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Research Report

Neuroprotective mechanisms activated in non-seizing rats exposed to sarin

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ABSTRACT

Exposure to organophosphate (OP) nerve agents, such as sarin, may lead to uncontrolled seizures and irreversible brain injury and neuropathology. In rat studies, a median lethal dose of sarin leads to approximately half of the animals developing seizures. Whereas previous studies analyzed transcriptomic effects associated with seizing sarin-exposed rats, our study focused on the cohort of sarin-exposed rats that did not develop seizures. We analyzed the genomic changes occurring in sarin-exposed, non-seizing rats and compared differentially expressed genes and pathway activation to those of seizing rats. At the earliest time point (0.25 h) and in multiple sarin-sensitive brain regions, defense response genes were commonly expressed in both groups of animals as compared to the control groups. All sarin-exposed animals activated the MAPK signaling pathway, but only the seizing rats activated the apoptotic-associated JNK and p38 MAPK signaling sub-pathway. A unique phenotype of the non-seizing rats was the altered expression levels of genes that generally suppress inflammation or apoptosis. Importantly, the early transcriptional response for inflammation- and apoptosis-related genes in the thalamus showed opposite trends, with significantly down-regulated genes being up-regulated, and vice

Abbreviations: 2-PAM, 2-pyridine aldoxime methylchloride; ACh, acetylcholine; AChE, acetylcholinesterase; Avp, arginine vasopressin; Bcat1, branched chain amino-acid transaminase; Btg2, B-cell translocation gene 2; Dbp, D site of albumin promoter binding protein; DEG, differentially expressed gene; Dusp1, dual specificity phosphatase 1; Ephx2, epoxide hydrolase 2; FC, $\log_2$ fold-change; FDR, false discovery rate; Gja1, gap junction protein, α1; GO, Gene Ontology; Homer1, Homer protein homolog; Ier2, immediate early response 2; Ilf3, interleukin enhancer binding factor 3; KEGG, Kyoto Encyclopedia of Genes and Genomes; NMDA, N-methyl-D-aspartate; Nqo2, NAD(P)H/quinone dehydrogenase 2; OP, organophosphate; Plekhb2, pleckstrin homology domain containing, family B member 2, evectins; Ppp3r1, protein phosphatase 3, regulatory subunit B, alpha isoform, calcineurin B type I; Prim1, DNA primase, p49 subunit; Prkacb, protein kinase, cAMP-dependent, catalytic, beta; Retsat, retinol saturase; Rnf6, ring finger protein 6, C3H2C3 type; sEH, soluble epoxide hydrolase 2; Ttr, transthyretin; Zeb2, zinc finger E-box binding homeobox 2; Zfp36, zing finger protein 36

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versa, between the seizing and non-seizing rats. These observations lend support to the hypothesis that regulation of anti-inflammatory genes might be part of an active and sufficient response in the non-seizing group to protect against the onset of seizures. As such, stimulating or activating these responses via pretreatment strategies could boost resilience against nerve agent exposures.

1. Introduction

Highly toxic organophosphate (OP) nerve agents, such as sarin (O-isopropyl methylphosphonofluoridate), inhibit acetylcholinesterase (AChE), an enzyme that hydrolyzes and regulates the neurotransmitter acetylcholine (ACh). The inactivation of AChE causes a toxic buildup of postsynaptic ACh and results in the overstimulation of muscarinic and nicotinic ACh receptors (McDonough and Shih, 1997). Systemic damage follows a three-phase neuropharmacological process in which 1) the initial hyperstimulation of the cholinergic receptors by excess ACh initiates seizures, followed by 2) a predominantly glutamatergic phase that sustains the seizure, and 3) a final state where the excessive stimulation of ionotropic glutamate receptors causes excessive elevations in intracellular sodium and calcium concentrations. This imbalance of ions, especially the increase in intracellular free calcium, produces a harmful cascade of pathological processes leading to excitotoxic cell death (McDonough and Shih, 1997). Seizure duration and intensity are correlated with neuronal injury and irreversible brain damage (Tanaka et al., 1996). In animal models, signs of neuropathology are evident after 20 min of seizures, and seizures lasting 40 min are more difficult to terminate (Myhrer, 2007). Thus, understanding how seizures are initiated or inhibited at the cellular level is critical for understanding damage prevention and countermeasure development.

