N-acetylcysteine aggravates seizures while improving depressive-like and cognitive impairment comorbidities in the WAG/Rij rat model of absence epilepsy

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Abstract

N-acetylcysteine (NAC) is an antioxidant with some demonstrated efficacy in a range of neuropsychiatric disorders. NAC has shown anticonvulsant effects in animal models. NAC effects on absence seizures are still not uncovered and considering its clinical use as a mucolytic in patients with lung diseases, people with epilepsy are also likely to be exposed to the drug. Therefore, we aimed to study the effects of NAC on absence seizures in the WAG/Rij rat model of absence epilepsy with neuropsychiatric comorbidities. The effects of NAC chronic treatment in WAG/Rij rats were evaluated on: absence seizures at 15 and 30 days by EEG recordings and animal behaviour at 30 days on neuropsychiatric comorbidities. Furthermore, the mechanism of action of NAC was evaluated by analysing brain expression levels of some possible key targets: the excitatory amino acid transporter 2, cystine–glutamate antiporter, metabotropic glutamate receptor 2, the mechanistic target of rapamycin and p70S6K as well as levels of total glutathione. Our results demonstrate that in WAG/Rij rats, NAC treatment significantly increased the number and duration of SWDs, aggravating absence epilepsy while ameliorating neuropsychiatric comorbidities. NAC treatment was linked to an increase in brain mGlu2 receptor expression with this being likely responsible for the observed absence seizure-promoting effects. In conclusion, while confirming the positive effects on animal behaviour induced by NAC also in epileptic animals, we report the aggravating effects of NAC on absence seizures which could have some serious consequences for epilepsy patients with the possible wider use of NAC in clinical therapeutics.

Keywords: N-acetylcysteine (NAC); absence epilepsy; Depression; Cognition; Seizure aggravation.

Introduction

N-acetylcysteine (NAC) is a thiol which can act as a precursor for glutathione synthesis as well as a stimulator of the cytosolic enzymes involved in glutathione (GSH) regeneration [1, 2]. Besides its known antioxidant effects, NAC can cross the blood-brain barrier for example, increasing brain GSH levels in rats [2, 3]. Moreover, NAC has been reported to: protect against oxidative stress, chelate heavy metals, impact glutamate neurotransmission, reduce markers of inflammation, protect against multiple models of mitochondrial dysfunction, inhibit apoptosis, enhance neurogenesis, and promote neuronal survival in a variety of neurological preclinical models [4].

There has also been growing evidence for the use of NAC in treating a variety of psychiatric and neurological disorders *e.g.* cocaine, cannabis, and smoking addictions, Alzheimer's and Parkinson's diseases, autism, schizophrenia, depression, bipolar disorder [5] as well as epilepsy, considering its role in attenuating pathophysiological processes associated with these disorders, including oxidative stress, apoptosis, mitochondrial dysfunction, neuroinflammation and glutamate and dopamine dysregulation [1, 4, 6, 7]. NAC effects have been particularly studied in psychiatry with several studies demonstrating efficacy against depressive symptoms in animal studies [8–10] and clinical trials [2, 7, 8]. In addition, NAC ameliorates cognitive deficits in several animal models of neurodegenerative diseases; the antioxidant effects of NAC and its potential anti-inflammatory action have also been suggested in these studies[11–13].

Regarding convulsive epilepsy, NAC attenuated aminophylline-induced seizures and mortality in mice [14] and increased the threshold for flurothyl ether-induced seizures [15]. Additionally, this antioxidant seems to possess anticonvulsant effects in acute and chronic use in pentylenetetrazole (PTZ)-induced seizures [6, 16, 17]. Compounds effective in the PTZ model of seizures seem to be effective against absence seizures in humans. Therefore, NAC may have a potential clinical use in absence epilepsy; clinically, NAC, when used as add-on treatment in patients with myoclonus epilepsies, was able to further decrease generalized tonic-clonic seizure, myoclonic jerks and absences, stabilizing also the neurological condition of these patients [18]. Antioxidant effects may contribute to the potential anticonvulsant effects of NAC; the bidirectional correlation between oxidative stress and epilepsy has been demonstrated both in experimental and clinical studies [19, 20]. However, it is generally accepted that NAC influences glutamate neurotransmission by indirectly acting on metabotropic glutamate receptors (mGluRs) through its action on cysteine-glutamate exchange [21–23] and this mechanism has been indicated as a major factor responsible for its neurological effects [21, 24].

