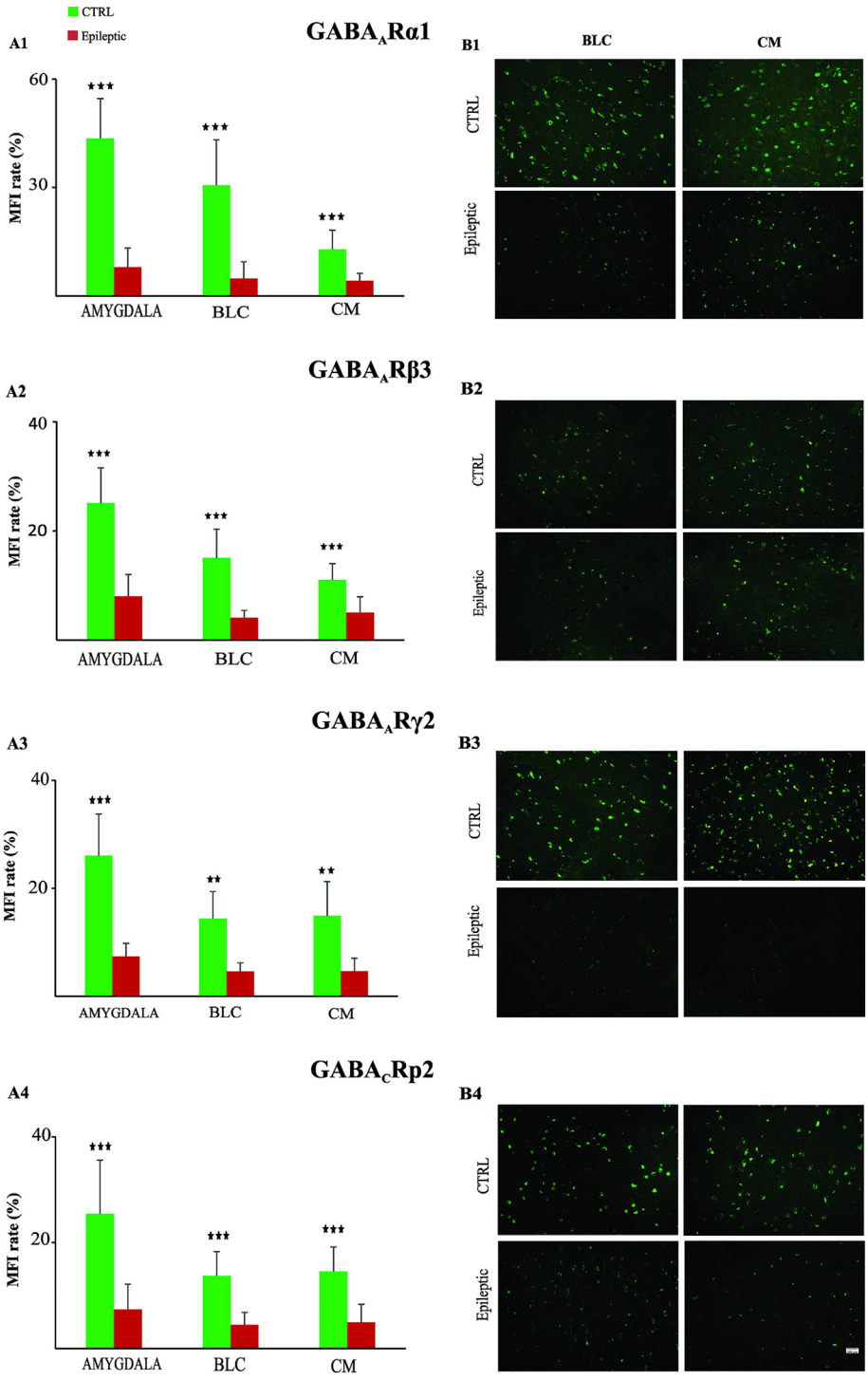

Fig. 3. The expression of the ionotropic GABA$_A$Rα1, β3, γ2 and GABA$_C$Rp2 receptor subunits in the resected human amygdala obtained during epilepsy surgery and in control autopsy tissues. A1-A4: Bar charts summarizing the mean fluorescence intensities (MFI) of the GABAARα1, β3, γ2 and GABACRp2 receptors subunits in the basolateral complex (BLC) and the centromedial nuclei (CM) of the amygdala in the epileptic and autopsy brain tissues. B1-B4: Representative micrographs of immunohistological sections of the GABA$_A$Rα1, β3, γ2 and GABA$_C$Rp2 staining in the BLC and CM areas of the epileptic and control groups. ** and *** indicate $P \leq .05$ and, $P \leq .001$, respectively.

unlabeled areas (Karimzadeh et al., 2017).

A digital camera attached to the microscope was used for imaging. The Mean fluorescence intensity of inhibitory GABA$_A$ (Rα1, Rβ3, and Rγ2), GABA$_B$ (R1 and R2), GABA$_C$ (Rp2)and GAD-65 as well as excitatory glutamate receptors NMDA (NR2B, NR1, and mGluR1α) and AMPA (GluR1, GluR2) subunits were determined for both epileptic and control tissues in the amygdala BLC and CM areas.