To understand the mechanism of seizures, previous studies (Spradling et al., 2011a, 2011b) have analyzed the transcriptomic responses in five nerve agent-sensitive brain regions (Aroniadou-Anderjaska et al., 2009; Myhrer, 2007; Myhrer et al., 2007) at different time intervals (0.25 h, 1 h, 3 h, 6 h, and 24 h) in rats following sarin-induced seizures. In these experiments, approximately half of the rats exposed to a $1 \times LD_{50}$ (median lethal dose) concentration of sarin developed seizures (Fig. 1a). The extensive activation of inflammatory responses in the seizing rats, in particular by neurotoxic and pro-inflammatory cytokines, such as Il-1β, Tnf-α, and Il-6, was observed in all brain regions (Spradling et al., 2011a, 2011b), consistent with findings from previous studies (Chapman et al., 2006; Dhote et al., 2007; Dillman et al., 2009; Johnson and Kan, 2010; Svensson et al., 2001, 2005; Williams et al., 2003).

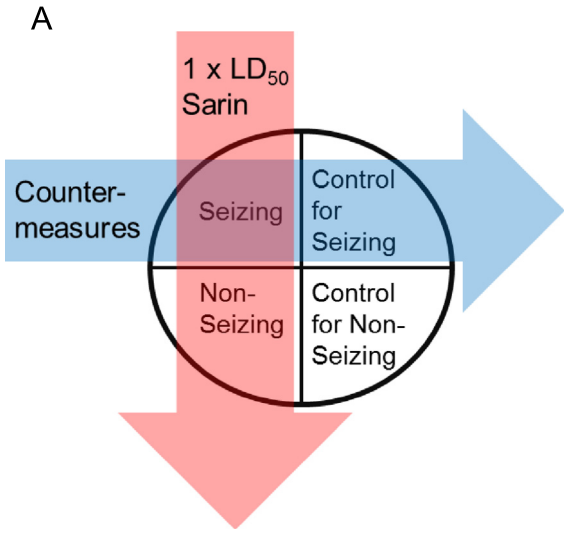
In contrast, our study was focused on the roughly half of the sarin-exposed rats that did not develop seizures (Spradling et al., 2011a). Even though half of the animals given a dose of $1 \times LD_{50}$ are affected by seizures leading to death (if left untreated), the remaining non-seizing animals will be affected by sarin at these exposure levels, and non-lethal damage and subsequent long-term sequelae cannot be ruled out for this group. The differences in seizure onset are part of the natural variability in the biological response capacity to overcome the sarin insult and do not represent an experimental artifact. Thus, this study addresses the difference in this biological response and attempts to identify the underlying molecular pathways responsible for avoiding the onset of seizures in the non-seizing group.

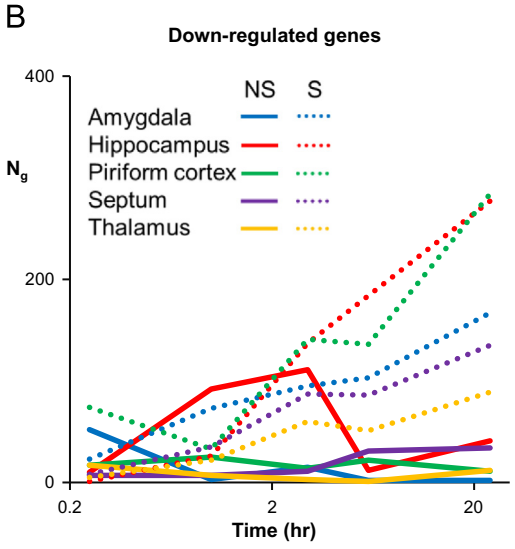
We hypothesized that endogenous neuroprotective mechanisms helped this group respond to sarin intoxication and that this response was reflected in global changes in RNA levels from non-seizing rats. Thus, a comparison between seizing and non-seizing sarin-exposed rats could help us better understand both pathological and defense mechanisms involved in the response to OP nerve agents. These mechanisms could further be exploited for implementing novel potential neuroprotective therapeutic strategies that could mediate the cascade of secondary events leading to brain damage (Tang et al., 2011). Importantly, our analysis determined that there were both commonalities and substantial differences between the seizing and non-seizing rat responses, and that the non-seizing response was specifically tied to neuroprotection via the differential activation of anti-inflammatory and anti-apoptotic pathways.