NAC effects on absence seizures are not presently known and considering its current clinical use as a mucolytic in patients with lung diseases like chronic bronchitis, people with epilepsy (convulsive

or absence) may also be exposed to the drug; likewise, future development may indicate the use of NAC in psychiatric disorders and therefore its use in people with epilepsy. Therefore, we decided to investigate the potential pharmacological effects of NAC in the WAG/Rij rat model of absence epilepsy with additional recognised depressive-like behaviour, and cognitive impairment comorbidities [25, 26]; further value to this choice is added by the known and not completely clarified involvement of mGluRs on absence seizure incidence in this animal model [27–30].

Surprisingly, we found that NAC treatment in WAG/Rij rats aggravated absence seizures although its beneficial effects on the comorbid depressive-like behaviour and cognitive functions in this model were confirmed. We suggest that this seizure-aggravating effect may be linked to an increased expression of brain mGlu2 receptors.

Materials and methods

Animals

All the experiments were performed in male WAG/Rij rats of 6 months of age (*n = 36*) that were originally purchased from Charles River Laboratories s.r.l. (Calco, Lecco, Italia). Rats were housed three/four per cage and maintained under fixed environmental conditions (60 ±5% humidity; 22 ± 2 °C; 12/12 h reversed light/dark cycle; lights on at 20.00). Rats were allowed free access to standard laboratory chow and tap water. WAG/Rij rats were tested, as previously reported [31], to identify their potential vulnerability to audiogenic stimuli. Subsequently, only rats without reflex seizures were used in experiments, considering that WAG/Rij rats expressing audiogenic susceptibility show anxiety-like behaviour compared to nonaudiogenic rats [31]. Animal care and procedures involving rats were accomplished in agreement with the international and national laws and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept). The experimental protocols and the procedures reported herein were approved *(Authorization n° 491/2016-PR*) by the Animal Care Committee of the University of Catanzaro, Italy. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Treatment schedule and drug administration

WAG/Rij rats (*n=36*) were randomly distributed into 2 subgroups (Fig.1); the first subgroup of WAG/Rij rats (*n=8* for treatment and vehicle) were subjected to EEG recordings at three different time points: before drug administration (T0), after 15 and 30 days of treatment for the quantification of absence seizures and the study of NAC effects (see section Surgery and EEG recordings). Successively, these rats were sacrificed to perform biochemical analysis (see sections Behavioural

Tests). Furthermore, the second subgroup of WAG/Rij rats (*n=10* for treatment and vehicle) was used to study drug effects on different behavioural tests: forced swimming test (FST), elevated plus-maze test (EPM) and novel object recognition test (nORT), after 30 days of treatment. At the end of behavioural tests, the brains of treated and untreated rats were collected to carry out immunohistochemical assays (see section Immunohistochemistry). NAC (Fluimucil® syrup 600 mg/15 ml; Zambon Srl, Italy) was orally administered in WAG/Rij rats (*n=18*) for 30 consecutive days. The dose of NAC (500 mg/Kg/day) was chosen according to previous experiments [6]. In detail, NAC was delivered in the drinking water by solubilizing the desired dose into 120 ml of tap water, as previously reported [31, 32]. Age-matched vehicle WAG/Rij rats (*n=18*) were maintained under the same housing conditions during the experimental period. During this period, rats were weekly weighed and the possible appearance of any obvious drug side-effects was monitored.