2.5. Statistical analysis

All data are expressed as mean ± standard deviation as well as median (25th–75th percentile). For two-group comparisons, statistical study was performed using the two-tailed Student's unpaired or paired *t*-test for normally distributed data or the non-parametric Mann-Whitney *U* test for data not distributed normally. The Shapiro-Wilk test was used to test for normality. To compare the level of cell death between different brain regions, we used “repeated measures ANOVA” followed by Bonferroni post-hoc. With regard to the assumptions for repeated measures ANOVA, we did perform both Levene’s test for equality of variances for ‘between subject’ factors, as well as Mauchly’s test of sphericity for “within subject” variables. Spearman's Rho correlation test was performed to evaluate a relationship between receptor expression and apoptotic cells in the amygdala. Scatter plots were drawn to ensure the monotonicity of relationship between variables,

Table 3
The mean percentage of dark cells and apoptotic cells in the epileptic and autopsy (control) amygdala tissues. BLC, the basolateral complex; CM, the centromedial nucleus; SD; standard deviation. Data are presented as median (25th, 75th %).

			Median (25th, 75th %)
Dark cells	Total Amygdala	Control	5.5 (4.3, 8.4)
		Epileptic	32.3 (24.7, 36.2)
	BLC	Control	5.4 (2.8, 9.8)
		Epileptic	37.5 (26.5, 45.5)
	CM	Control	5 (2.9, 8.9)
		Epileptic	20.9 (18.8, 30.6)
Apoptotic cells	Total Amygdala	Control	2.3 (0.7, 6.8)
		Epileptic	52.4 (41, 71.1)
	BLC	Control	5.7 (4.4, 13.2)
		Epileptic	57.7 (42.1, 71.1)
	CM	Control	3.3 (2.5, 5.6)
		Epileptic	56.3 (40.1, 64.2)

before doing the correlation analyses. The value of $P < .05$ was considered as statistically significant.

3. Results

3.1. Number of dark cells

Cell shrinkage with surrounding spongiosis and eosinophilic cytoplasm as well as nuclear pyknosis are characteristics of dark cells (Söderfeldt et al., 1983; Jafarian et al., 2010). In our study the mean percentage of dark cells was significantly higher in the amygdala of epileptic patients compared to the control group ($P < .001$; Fig. 2 A1 and B1). Analyzing the occurrence of dark cells in the BLC and CM areas revealed that the mean percentage of dark cells in both regions was significantly higher in the epileptic tissues compared to the autopsy tissues ($P < .001$; Fig. 2 A1 and B1, Table 2). In addition, the mean percentage of dark cells in the CM nucleus was significantly lower compared to the BLC ($P < .002$; Fig. 2 A1 and B1, Table 2). There was no significant correlation between the length and severity of seizures in epileptic patients and the mean percentage of dark cells in the epileptic amygdala. In addition, there was no significant difference between the mean percentage of dark cells in the amygdala of patients with and without psychiatric disorders as well as in the seizure-free patients vs. patients who were not initially seizure free. There was no statistical difference in the mean number of dark cells between autopsy tissues obtained with delay by < 6 h and > 6 h.

3.2. Neuronal apoptosis

Micrographs of TUNEL-positive apoptotic cells in different regions of the amygdala of epileptic patients and control group are presented in Fig. 2. The mean percentage of apoptotic cells was significantly higher in the amygdala of the epileptic patients compared to the control tissues ($P < .001$; Fig. 2 A2 and B2). Furthermore, the mean percentage of TUNEL-stained apoptotic cells in the BLC and CM regions was significantly higher in the epileptic amygdala compared to the control tissues ($P < .001$; Fig. 2 A2 and B2, Table 2). There was no significant correlation between the length and severity of seizures in patients and the mean percentage of apoptotic cells in the epileptic amygdala. Furthermore, there was no significant difference between the mean percentage of apoptotic cells in the amygdala of patients with and without psychiatric disorders as well as in the seizure-free patients vs. patients who were not initially seizure free. There was no statistical difference in the mean number of apoptotic cells between autopsy tissues obtained with delay by < 6 h and > 6 h.

3.3. Immunohistochemistry studies

To identify the regional distribution of specific alterations of inhibitory and excitatory receptors, we carried out immunohistochemistry investigations in different regions of the epileptic amygdala tissues and compared these with controls. Distribution of some subunits of $GABA_A$ and $GABA_C$ receptors, members of transmitter-gated ion channels, including $GABA_AR\alpha1$, $GABA_AR\beta3$, $GABA_AR\gamma2$, $GABA_CRp2$ receptor subunits was investigated. The expression of these receptors was significantly lower in the BLC and CM regions of the epileptic amygdala compared to autopsy control tissues ($P < .001$; Fig. 3 A1-A4 and B1-B4, Table 3). Furthermore, studying of the expression of G-protein coupled $GABA_B$ receptors subunits $GABA_BR1$ and $GABA_BR2$ in the epileptic amygdala revealed a significant reduction compared to control tissues ($P < .001$; Fig. 4 A1-A2 and B1-B2, Table 3). GAD_{65} catalyzes the majority of brain GABA during seizure activities (Patel et al., 2006). The expression of GAD_{65} was also significantly lower in the epileptic amygdala compared to the control autopsy group ($P < .001$; Fig. 4 A3 and B3, Table 3).