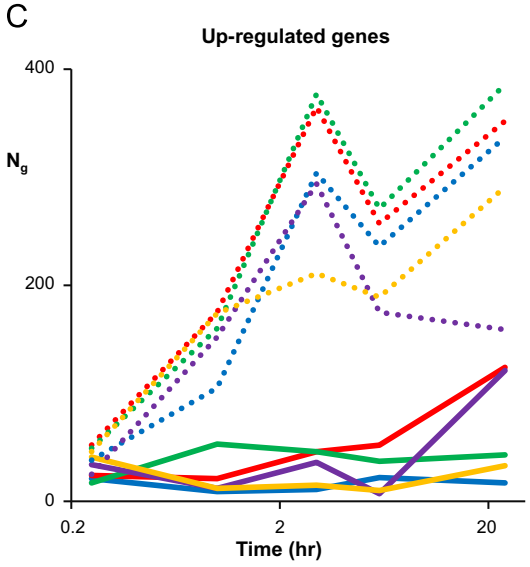
2. Results

We determined the differentially expressed genes (DEGs) from the data of Spradling et al. (2011b), using a false discovery rate (FDR)-corrected *p*-value cutoff of 0.05 for each of the five studied brain regions (amygdala, hippocampus, piriform cortex, septum, and thalamus) and at each time point (0.25 h, 1 h, 3 h, 6 h, and 24 h). The determination of the DEGs from each cohort of seizing and non-seizing rats employed separate control groups due to differences in countermeasure administration. Seizing rats and their controls were given the countermeasures atropine sulfate and 2-pyridine aldoxime methylchloride (2-PAM) within one minute of seizure onset, followed by the anticonvulsant diazepam 0.50 h later. Non-seizing rats and their controls received no countermeasures (Fig. 1a). Fig. 1b and c shows the total number of up- and down-regulated genes in either group as a function of time and brain region. Although the number of DEGs was comparable at 0.25 h, the continued transcriptional response in the seizing group was substantial and still increasing at 24 h. In

order to characterize the initial differences and similarities between the seizing and non-seizing group, we focused on the 0.25-h time point, i.e., before the anticonvulsant was administered.







2.1. *Seizing and non-seizing rat transcriptional response at 0.25 h*

Table 1 shows the number of different and common down- and up-regulated genes and their distribution in the investigated brain regions for the seizing and non-seizing groups. In general, the number of up-regulated genes was larger than the number of down-regulated genes (179 versus 96 in seizing rats and 121 versus 97 in non-seizing rats). The gene overlap between the seizing and non-seizing groups ranged from 13% for the down-regulated genes to 17–25% among the up-regulated genes. Among the down-regulated genes, the amygdala had the largest number of common genes between the two groups; i.e., out of the 17 (47) genes for seizing (non-seizing) animals, 11 were common to both groups. Table 2 lists the identity of these genes and highlights the commonality of the up-regulated genes among the brain regions. Five genes, Btg2, Dusp1, Ier2, Ilf3, and Zfp36, were common to at least four brain regions between the seizing and non-seizing groups. The immediate early response gene Fos was also up-regulated in the hippocampus and thalamus of both groups, although the intensity of this up-regulation became signifi-

Table 1 – Total number of down-regulated and up-regulated genes in the sarin-sensitive brain regions that are unique to each group of animals as well as the differentially expressed genes found to be common to both groups at 0.25 h.

	Seizing	Common	Non-seizing
Down-regulated genes			
Amygdala	17	11	47
Hippocampus	1	0	10
Piriform	65	2	17
Septum	8	0	7
Thalamus	5	0	16
Up-regulated genes			
Amygdala	32	5	18
Hippocampus	43	8	22
Piriform	41	4	15
Septum	21	4	30
Thalamus	42	10	36

Fig. 1 – Definition of seizing/non-seizing animal groups and number of differentially expressed genes. (A) A diagram of the different groups of rats in the experiment (Spradling et al., 2011a). Rats were challenged with 1 × LD_{50} sarin. Approximately half of the sarin-exposed rats developed seizures (seizing rats) and were injected with countermeasure treatments. The corresponding control group for seizing rats was also injected with countermeasure medicines. The sarin-exposed rats that did not develop seizures (non-seizing rats) and their corresponding controls did not receive countermeasure treatments. All control rats received saline injections in lieu of sarin. (B) The number of genes differentially down-regulated, and (C) up-regulated in the five sarin-sensitive brain regions of seizing and non-seizing rats. N_g: number of genes; S: seizing; NS: non-seizing.

cantly different as the experiments continued. Thus, among all brain regions at 24 h, Fos was up-regulated in seizing rats, with an average $\log_2$ fold change (FC) of 4.47 (σ=0.70), compared to 1.46 (σ=0.40) for the non-seizing group.

2.1.1. *Activation of cholinergic and glutamatergic stress-related genes*

We determined the enriched Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were associated with the DEGs at the earliest time point and were related to the different phases (McDonough and Shih, 1997) for sarin-induced seizures. Fig. 2 shows the gross difference in activation of these phases for the seizing versus non-seizing animals, in particular the overall low activation of downstream inflammatory signaling pathways for the non-seizing animals.