Surgery and EEG recordings

A standardized surgical procedure in our laboratory for EEG-recordings with some modifications was used [33]. Briefly, adult WAG/Rij rats, of 6 months of age, under general anaesthesia (tiletamine/zolazepam 1:1; Zoletil 100®; 50 mg/kg i.p.; VIRBAC Srl, Milan, Italy) were chronically implanted, using a Kopf stereotaxic apparatus, with 3 cortical electrodes fastened to a 3-channel rat headmount (8239-SE3; Pinnacle Technology, Stoke-on-Trent, UK). Three stainless-steel screw electrodes were implanted on the dura mater over the cortex: two in the frontal region (AP = −2; L = ±2.5), and the ground/reference electrode over the cerebellum. After surgery, rats were allowed at least 7 days of recovery and then connected to preamplifiers [PinnacleTechnology's8400–9000 video/EEG system with Sirenia Software (Lawrence, KS, USA)], for at least 3 days before the experiments, to habituate animals to the recording procedures. To avoid circadian alterations within groups, every electroencephalographic session started at 9:00 am and groups. EEG signals were amplified and conditioned by analog filters (filtering: < 1 Hz and > 30 Hz) and exposed to analog-to-digital switching with a sampling rate of 300 Hz. Quantification of epileptic seizures, performed by two trained investigators blinded for the experimental protocol, was based on the number and the duration of EEG SWDs according to well established criteria. Briefly, the number and duration of SWDs for each rat were summarized in 30-min intervals (epochs) and scored by visual inspection of the EEG recordings; all recordings were analysed by two independent researchers that were blinded to the treatment. An SWD was considered as an EEG background deflection characterized by a 7.5–9.5Hz frequency and an amplitude at least double that of the background with a minimal duration of 1s. To study the effects of NAC treatment, every recording session was divided into 30-min epochs;

the cumulative SWD duration and number per epoch were estimated and displayed as means ± standard error of the mean (SEM) in Fig. 2 [48, 49].

Behavioural Tests

NAC effects on neuropsychiatric comorbidities in adult WAG/Rij rats were evaluated after the end of treatment (30 days) for both vehicle and NAC-treated groups. All behavioural tests were performed under controlled environmental conditions such as temperature, humidity and illumination (red light), and with the support of video-tracking software (EthoVision XT15; Noldus Information Technology, Wageningen, the Netherlands). All behavioural tests were carried out between 09:00 and 11:00 am, to preclude possible circadian modifications of test results [50, 51]. Furthermore, every maze was systematically cleaned to remove olfactory cues, after each rat was tested. For this purpose, in the nORT, also the objects were cleaned after each trial.

Forced swimming test (FST)

The FST is a widely used tool to evaluate the depressive-like symptom in both mice and rats; we performed a FST protocol previously standardized in our laboratories [52, 53]. Briefly, rats were separately forced to swim for 6 min into an acrylic cylinder (height 47 cm, diameter 38 cm) filled with water kept at 23°C to 25°C. The duration of immobility (immobility time; IT) including passive swimming, was calculated during the last 4 min of the 6-min testing period. Immobility, and/or passive swimming, was defined by the absence of any movement apart from those required to keep the head above water, which is directly proportional to depression-like behaviour [52, 54].

Elevated plus-maze test (EPM)

The test was performed as previously reported for WAG/Rij rats [40]. Briefly, EPM apparatus comprised two opposing open arms (50 cm × 10 cm) and two opposing closed arms of the same size with roofless walls 40 cm high. These arms were connected by a central platform (10 cm × 10 cm). The apparatus was raised 80 cm above the ground. Rats were individually placed in the center of the maze facing a closed arm and the time spent in the four arms and in the central platform was recorded. The shorter the time spent in open arms and in central platform the higher the anxiety and *vice versa*. Mean velocity and total distance moved were also measured as previously described [52, 55].

Novel object recognition test (nORT)

The nORT was carried out as previously described [50]. Briefly, the day before testing, each animal was habituated to freely explore an open field arena (100×100×40 cm) for 5 min. The day after, a single session of two trials (T1 and T2), separated by a retention interval of 60 min, was carried out. In the familiarization trial (T1) each rat was returned to the arena with two identical objects placed on opposite sides of the box. The familiarization session lasted until the rat explored both identical objects for 20 s in 5 min. Exploration was considered when rats touched the object while looking directly at it or directed snout/nose for a distance < 2 cm. After an intertrial period of 60 min, the test phase (T2) was performed and each rat was placed again in the arena in which one of the familiar objects had been replaced with a new object. Moreover, in order to avoid place preference, the role (familiar or new object) and the object's allocation during the T2 were randomly changed. Rats explored the arena for 5 min, and total time spent exploring each object was acquired. The time spent for the exploration of the familiar (F) and the new object (N) was detected separately and the difference between the two exploration times was calculated as the discrimination index (DI = N-F/N+F). Mean velocity and total distance moved were also measured as previously reported [56].