Analyses of the expression of glutamate excitatory receptor subunits NR1, NR2B, mGluR1α, GluR1, and GluR2 revealed no significant differences between the amygdalae obtained during epilepsy surgery and autopsy tissues (Fig. 5 A1-A4 and B1-B4, Table 3). The data indicate a decrease of GABA inhibitory signals in the epileptic amygdala compared to controls, whereas excitatory neurotransmission seems to be intact. There was no statistical difference in the expression of studied inhibitory and excitatory receptors between autopsy tissues obtained with delay by < 6 h and > 6 h.

3.4. Correlation between apoptotic cells and receptor expression

We found a significant negative correlation between the increased incidence of apoptotic cells and the expression of various receptors in the amygdala tissues of the epileptic patients and autoptic controls. An enhancement of apoptotic cells was accompanied by a decrease in the expression of $GABA_AR\alpha1$ ($R^2 = 0.39$, $P = .03$), $GABA_AR\beta3$ ($R^2 = 0.72$, $P = .009$), $GABA_BR1$ ($R^2 = 0.37$, $P = .02$), and GAD_{65} ($R^2 = 0.44$, $P = .01$; Fig. 6). No significant correlation was found between apoptotic cell death and the expression of $GABAAR\gamma2$, $GABA_CRp2$, $GABA_BR1$, NR1, NR2B, mGluR1α, GluR1, and GluR2 receptor subunits (Table 4).

4. Discussion

The present data reveal an increased rate of cell damage as well as decreased expression levels of several GABAergic receptor subunits and GAD_{65} in the amygdalae obtained during epilepsy surgery compared to autopsy specimens. Furthermore, the increased occurrence of apoptotic cells in the amygdala is negatively correlated with the reduced expression of some GABAergic receptor subunits and GAD_{65} but is not correlated with the expression of excitatory receptors.

There are different types of neuronal populations with diverse morphology and functions in the amygdala (Tosevski et al., 2002; Schiess et al., 1999; Sah et al., 2003). The BLC, the principal source of afferent inputs to the amygdala, activates the CM area directly via glutamatergic pathways and inhibits the CM nucleus through activation of a relay of inhibitory GABAergic interneurons. The inhibitory GABAergic neurons of the CM area, which comprises the major output