For the cholinergic-related pathways, only the thalamus of non-seizing rats showed a transcription response by up-regulation of Chrna3 and the choline transporter Slc5a7 (p-value=0.02). On the other hand, only the thalamus of seizing rats showed a consistent up-regulation of glutamate secretion through expression of Avp and Il-1β (p-value=0.04). The thalamus of seizing rats was also associated with an elevated cytosolic calcium ion concentration through up-regulating transcription of Avp, Ccl3, Hcrt, Il-1β, and Pmch (p-value=8×10^{-6}). The most notable difference between the two groups was the large number of KEGG pathways associated with inflammation activated in the seizing group (Fig. 2). The exception in the non-seizing group was the partial activation of the MAPK signaling pathway in two brain regions in non-seizing rats.

2.2. *MAPK signaling pathway activation*

We examined the number of DEGs involved with the MAPK signaling pathway as a function of time and brain region for

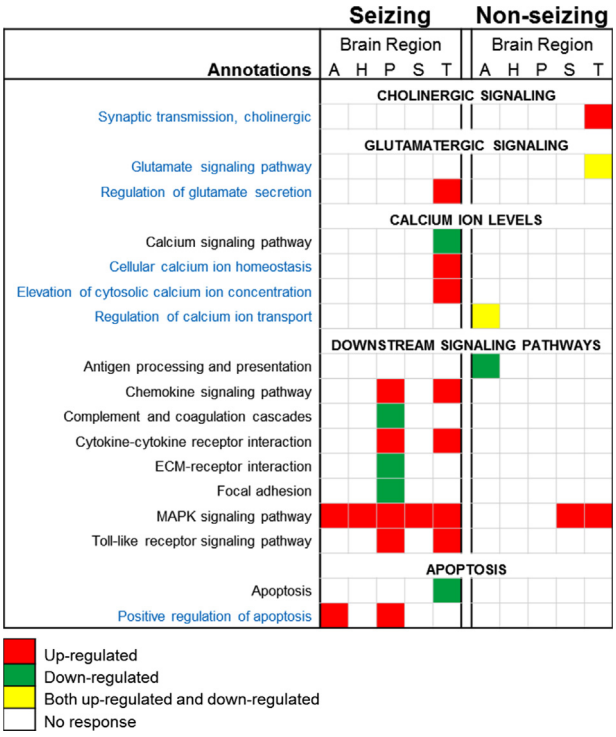


Fig. 2 – Activated and suppressed Gene Ontology (GO) terms (blue text) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (black text) related to the three-phase model of sarin-induced seizure. Based on the differentially expressed genes in the brain regions at 0.25 h, a number of GO terms and KEGG pathways may be involved in the cholinergic, intermediate, and glutamatergic phases of seizure, as well as in the consequences from the activation of receptors in those phases (i.e., calcium ion levels, downstream signaling pathways, apoptosis). Each observation is statistically significant, with an associated p-value<0.05. A: amygdala; H: hippocampus; P: piriform cortex; S: septum; T: thalamus.

Table 2 – Common down-regulated and up-regulated genes at 0.25 h in each brain region. Five genes (shown in bold text) were common in at least four brain regions: Btg2, Dusp1, Ier2, Ilf3, and Zfp36.

Amygdala	Hippocampus	Piriform	Septum	Thalamus
Down-regulated genes				
Coch	–	Nqo2	–	–
Col1a2	–	–	–	–
Colec12	–	–	–	–
Cp	–	–	–	–
Fmod	–	–	–	–
Gjb2	–	–	–	–
Igf2	–	–	–	–
Ogn	–	Ogn	–	–
Serping1	–	–	–	–
Slc6a13	–	–	–	–
Slc6a20	–	–	–	–
Up-regulated genes				
–	–	–	–	Atf3
Btg2	**Btg2**	–	**Btg2**	**Btg2**
Dusp1	**Dusp1**	**Dusp1**	**Dusp1**	**Dusp1**
–	Fos	–	–	Fos
Ier2	**Ier2**	**Ier2**	–	**Ier2**
–	–	–	–	Ifit3
Ilf3	**Ilf3**	**Ilf3**	**Ilf3**	**Ilf3**
–	Ogn	–	–	Junb
–	Ttr	–	–	Oxt
Zfp36	**Zfp36**	**Zfp36**	**Zfp36**	**Zfp36**

both the seizing and non-seizing groups. Fig. 3a shows the increased number of up-regulated MAPK-associated genes for the seizing group as a function of time; in the non-seizing group, the number of MAPK-related genes was roughly similar at the earliest and latest time points.