Western Blot Analysis

Rats were sacrificed and the brains (without cerebellum) were immediately removed and submerged in ice-cold artificial cerebrospinal fluid. Afterwards, brain tissue was homogenized, using the Gentle MACS Dissociator (Miltenyi Biotech, Germany), in ice-cold lysis buffer (100 mM Tris pH 7,6; 150 mM NaCl; 0,1% Triton X 100; 0,1% SDS; 0,5% Sodium deoxycholate; 10 µL/mL freshly added protease and phosphatase inhibitor cocktails), and then centrifuged at 13,362 rcf for 30 min at 4 °C to remove tissue debris [51, 57]. Supernatant was collected to determine the total protein concentration through a BCA protein assay (BCA protein Assai Kit, Thermo Scientific, #23225, Rockford, IL, USA). About 20 µg of lysate were resolved on SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Protan 0.2 µm NC 10600001, Little Chalfont, United Kingdom). Membranes were blocked for 1h with phosphate-buffered saline (PBS) containing 5% BSA and 0.1% Tween with 5% nonfat dry milk /PBS–Tween 0.05% (Bio-Rad, Hercules, CA, USA) and incubated overnight at 4°C with the following primary antibodies: anti-phospho mTOR (Cell Signaling #5536, Danvers, Ma USA), anti-mTOR (Cell Signaling #2983, Danvers, Ma USA), anti-phospho p70s6 kinase (Cell Signaling #9208, Danvers, Ma USA), anti-p70s6 kinase (Cell Signaling #9202, Danvers, Ma USA), anti-xCT (Abcam, [EPR8290(2) ab175186, Cambridge, UK), anti-mGlu2 (Abcam, [EPR8975]

ab150387, Cambridge, UK) and anti-EAAT2 (GeneTex, GTX134062, USA). Then, membranes were washed in PBS with 0.1% Tween-20 and incubated with goat anti rabbit IgG (Bioss bs-0295G, Beijin, China) and goat anti-mouse IgG (Bioss, bs-0296G, Beijin, China) for 1 h at room temperature, and immune complexes were visualized by a chemiluminescent kit (ECL, Amersham) using an imaging system (ChemiDoc MP Imaging Systems, Bio-Rad Laboratories). Western blots were normalized to beta-actin as loading control proteins and densitometric analysis was carried out by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Total glutathione determination

Brain oxidative stress was study by assessing the total glutathione (tGSH) in whole rat brains [58]. Glutathione reductase, ß-$NADPH_2$, and DTNB (5,50−dithiobis−2−nitrobenzoic acid), used for the determination of glutathione were purchased from Oxford Biomedical Research (GT20, Oxford, MI, USA) and tGSH was measured following the manufacturer's protocol. Briefly, 0.1 mg of brain tissue was homogenized in 1 ml of ice-cold 5% metaphosphoric acid (MPA) solution. Then, the homogenate was centrifuged at 3,000 x rcf at 4°C for 10 minutes and the supernatant was collected. Subsequently, the reagent NADPH, oxidoreductase, DTNB and GSH working stock were reconstituted and the standard curve was prepared. Later, about 50 µL of DNTB and oxidoreductase solutions were added to each sample and the plate was incubated for 10 minutes at room temperature. Then, 50 µL of ß-$NADPH_2$ solution was added to each well to start the reaction and, the fluorescence was measured at 405 nm.

Immunohistochemistry

For the immunohistochemical analysis, rats were anesthetized and sacrificed by transcardiac perfusion with cold phosphate-buffered saline (PBS), pH 7.4 and subsequently with cold 4% paraformaldehyde, containing 0.2% saturated picric acid in PBS, as previously reported [54]. Sections of 10 µm thickness were collected from paraffin embedded brain tissue, and subsequently the tissue sections were deparaffinized in xylene and dehydrated using different grades of alcohol. The sections were pretreated in 10 mM citrate buffer (pH 6), using a heat-induced epitope retrieval method for 30 min, and then incubated in hydrogen peroxide for 10 min to inactivate endogenous peroxidases. After being washed with PBS to block nonspecific background reactions, the sections were exposed to protein block buffer for 10 min, and then incubated overnight at 4 °C with monoclonal rabbit anti-mGluR2 antibody (1:100 Abcam, [EPR8975] ab150387, Cambridge, UK) and (or) polyclonal rabbit anti-EAAT2 (1:500 GeneTex, GTX134062, USA) antibody. After a washing step, sections were further incubated with biotinylated secondary antibody, for 10 min at RT, and then incubated with