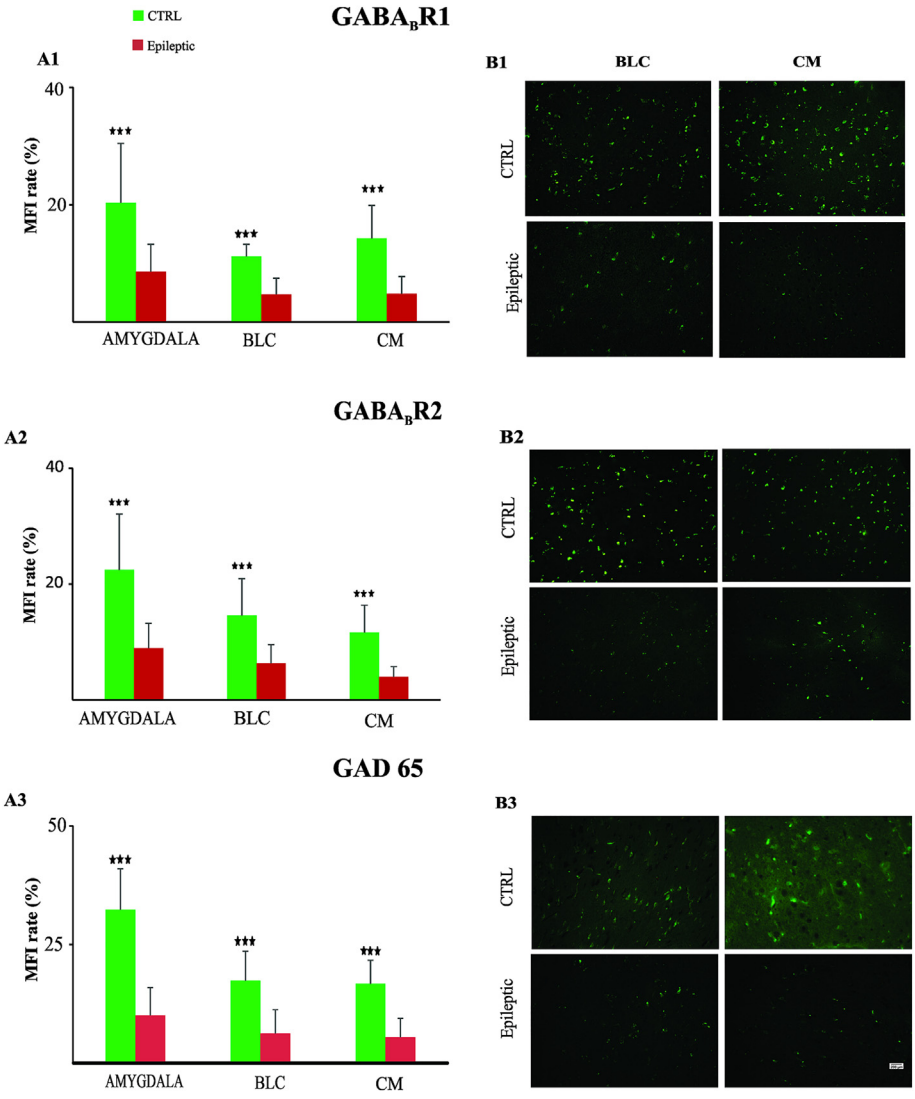

Fig. 4. The expression of the GABA$_B$R1 and GABA$_B$R2 receptor subunits as well as GAD$_{65}$ in the resected human amygdala obtained during epilepsy surgery and in control autopsy tissues. A1-A3: Bar charts summarizing the mean fluorescence intensities (MFI) of the GABA$_B$R1 and GABA$_B$R2 subunits as well as GAD$_{65}$, an enzyme that catalyzes the decarboxylation of glutamate to GABA, in the basolateral complex (BLC) and the centromedial nuclei (CM) of the amygdala in the epileptic and autopsy brain tissues. B1-B3: Representative micrographs of immunohistological sections of the GABA$_B$R1 and GABA$_B$R2 subunits as well as GAD$_{65}$ in the BLC and CM regions of the epileptic and control groups. *** $P \leq .001$.

pathway from the amygdala, project to the neighboring structures, including the hypothalamus, striatum, and brainstem (Pitkänen and Amaral, 1994; Capogna, 2014; Gilpin et al., 2015). Various populations of GABAergic neurons are located in the BLC and at the border between the BLC and the CM nucleus and maintain a strong inhibitory feedforward control on the signal flow at the entry and the exit of the amygdala (Sotres-Bayon et al., 2012) as well as a feedforward inhibitory gate for signals between different nuclei of the amygdala (Royer et al., 1999). Experimental studies have suggested that epileptic activities may cause damage to the GABAergic neuron population as well as to the glutamatergic (pyramidal) principal neurons in the amygdala (Tuunanen et al., 1996). However, the damage of GABAergic neurons in the amygdala following seizure is significantly greater than that of principal neurons (Fritsch et al., 2009). The loss of 35% to 64% of GABAergic interneurons in the BLC has been reported in different animal models of epilepsy (Fritsch et al., 2009; Prager et al., 2016). In the present study, a significant reduction in the expression of various GABAergic receptor subunits and GAD$_{65}$ associated with enhancement of apoptotic cells is observed in the epileptic amygdala. It has been reported that increased neuronal and glial cell death in the amygdala may be in part due to lower levels of GABAergic signaling (Kim et al., 2016). The loss of GABAergic neurons and reduction in GAD$_{65}$ levels could lead to deficits in the presynaptic release of GABA (Benini and Avoli, 2006), reduction in feedforward GABAergic synaptic inhibition (Rainnie et al., 1992), and amygdala hyperexcitability (Aroniadou-Anderjaska et al., 2008). In addition, a decrease of feedforward inhibition by disinhibition of GABAergic receptors in the amygdala contributes to the generation and propagation of seizure activity in associated neural networks (Gean and Chang, 1991). In line with our results, previous studies have revealed a loss of GABAergic neurons in the amygdala nuclei of patients with MTLE (Yilmazer-Hanke et al., 2007; Fritsch et al., 2009; Graebenitz et al., 2011). It has been suggested that GABAergic disinhibition plays a contributing and/or causative role in the development of human epilepsy (Lerche et al., 2005). Impairment in the function of the GABAergic system in the amygdala leads to decreased phasic and tonic inhibition, and subsequently results in increased anxiety and fear, which are common in patients with epilepsy (Muller et al., 2015; Prager et al., 2016). Therapeutic effects of anticonvulsive tiagabine have been suggested to be associated with an enhancement of endogenous synaptic GABA levels in the human amygdala (Stokes et al., 2014). The establishment of epileptiform discharges in other human brain regions, such as the subiculum and neocortex, is also associated with a collapse of inhibitory synaptic transmission (Huberfeld et al., 2011).