2.2.1. *Differential activation of MAPK sub-pathways*

We further analyzed the DEGs in the MAPK signaling pathway in terms of differential activation of the classical versus the distinct JNK and p38, and ERK5 sub-pathways. Fig. 3b shows the average number of DEGs from all brain regions for

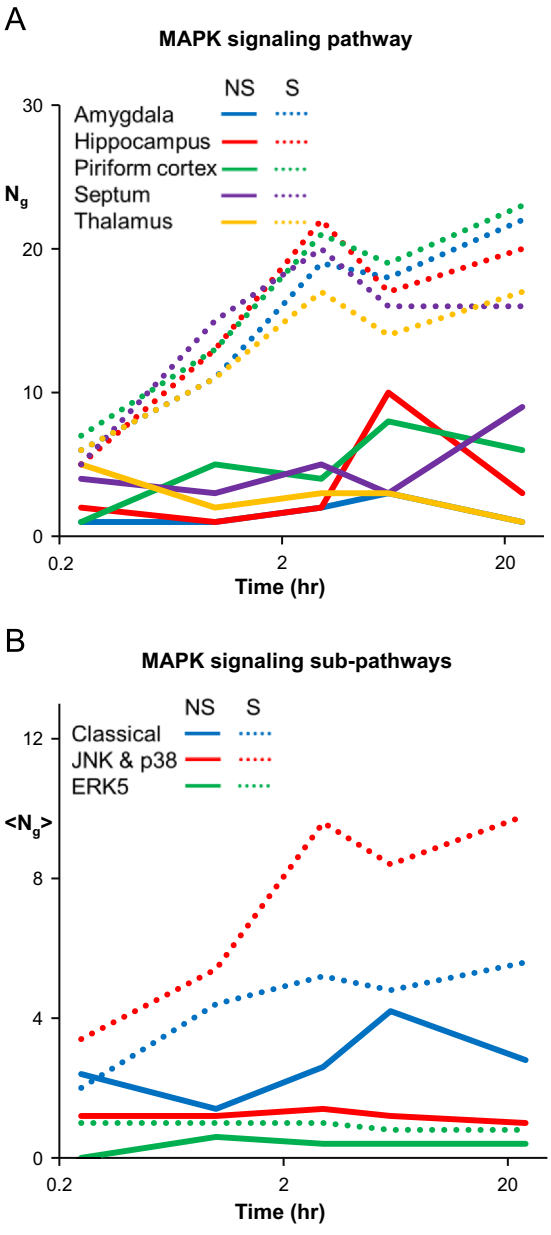


Fig. 3 – Up-regulated genes in the MAPK signaling pathway. (A) The number of genes up-regulated in the MAPK signaling pathway in the five sarin-sensitive brain regions of seizing and non-seizing rats. (B) The average number of up-regulated genes in the three MAPK signaling sub-pathways. N_g: number of genes; S: seizing; NS: non-seizing; $<N_g>$: average N_g.

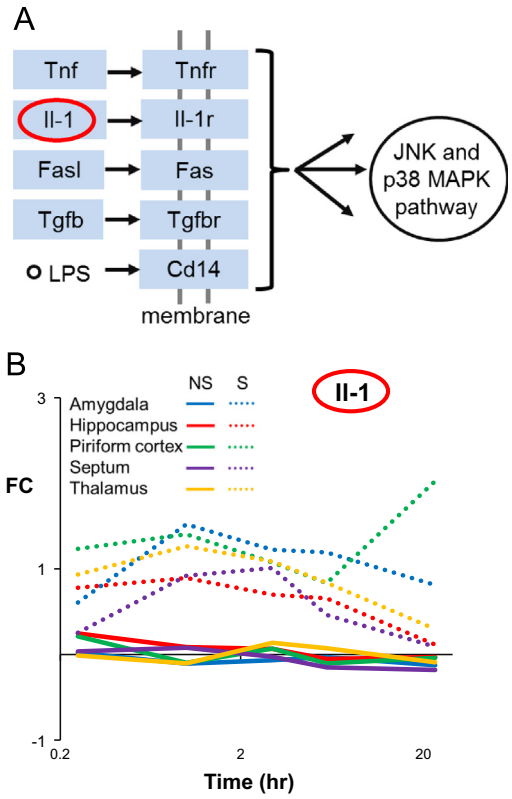


Fig. 4 – A representation of part of the JNK and p38 MAPK signaling pathway. (A) The binding of cytokines such as interleukin-1β to its receptor can trigger the activation of the JNK and p38 MAPK signaling pathway. (B) The $\log_2$ fold-change of Il-1β as a function of time in the different sarin-sensitive regions of the brain in seizing and non-seizing rats. Il-1β: interleukin-1β; FC: $\log_2$ fold-change; NS: non-seizing; S: seizing.