peroxidase-conjugated streptavidin for another 10 min. Negative control staining was performed without the primary antibodies. The signal was detected using 3',3'-diaminobenzidine tetrachloride (DAB) immunostaining. Images were obtained at magnification (10x) using an optical microscope (Olympus BX53, Shinjuku, Tokyo, Japan), and the immunoreactivity was quantified by measuring the relative optical densities of the hippocampus, hilus of the dentate gyrus, somatosensory area, reticular thalamus nuclei, and ventrolateral thalamus nuclei in the stained sections in each side of the brain region, using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All statistical procedures were performed using GraphPad Prism 9.0 software (GraphPad Software Inc., La Jolla, CA, USA). With the aim of evaluating NAC effects on absence seizures parameters, EEG recordings were subdivided into 30 min epochs, and both total and single duration and number of SWDs were evaluated separately for every epoch. Such values were averaged, and data obtained were expressed as mean ± S.E.M. Results were compared by two-way analysis of variance (ANOVA; effect size = 0.97), time and treatment as factor, with repeated measures followed by a post-hoc Tukey's test to analyse and compare EEG data acquired from NAC-treated and age-matched control untreated WAG/Rij rats. Data obtained by behavioural tests, Western blotting analysis and GSH determination were analysed and compared by the Student's t-test (effect size = 1.32), in order to define NAC effects on depressive-like behaviour, anxiety and memory and define a potential mechanism of action. Normality tests (i.e., Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov) were carried out and in all cases the test was passed; all data have been presented in graphs. All tests used were 2 sided and $P \leq 0.05$ was considered significant.

Results

Effects of oral NAC treatment on absence seizure in WAG/Rij rats

Statistical analysis revealed that, before treatment with NAC (T0), no significant differences on SWD parameters were recorded between the WAG/Rij rat groups. Afterwards, when the same rats were recorded after 15 days (T15) of NAC treatment, a significant worsening of SWD parameters (but not sSWD), namely the number ($F_{(1, 14)} = 99.62$; $P<0.0001$) and total duration ($F_{(1, 14)} = 47.25$; $P<0.0001$), was noticed in NAC-treated WAG/Rij rats compared to age-matched control (vehicle) rats. In detail, EEG recording analysis obtained from vehicle 6-month-old control WAG/Rij rats (*n=8*) showed a mean number of SWDs (nSWDs) of 8.81 ± 0.50, with a mean total duration (dSWDs) of 63.45 ± 9.25 s and a mean single duration (sSWD) of 7.24 ± 1.33 for a 30-min epoch (Fig. 2a, b). In contrast, a short term-treatment with NAC (500 mg/kg/day *per os*) in WAG/Rij rats (*n=8)* for 15 days produced

a significant increase of the mean nSWDs and dSWDs by ~45% (P= 0.0001) and 25% (P<0.0001), respectively (Fig. 2a, b). Similarly, when the same rats were recorded after 30 days (T30) of treatment, a significant increment in the SWD parameters appeared in NAC-treated WAG/Rij rats in comparison with age-matched control rats (P<0.0001 for nSWDs and dSWDs), suggesting a worsening in absence epileptic activity (Fig 2a, b). In detail, NAC treatment increased the nSWDs (14.25. ± 0.99; 60%; P<0.0001) and total duration (101.22 ± 22.5 s; 37%; P<0.0001). The mean sSWD value was not significantly changed between NAC-treated and untreated WAG/Rij rats, at the three time points evaluated (Table S1). No significant modification was detected on SWDs parameters in NAC-treated WAG/Rij rats at the two different time points considered.

Effects of NAC on depressive- and anxiety-like behaviour in WAG/Rij rats

Previous studies prove that WAG/Rij rats, from 4 months of age onwards, show increased immobility time in the FST, which is directly related to a depressive-like behaviour [27, 50]. 30 days of chronic treatment with NAC significantly reduced the IT in adult WAG/Rij rats in comparison to their age-matched vehicle control rats (172.39 s vs 136.96 s respectively; $F_{(9,9)} = 1.167$; P =0.0006), suggesting a reduced depressive-like behaviour (Fig. 3a). At odds, NAC treatment had no effect on anxiety-like behaviour, assessed by EPM, in WAG/Rij rats (Fig. S1). No significant modification was reported on mean velocity and total distance moved (P >0.05) between groups (Fig. S2a, b).