The occurrence of dark cells has been observed in different animal models of epilepsy (Söderfeldt et al., 1983; Jafarian et al., 2015). The process of formation of repeated seizure-induced dark cells displays a dramatic compaction of the ultrastructural cellular elements, which are capable to recover (Gallyas et al., 2008). Significant alterations included a decrease of neuronal soma size and reduction of the mean

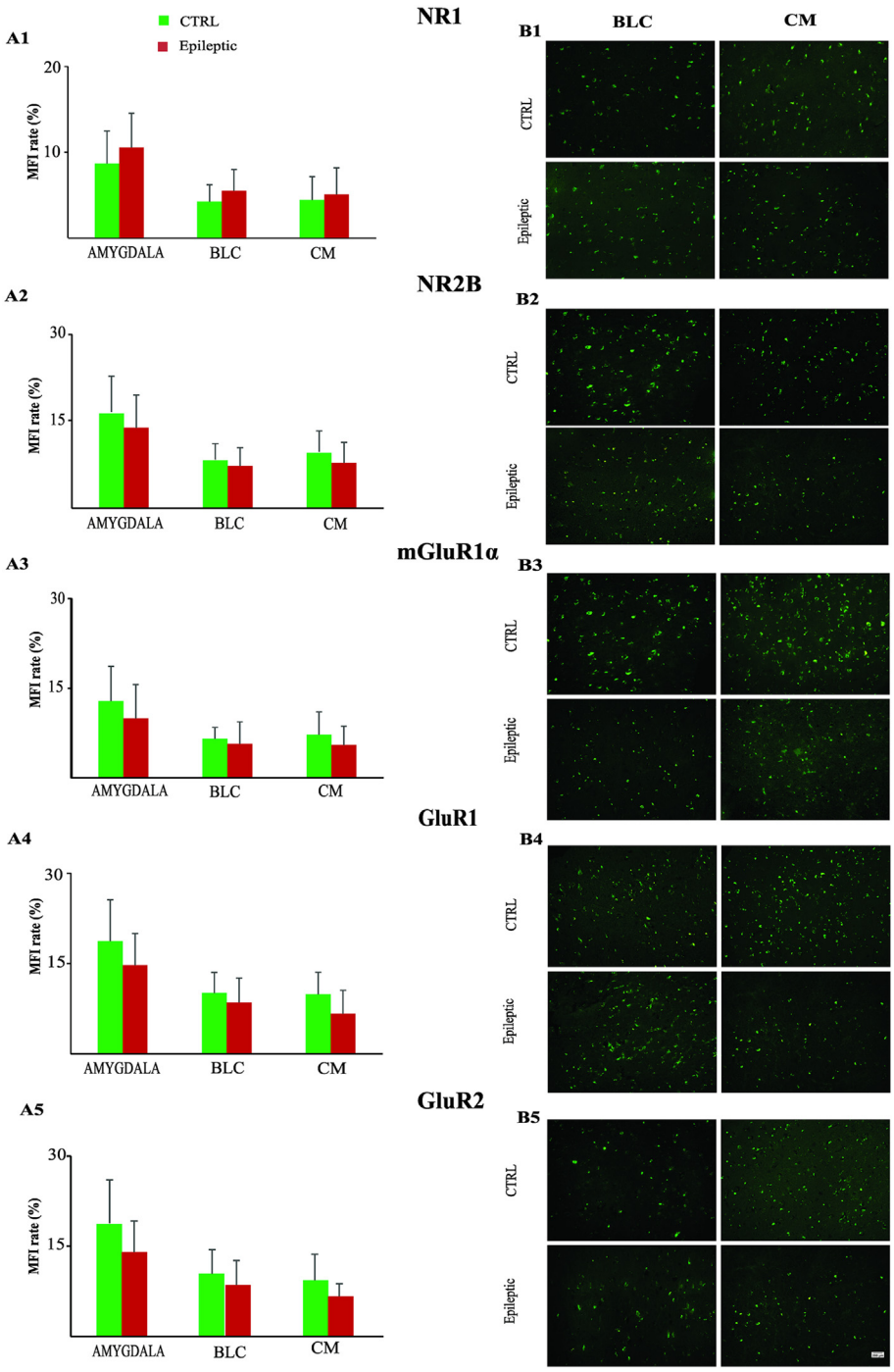

Fig. 5. The expression of the NMDA receptor NR1 and NR2B subunits as well as the AMPA receptor GluR1 and GluR2 subunits in the resected human amygdala obtained during epilepsy surgery and in control autopsy tissues. A1-A4: Bar charts summarizing the mean fluorescence intensities (MFI) of the NR1, NR2, GluR1 and GluR2 receptor subunits in the basolateral complex (BLC) and the centromedial nuclei (CM) of the amygdala in the epileptic and autopsy brain tissues. B1-B3: Representative photomicrographs of immunohistological sections of the NR1, NR2, GluR1 and GluR2 receptor subunits in the BLC and CM areas of the epileptic and control tissues.

number of dendrites has been consistently observed in the amygdala of MTLE patients (Aliashkevich et al., 2003). Our data reveal greater numbers of dark cells in the BLC compared to the CM nucleus in the epileptic amygdala. Previous human and animal studies have shown that the lateral amygdaloid nucleus is the region of the amygdala most sensitive to seizure-induced cellular damage (Hudson et al., 1993; Apland et al., 2010). Several investigations indicate that apoptosis may contribute to repeated seizure-induced cell damage in the lateral amygdala (Pitkänen et al., 1998; Wiese et al., 2005). However, our results do not show any differences in the mean percentage of apoptotic cells between the BLC and the CM nucleus in the epileptic amygdala.