these pathways for both the seizing and the non-seizing groups. There was no difference in the time profile between the groups in the ERK5 pathway, and, with the exception of the 1-h and 3-h time points (with p-values of 0.02 and 0.05, respectively), there was also no significant difference between the number of DEGs in the classical MAPK signaling sub-pathway in seizing and non-seizing rats. In contrast, the transcriptional response in the non-seizing group showed no evidence of elevated levels of genes from the JNK and p38 MAPK signaling sub-pathway, whereas the number of genes for the seizing group was significantly elevated for all time points (p-value <0.05). Supplementary Fig. 1 provides the FC for all genes activated by seizing rats in this sub-pathway.

We also examined the genes that could trigger the JNK and p38 MAPK signaling sub-pathway (Fig. 4a) and observed the up-regulation of Il-1β in seizing rats at 0.25 h (Fig. 4b). The overexpression of Il-1β in seizing rats at 0.25 h was compatible with the activation of apoptosis through the JNK and p38 pathways (Abraham and Clark, 2006). The onset of apoptosis in this group was evident at later time points by the up-regulation of several gene transcripts associated with apoptosis-related genes: Caspase 3, Myc, Hmox1, and Bcl3 were up-regulated in multiple brain regions at later time

points (Supplementary Figs. 1 and 2). Importantly, we found no evidence of up-regulation or activation of apoptosis in the non-seizing animals.

2.3. *Altered gene transcription levels between non-seizing and seizing animals*

To investigate the differences between seizing and non-seizing rats, we identified 1) sets of genes common to at least three brain regions, whose transcription levels significantly increased or decreased compared to controls, and whose levels did not change among the seizing animals at the earliest time point; 2) sets of "opposing" genes in specific brain regions that exhibited opposite up/down or down/up transcriptional levels among the seizing/non-seizing groups; and 3) GO terms related to either immune response or neurological function.

2.3.1. *Common genes differentially expressed in brain regions of non-seizing rats*

Table 3 shows the three genes that were differentially over-expressed at 0.25 h in at least three of the five sarin-sensitive brain regions of non-seizing rats: Homer1, Bcat1, and Ttr. In seizing rats, the expression levels of these genes were generally not significantly different from the levels in the control group. Fig. 5a and Supplementary Fig. 3a show the detailed time course and brain-region-specific expression-level changes for these genes. The initial increased mRNA expression levels of Homer1 in all brain regions in the non-seizing animals were followed by a decrease at 1 h, then an increase. The seizing animals lacked the initial onset, but showed stronger and sustained levels of Homer1 expression beyond the 3-h time point.

Table 4 shows the five genes that were differentially under-expressed at 0.25 h in at least three of the five sarin-sensitive brain regions of non-seizing rats: Ephx2, Retsat, Nqo2, Dbp, and Zeb2. In seizing rats, the expression levels of these genes were not significantly different from levels in the control group. Fig. 5b and Supplementary Fig. 3b show the corresponding brain region and time-dependent mRNA expression-level changes relative to the control groups beyond the 0.25-h time point. Interestingly, the pattern for expression of Ephx2 was negatively correlated between the seizing and non-seizing groups (Fig. 5b), with an average Pearson coefficient of −0.93 for all the brain regions, with individual values ranging from −0.87 to −0.98. Ephx2 expression levels were down in the non-seizing group until 3 h, after which the expression levels returned to control group levels. We also observed moderately anti-correlated time-dependent expression levels of Retsat in the amygdala ($r=-0.44$) and hippocampus ($r=-0.49$), Dbp in the amygdala ($r=-0.45$) and hippocampus ($r=-0.53$), and Zeb2 in the hippocampus ($r=-0.40$) (Supplementary Fig. 3b). Anti-correlated behavior indicated the opposing regulation of gene expression between the two groups over the entire duration of the experiment.