Effects of NAC on memory performance in WAG/Rij rats

The nORT was carried out to study the working-episodic memory performance in NAC-treated and vehicle WAG/Rij rats. As previously described, WAG/Rij rats, at 6 months of age, exhibited a smaller DI compared to age-matched controls [50]. *Post-hoc* analysis revealed that NAC treated WAG/Rij rats had a higher DI compared with their respective untreated (vehicle) controls (0.4 vs 0.239 respectively; $F_{(9,9)} = 2.418$; P<0.0001, Fig. 3b) indicating an improved memory performance.

Effects of NAC treatment on brain EAAT2, xCT, mGlu2, p-mTOR, p-p70S6K expressions and GSH levels.

No significant difference (P>0.05; Fig 4a, b) was recognized on brain excitatory amino acid transporter 2 (EAAT2) and cystine–glutamate antiporter (xCT) protein levels between vehicle and NAC-treated WAG/Rij rats. On the contrary, NAC treatment, lasting 30 days, significantly increased ($F_{(7,7)} = 1.415$; P < 0.0001; Fig 4c) metabotropic glutamate receptor 2 (mGlu2) expression levels in WAG/Rij rats compared to their control untreated rats. Additionally, a slight, but not significant (P>0.05; Fig 4d, e) decrease of mechanistic target of rapamycin (mTOR) and p70S6K

serine/threonine-protein kinase (that acts downstream of mTOR signalling) protein levels was observed in the NAC-treated WAG/Rij rats group. *Post-hoc* analysis revealed that after 30 days of treatment with NAC there was a significant ($F_{(7,7)}$ = 1.367; P<0.0001; Fig 4f) increase in the total glutathione (tGSH) brain levels in treated WAG/Rij rats compared to vehicle control group.

Immunohistochemical analysis

Immunohistochemical assays revealed a significant ($F_{(1,108)}$ = 6462; Fig. 5; P<0.001) increase of mGlu2 receptor expression in all considered areas (*e.g.*, hippocampus, reticular thalamus nuclei, and ventrolateral thalamus nuclei) and more markedly in the hilus of the dentate gyrus and peri-oral region of the somatosensory cortex (S1po) of NAC-treated WAG/Rij rats. The increased expression of these receptors was observed in cells with neuronal and glia morphology. Conversely, no differences in the expression of EAAT2 transporter were observed in any brain region of NAC-treated WAG/Rij rats as compared to untreated rats.

Discussion

Our findings highlight for the first time, NAC's worsening effects on absence seizures in the WAG/Rij genetic rat model accompanied by a surprisingly positive effect on animal behaviour with an improvement in known comorbid depressive-like behaviour and cognitive functions in this animal. Accordingly, this is the first report of proseizure effects of NAC which was previously demonstrated to be *anti*convulsant in some animal models and in patients with progressive myoclonic epilepsy [6, 16–18]. Notably, NAC was previously found to be partially effective, in a non dose-dependent manner, against PTZ-induced seizures which is generally believed to be predictive of efficacy against absence seizures [17, 59]. Therefore, NAC's effect of seizures may depend on seizure type as in the case of some other antiseizure medications such as the aggravating effects on absence seizures of carbamazepine [60–62].

On the other hand, our results confirm the positive effects of NAC on depressive-like behaviour and cognitive functions [7, 8]; however, this represents a peculiar result. WAG/Rij rats are known to display depressive-like behaviour and cognitive impairment which are generally accepted to be strictly linked to absence seizures. In other words, it is known that their behaviour worsens with increasing seizures and is improved by treatments reducing absences [50, 63, 64]. This has also recently been demonstrated for the glucagon-like peptide-1 (GLP-1) receptor agonist exendin-4 which dose-dependently increased absence seizures in this strain and this effect was accompanied by

an increased immobility time in the FST [65]; however, some other exceptions were also previously reported [54, 64, 66]. Therefore, NAC may also be useful for the treatment of epilepsy comorbidities, despite an effect on seizures, at least in these patients in which seizures are not aggravated.