The study of receptor alteration occurring in certain neuronal brain circuits in patients with epilepsy provides the opportunity for developing integrative therapeutic approaches that may prevent or decrease the severity and/or frequency of seizures. Our data indicate a significant lower expression level of ionotropic $GABA_AR\alpha1$ and $GABA_AR\beta3$ receptors in the epileptic amygdala compared to control autopsy tissues, which correlates with the mean percentage of apoptotic cells. Several lines of evidence point to the role of various ionotropic GABA receptors in cell injury (Fritsch et al., 2009; Prager et al., 2016) and epileptogenesis (Gorji et al., 2006) in the amygdala as well as in other brain regions. When binding sites are visualized by the receptor antagonist SR95531, the density of $GABA_A$ receptors is significantly downregulated in the lateral nucleus of the human epileptic amygdala (Graebenitz et al., 2011). The $GABA_AR\alpha1$ subunit is crucial for the sedative and anticonvulsant effects of benzodiazepines. Downregulation of $GABA_AR\beta3$ expression in different brain regions has been shown to be related to cell injury and death in other neurological

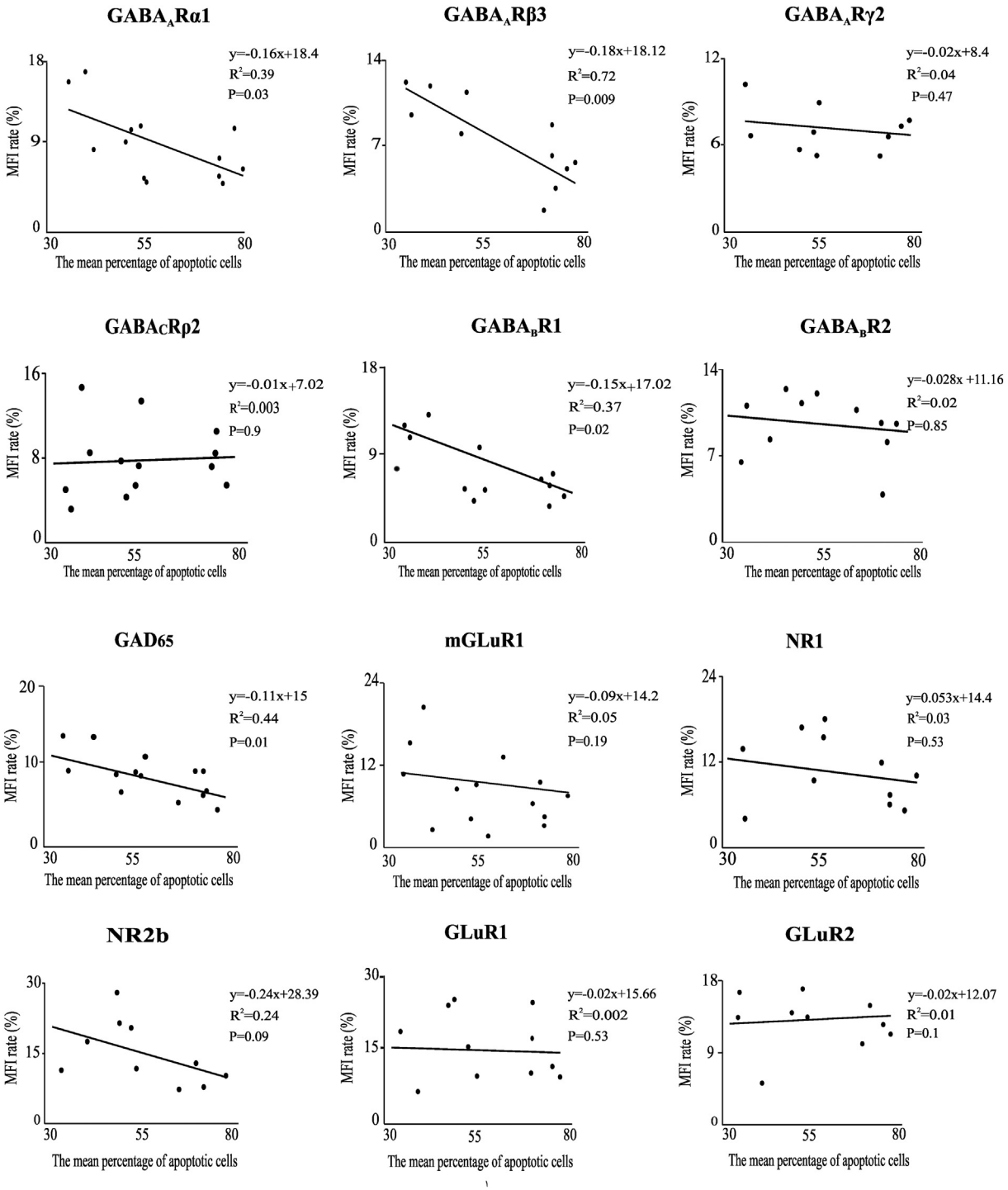

Fig. 6. Correlation between the total mean percentage of apoptotic cells and receptor distribution in the amygdala tissues obtained during epilepsy surgery and autopsy. Correlation plots of different receptor subtypes as indicated above the correlation plot. Note that a significant correlation was found for some inhibitory receptor subunits whereas no significant correlation was found between the mean number of apoptotic cells and the expression of excitatory receptor subunits. MFI, mean fluorescence intensities.