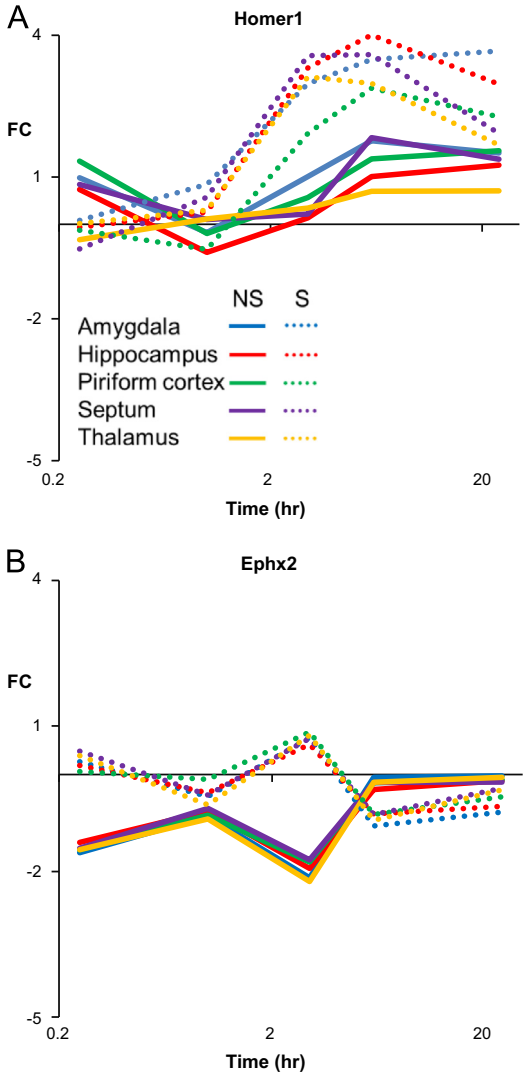


Fig. 5 – Expression of Homer1 and Ephx2. (A) The log_2 fold-change of Homer1 at different time points in the five sarin-sensitive brain regions in seizing and non-seizing rats. (B) The time-dependent log_2 fold-change of Ephx2. FC: log_2 fold-change; NS: non-seizing; S: seizing.

Table 3 – Up-regulated genes found at 0.25 h in at least three brain regions in non-seizing rats but not in seizing rats.

Gene	Possible neuroprotective function	Reference
Homer1	– Regulates apoptosis – Strongly attenuates calcium mobilization and MAPK activation induced by glutamate receptors	(Luo et al., 2012; Tappe et al., 2006)
Bcat1	– Key enzyme of glutamate metabolism	(Madeddu et al., 2004)
Ttr	– Inhibits interleukin-1 production – Contributes to control neuronal cell death and inflammation	(Borish et al., 1992; Santos et al., 2010)

Table 4 – Down-regulated genes found in at least three brain regions in non-seizing rats (but not in seizing rats) at 0.25 h.

Gene	Possible neuroprotective function	Reference
Ephx2	– Inhibition decreased plasma levels of pro-inflammatory cytokines – Suppression partially explains anti-inflammatory effect of estrogen – Inhibition enhances the anti-inflammatory effects of aspirin and MK886 – *Ephx2*$^{-/-}$ mice attenuate endotoxin-induced activation of nuclear factor (NF)-κB signaling, chemokine and cytokine expression, and neutrophil infiltration in the lung – Inhibition reduces renal inflammation and injury	(Deng et al., 2011; Elmarakby et al., 2011; Koerner et al., 2008; Liu et al., 2010; Schmelzer et al., 2005)
Retsat	– Reduced expression strongly protects cells from oxidative stress	(Nagaoka-Yasuda et al., 2007)
Nqo2	– Loss of Nqo1 and Nqo2 leads to altered intracellular redox status, decreased expression and activation of NF-κB, and altered chemokines – Inhibition could prevent oxidative damage of membrane structures, proteins, and nucleic acids (neuroprotective effect)	(Iskander et al., 2006; Kadnikov et al., 2014)
Dbp	– Down-regulation occurs during chemically induced liver regeneration	(Mueller et al., 1990)
Zeb2	– *Zeb2*$^{+/-}$ mice have a hypoalgesic phenotype in inflammatory pain	(Pradier et al., 2014)

Table 5 – Genes with opposing $\log_2$ fold-change between seizing and non-seizing rats at 0.25 h.

Gene	FC Seizing	FC Non-seizing	Possible neuroprotective action	Reference
Amygdala				
Avp	1.57	−1.13	– Up-regulated in rat hypothalamus after kainic-acid-induced seizures	(Iwanaga et al., 2011)
Prim1	−0.97	0.78	– Mutation causes extensive apoptosis	(Yamaguchi et al., 2008)
Thalamus				
Gja1	−1.24	1.38	– A mixture of tumor necrosis factor-α and interferon γ decreases Gja1 expression	(Zhang et al., 2013)
Plekhb2	−1.28	1.39	– Depletion of Plekhb2 suppresses membrane traffic	(Uchida et al., 2011)
Ppp3r1	−1.15	1.31	– In cardiomyocytes, pretreatment with Ppp3r1 reduces apoptosis in response to hypoxia – Down-regulated in traumatic brain injury – Has inverse relationship with p38MAPK-diP	(Bodega et al., 2007; Guo et al., 2012; Wu et al., 2013)
Prkacb	−1.17	1.32	– Injury to hypoglossal motor neurons decreases Prkacb expression	(Kiryu et al., 1995)
Rnf6	−1.45	1.03	– High Rnf6 protein levels play a role in developing axonal projections	(Tursun et al., 2005)