In an attempt to clarify the mechanisms involved in the observed effects of NAC, we focused on some known mechanisms of NAC of which some were already known to be involved in the pathophysiology of absence seizures in WAG/Rij rats. Here, we confirmed the antioxidant properties of NAC by demonstrating its ability to increase GSH levels in the brain as previously demonstrated in other animal models [67, 68]. This effect has often been indicated as a relevant mechanism for its neuronal effects and in particular being linked to an anti-inflammatory effect due to the reduction of cytokine production [69, 70]. Indeed, oxidative stress (OS) and excessive reactive oxygen species (ROS) production are a contributing factor to acute seizures and epilepsy [19, 69]; accordingly, GSH is the most abundant antioxidant in mammalians, acting as a scavenger against reactive oxidative species (ROS) released from mitochondria [71]. Decreased levels of brain GSH were reported in chronic models of epilepsy [72, 73]. This represents the background leading to the clinical evaluation of NAC in patients with progressive myoclonus epilepsy (Unverricht–Lundborg disease, EPM1) in which NAC demonstrated some improvement in seizures, ataxia and symptom progression, although with a variable response [74–76]. However, oxidative stress does not have the same pattern in all seizure models [77]. Although it has been suggested that oxidative mechanisms and inflammatory mechanisms do not cause absence epilepsy [78] and there are no inflammatory or neurodegenerative processes in WAG/Rij rats, it has been found that decreased levels of GSH in the occipital cortex is related to seizure generation in tg/tg mice, another recognised mouse model of absence epilepsy [79]. Further studies are needed to better define the role of oxidative stress in this specific model and whether this has a role on absence seizures.

NAC is known to indirectly increase extrasynaptic glutamate release through activation of the cystine–glutamate antiporter [1]; NAC regulates glutamate via the cysteine: glutamate antiporter (system Xc-) and glial glutamate transporter (GLT1 also termed EAAT2), both essential components of glutamate homeostasis. System Xc-exchanges extracellular glutamate for intracellular cysteine on a 1:1 ratio, promoting the activation of mGlu2/3 receptors and inhibiting presynaptic release of glutamate [5, 80]. NAC also induces the expression of the glutamate transporter-1 (GLT1) in astrocytes, which clears synaptic and extra-synaptic glutamate and enhances the maintenance of glutamate homeostasis. The nonvesicular glutamate released into the extracellular space stimulates the type 2/3 metabotropic glutamate receptors (mGluR2/3), which, in turn, inhibit the vesicular release of glutamate, thereby resulting in a decrease in glutamatergic neurotransmission [23, 80]. In agreement, we studied the effects of NAC on the expression of some key components of this system

and found that NAC administration increased mGlu2 receptor expression in WAG/Rij rat brain with no major apparent differences between different brain areas, while it did not affect the expression of EAAT2 and xCT.

Activation of group II metabotropic glutamate (mGlu2/3) receptors reduces excessive glutamate release that is often associated with epilepsy and psychiatric disorders. The role of this receptor has been previously studied in WAG/Rij rats and it was demonstrated that administration of the mGlu2/3 receptor agonist LY379268 increased the number of absence seizures, while the mGlu2/3 receptor antagonist possessed antiabsence effects [30]. It is worth noticing that mGlu2/3 receptor signalling was reduced in the somatosensory cortex of 6-month-old WAG/Rij rats. This reduction was measured by the ability of LY379268 to inhibit forskolin-stimulated cAMP formation, while this brain region showed an increase in mGlu2/3 receptor expression [30]. Furthermore, symptomatic 6-month-old WAG/Rij rats showed an increased expression of mGlu2/3 receptors in the ventrolateral portion of the somatosensory cortex, which includes the putative ‘‘triggering zone’’ of SWDs [25, 81]. Our results are in agreement with previous findings indicating that NAC acts through the activation of mGlu2 receptors, which in this specific case would be proabsence but are linked to a favourable outcome on some comorbidities such as depressive-like behaviour and cognitive impairment.

Finally, considering the known role of the mTOR pathway in this model, depression, cognitive functions [66, 82–85] and potentially in the effects of NAC [86, 87], we studied treatment effects on this pathway but found no significant modifications.

In conclusion, while we confirm that NAC possesses positive effects on depressive-like symptoms and cognitive functions also in epileptic animals, we found a time dependent worsening of absence seizures, linked to an increased expression of brain mGlu2 receptors. Therefore, it seems that this receptor may modulate both these aspects playing an opposite role in different pathologies. Furthermore, our results suggest that NAC may positively influence epilepsy comorbidities such as depression or cognitive impairment; however, it may aggravate absence seizures with possible serious consequences in epilepsy patients. Further studies may therefore be needed to understand how other seizure types are influenced by this agent.