disorders, such as traumatic brain injury (Drexel et al., 2015) and Huntington's disease (Rosas-Arellano et al., 2018). Amygdala-specific reduction of GABA$_A$Rα1 subunits in mice inhibits the normal sedative-locomotor inhibition and anticonvulsive action of diazepam and zolpidem (Heldt and Ressler, 2010). Mutations in genes encoding GABA$_A$Rβ3 and GABA$_A$Rα1 subunits in patients with epilepsy are associated with a wide phenotypic spectrum of epilepsies, such as febrile seizures, early-onset absence epilepsy, and epileptic encephalopathies (Lachance-Touchette et al., 2011; Møller et al., 2017). Dysfunction of GABA$_A$Rβ3 reduces GABA-induced current amplitudes and decreased the seizure threshold (Vien et al., 2015; Møller et al., 2017). Induction of repetitive seizures by Kainic acid reduces the expression of GABA$_A$Rα1 and GABA$_A$Rβ3 subunits in mice (Naserpour Farivar et al., 2016). There are no data available concerning the changes of GABA$_C$ receptors in MTLE.

Although the different findings point to the downregulation of GABA$_A$ receptors Graebenitz et al., 2011; Froklage et al., 2017), so far there has been no evidence for changes in metabotropic GABA$_B$

Table 4
Densities of receptors and subreceptors. The mean fluorescence intensities as measured with immunohistochemistry in epileptic and control (autoptic) amygdala tissues. Data are presented as median (25th, 75th %). BLC, the basolateral complex; CM, the centromedial nucleus.

Biomarkers		Amygdala	BLC	CM
		Median (25th/75th %)	Median (25th/75th %)	Median (25th/75th %)
$GABA_AR\alpha1$	CTRL	39.47 (34.5,54.8)	25.8 (22, 42.8)	11.96 (8.6, 19.5)
	Patient	5.6 (4.9, 9.3)	3.82 (2.4, 5.2)	3.92 (2.7, 5.9)
$GABA_AR\beta3$	CTRL	26.84 (20.4, 30.3)	15.7 (12.2, 16.7)	10.3 (9.1,12.8)
	Patient	6.7 (5.4, 11.6)	4.3 (3, 5.2)	5.82 (2.4, 6.8)
$GABA_AR\gamma2$	CTRL	22.23 (20, 35.9)	12.9 (10.4, 17.6)	12.25 (9.7, 20.1)
	Patient	7.32 (5.4, 9.3)	4.5 (3.7, 5.9)	4.64 (3, 5.85)
$GABA_CRp2$	CTRL	25.7 (18.4, 30.3)	13.34 (9.3, 15.8)	14.5 (10.8, 17)
	Patient	5.75 (3.6, 11.3)	3.9 (3.1, 5.8)	4.2 (1.7, 7.7)
GAD 65	CTRL	32.19 (25.8, 39.2)	16.67 (11.6, 23.9)	15.43 (13, 22.4)
	Patient	8.54 (5.5, 13.7)	4.2 (2.9, 7.2)	4.11 (3.2, 6.4)
$GABA_BR1$	CTRL	22.34 (10.4, 24.9)	10.7 (9.1, 13.4)	12.22 (10.7, 15.7)
	Patient	6.8 (5.4, 12.1)	3.5 (2.9, 5.6)	4.23 (2.6, 6.8)
$GABA_BR2$	CTRL	23.08 (14.8, 24.9)	14.9 (8.5, 20)	10.8 (7.6, 14.5)
	Patient	9.15 (5, 12.8)	5.57 (3.4, 9.6)	4.13 (2.2, 5.6)
mGLuR-1	CTRL	11.7 (10.1, 14.9)	5.7 (5.1, 8.1)	6.24 (4.2, 9.1)
	Patient	8.54 (5.9, 14.8)	4.7 (3.7, 6.8)	4.71 (3.7, 6.8)
NR1	CTRL	8.67 (4.9, 11.9)	4.5 (2.6, 5.6)	4.2 (2.2, 6.1)
	Patient	8.72 (7.6, 14.4)	5.08 (3.7, 6.7)	3.82 (2.6, 6.8)
NR2B	CTRL	18 (11.2, 21.9)	8.71 (5.9, 9.8)	9.3 (7.2, 12.1)
	Patient	11.6 (9.9, 16.8)	7.09 (4.8, 9.2)	6.72 (5.2, 9.5)
GLuR-1α	CTRL	17.24 (14.3, 24.2)	10.08 (6.9, 12.6)	7.86 (6.9, 14.3)
	Patient	14.07 (10.9, 18.2)	7.96 (5.2, 10.1)	4.77 (4.3, 7.8)
GLuR-2	CTRL	16.7 (13.6, 16.2)	9.5 (7.8, 12.4)	8.32 (5.4, 13)
	Patient	13.44 (11, 15.8)	7.36 (5.9, 9.8)	6.3 (5.7, 97)