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Author declarations section

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Nothing to declare.

Conflict of interest

The authors declare that there are no conflicts of interest to be disclosed.

Ethics Approval

The experimental protocols and the procedures reported here were approved (Authorization n° 491/2016-PR) by the Animal Care Committee of the University of Catanzaro, Italy.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Authors' contributions

Conceptualization: Rita Citraro, Antonio Leo, Giovambattista De Sarro and Andrew Constanti; Formal analysis and investigation: Antonio Leo, Martina Tallarico, Emilio Russo, and Rita Citraro; Methodology: Martina Tallarico, Antonio Leo, Carmen De Caro, Maria Caterina Zito, Lorenza Guarnieri; Writing- Original draft preparation: Antonio Leo, Martina Tallarico, Andrew Constanti, Emilio Russo; Writing- Reviewing and Editing; Antonio Leo, Emilio Russo and Rita Citraro.

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Figure Captions

Fig. 1. Experimental scheme used in this study. Created by BioRender.com

Fig.2. Effects of NAC administration (500 mg/kg/day *per os*), recorded at baseline (before drug administration) and after 15 and 30 days of treatment, on the number a) and duration b) of characteristic SWDs in 6-month-old WAG/Rij rats (*n=8* per group). Data values are means ± SEM for every 30 min epoch. *Significantly different ($P \leq 0.05$) from age-matched untreated control rats. CTRL = control; NAC = N-acetylcysteine; nSWDs = number of spike–wave discharges; dSWDs = duration of spike–wave discharges; WAG/Rij = Wistar Albino Glaxo/Rijswijk.

Fig.3. Effects of chronic NAC administration on IT in the FST and on DI in the nORT in WAG/Rij rats. a) Bars indicate the IT, expressed in seconds, in the FST performed in WAG/Rij (*n=10* per group) following 30 days of treatment with NAC (500 mg/kg/day *per os*). b) Bars indicate the discrimination index (DI) in the nORT performed in WAG/Rij (*n=10* per group) following 30 days

of treatment with NAC (500 mg/kg/day *per os*). Values are means ± SEM (*n = 10* for every group). *Significantly different ($P \leq 0.05$) from age-matched untreated control rats. CTRL = control; DI = discrimination index; FST = forced swimming test; IT = immobility time; NAC = N-acetylcysteine; nORT = novel object recognition test; WAG/Rij = Wistar Albino Glaxo/Rijswijk.

Fig.4. Representative panel of Western blotting experiments on the effect of 30 days treatment with NAC, on the protein expression levels in the brain of WAG/Rij rats, of EAAT2 a); xCT b); mGlu2 c); phosphorylated mammalian target of rapamycin (p-mTOR) d), phosphorylated p70S6K (p-p70S6K) e) and tGSH levels f). a-e panel columns represent mean relative protein levels normalized to control (*n = 8* per group). Loading was normalized using actin levels. Data values are means ± SEM (*n = 8* for every group). *Significantly different ($P \leq 0.05$) from age-matched control untreated WAG/Rij rats. CTRL = control; NAC = N-acetylcysteine; tGSH =total glutathione; WAG/Rij = Wistar Albino Glaxo/Rijswijk.

Fig.5. a) Representative images (×10 magnification) of brain area sections (DG, CA1, CA3, NRT/VPL, S1po) obtained from a NAC-treated and an untreated control WAG/Rij rat immunostained for mGlu2. b) Densitometric analysis (by ImageJ) of mGlu2 staining in different brain regions of NAC-treated WAG/Rij or untreated control WAG/Rij rats. Scale bars in the representative panels = 100 μm for the brain regions. The densitometric analysis was performed in CA1, CA3, DG, S1po, NRT and VPL. Data values are means ± SEM and were calculated from the average of 3 coronal sections for each animal (*n=10* rats for every group). Densitometric data of different sections were expressed as percentage change relative to the DG of CTRL rats. *Significantly different ($P \leq 0.05$) from age-matched control untreated WAG/Rij rats. CTRL = control; CA1 = Cornus Ammonis 1; CA3 = Cornus Ammonis 3; DG = dentate gyrus; NAC = N-acetylcysteine; S1po = perioral region of somatosensory cortex; NRT = reticular thalamic nucleus; WAG/Rij = Wistar Albino Glaxo/Rijswijk; VPL = ventral posterolateral nucleus of the thalamus.