receptors in the human amygdala in patients with MTLE (Graebenitz et al., 2011). The present study, however, indicates a lower expression of $GABA_BR1$ subunits in the epileptic amygdala. Reduction of the sensitivity of presynaptic $GABA_B$ receptors after repeated seizures points to changes of the receptor affinity for the endogenous ligand in the amygdala, which may contribute to overexcitation accompanied by neuronal injury after seizures (Asprodini et al., 1992). In addition to its role in neuronal network excitability, $GABA_B$ receptors could protect brain cells from apoptosis and promote cell survival under pathological conditions (Zhang et al., 2007; Tu et al., 2010). Our data show a lower level of GAD_{65} expression in the epileptic amygdala. GAD_{65} is demanded for controlling the availability of extracellular GABA as well as GABAergic synaptic transmission and plasticity in the lateral amygdala, and if disturbed, affects neural network excitability (Lange et al., 2014). A reduction in the mean number of inhibitory axo-somatic synapses in GAD-negative projection neurons in the lateral amygdala of patients with MTLE in the presence of high peri-somatic fibrillary gliosis has been reported (Yilmazer-Hanke et al., 2007).

Variable data are available on the distribution level of glutamatergic receptors in the amygdala of patients with MLTE. Binding sites to ionotropic glutamatergic AMPA/kainate and mGluR2/3 receptors in the epileptic amygdala has been shown to increase, whereas NMDA binding densities remain unchanged (Graebenitz et al., 2011). Quantification of the expression of excitatory receptors in the human epileptic amygdala reveals an upregulation of NR1 subunits, whereas values of NR2A and NR2B subunits of the NMDA receptor remain unaffected (de Moura et al., 2012). It should be taken into consideration that while the post-mortem delay does not affect most receptor-mediated activities and receptor densities for at least 92 h (González-Maeso et al., 2002), the NR2A and NR2B subunits appear to degrade rapidly after death (Wang et al., 2000). The variability in glutamatergic receptor expression in the amygdala in different studies may be due to a number of factors, including variability in patient history and drug therapy as well as applied techniques. Data on the expression of different glutamate receptors in the hippocampus, in acute and chronic animal models of epilepsy as well as in human epileptic tissues, are also variable (Tang, 2005; Herold et al., 2018). Our immunohistochemical investigation does not find any significant changes in the expression of NR1, NR2B, mGluR1α, GluR1, and GluR2 in the human epileptic amygdala tissues compared to the amygdalae obtained from autopsy control subjects. Some evidence indicates that enhanced expression of glutamate receptors is not critical for epileptogenesis (Bradford, 1995). Although activation of glutamate receptors is observed in different animal models of epilepsy, repeated electrical stimulation does not increase expression of the mRNAs for AMPA/kainate and GluR1 subunits in the rat amygdala (Lee et al., 1994). Glutamatergic pathways do not play a crucial role in the onset and progression of epileptogenesis, since administration of different anticonvulsants (such as lamotrigine), which inhibit excitatory synaptic transmission, fail to inhibit epileptogenesis and status epilepticus-induced neuronal injury (Halonen et al., 2001; Tang, 2005; François et al., 2006; Aroniadou-Anderjaska et al., 2008). In contrast to glutamatergic transmission, substantial evidence suggests that alterations in the expression and function of GABA receptors are implicated in mechanisms for the epileptogenic process (Olsen and Avoli, 1997; González and Brooks-Kayal, 2011). After development of epilepsy, the main feature of the epileptic neuronal network is an abnormally enhanced glutamatergic transmission accompanied by an imbalance between glutamatergic and GABAergic synaptic activity (Bonansco and Fuenzalida, 2016).

More than half of patients with epilepsy develop psychiatric disorders (Marsh and Rao, 2002). The association of psychiatric disorders and seizure control has been shown to be bidirectional (Kanner, 2008). The existence of common underlying mechanisms between epilepsy and psychiatric disorders, such as the modulation of major neurotransmitter pathways, suggests possibilities for the development of novel therapeutic strategies in the management of emerging psychiatric symptoms in patients with epilepsy. Anticonvulsants which act via GABAergic pathways (such as valproic acid) exhibit anti-psychotic properties in humans (Bach et al., 2018). $GABA_A$ (Quadrato et al., 2014) and $GABA_B$ (Cryan and Slattery, 2010) receptors are targets for the therapy of emotional and psychiatric disorders. A detailed identification of the malfunction and maladaptation of inhibitory and excitatory receptors in different brain regions is crucial for understanding the mechanisms implicated in epilepsy and to develop effective preventive and therapeutic strategies. The present data elucidate the importance of GABAergic neurotransmission in seizure-induced cell injury in the amygdala and suggest several GABA receptor subunits as potential druggable target structures to control epilepsy and its comorbid disorders, such as anxiety.

Conflicts of interest

Nothing to report.

Acknowledgements

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