2.3.2. *Specific genes in the amygdala and thalamus show opposing expression levels in seizing and non-seizing rats*

We identified genes in specific brain regions that were up-regulated in one cohort but down-regulated in the other, or vice-versa. At the earliest time point of 0.25 h, reflective of the initial response prior to anticonvulsant administration, there were no genes in the hippocampus, the piriform cortex, or the septum that showed opposing fold changes between seizing and non-seizing animals. However, in the amygdala, Avp was up-regulated in seizing rats and down-regulated in non-seizing rats, whereas Prim1 showed the opposite up/down expression-level pattern (Table 5). Although Prim1 was not differentially expressed in any other brain region among the non-seizing animals, it was down-regulated in the hippocampus and the piriform cortex in the seizing group at 0.25 h.

We found a clear pattern of the up/down behavior for five genes in the thalamus. All five genes that were down-regulated at 0.25 h in the thalamus of seizing rats were up-regulated in non-seizing rats, namely: Gja1, Plekhb2, Ppp3r1, Prkacb, and Rnf6 (Table 5). Compared to their respective controls, non-seizing rats activated these genes by at least two-fold (FC > 1), while seizing rats suppressed their expression by more than two-fold (FC < -1).

2.3.3. *Differential expression of genes involved in immune, defense, and neurological pathways*

We analyzed the pattern of pathways impacted by the differential and opposite levels of mRNA transcripts in the seizing and non-seizing animal groups exposed to sarin at the initial 0.25-h time point. Fig. 6 shows the differential GO-term enrichment associated with different defense and immune responses, and with nerve and synaptic transmissions in the different brain regions.

The animals in the non-seizing group exhibited lowered expression of genes that belong to the defense and immune response categories in the amygdala and piriform cortex, whereas these categories were associated with increased expression levels in the piriform cortex and thalamus of the seizing rats. Seizing rats exhibited increased expression of genes related to the regulation of nerve impulse and synaptic

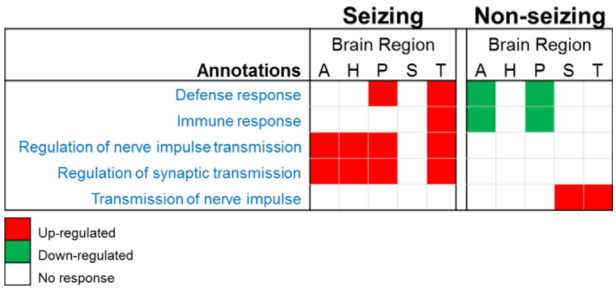


Fig. 6 – Activated and suppressed Gene Ontology (GO) terms related to immune response and nerve impulse transmission. Based on the differentially expressed genes in different brain regions at 0.25 h, a number of GO terms may be involved in immune response or nerve impulse transmission. Each observation is statistically significant, with an associated p-value <0.05. A: amygdala; H: hippocampus; P: piriform cortex; S: septum; T: thalamus.

transmission in all brain regions except the septum. These processes were absent in the non-seizing rats, except for the related term "transmission of nerve impulse," which was associated with increased expression levels in the septum and thalamus of the non-seizing rat.

3. Discussion

The data at the earliest time point (0.25 h) reflect the initial and early physiological response after sarin exposure in either non-seizing or seizing animal groups. The seizing phenotype indicates that the animals could not overcome the sarin intoxication using endogenous responses and that the countermeasures administered immediately after the onset of seizure served to reactivate non-aged AChE and protect muscarinic acetylcholine receptors from continued physiological damage, but did stop the seizures themselves. On the other hand, the non-seizing rats (and their control) received no countermeasures and represent a sufficient endogenous response to inhibit initial seizures. It was clear from the number of differentially expressed mRNA transcripts in both groups of animals that all studied brain regions were affected by sarin at the earliest time point (Fig. 1b and c, and Table 1). Here, we discuss the similarities and differences between these responses in order to characterize the response in the animals that did not experience seizures from the same level of sarin exposure.

3.1. *Similarities and differences between seizing and non-seizing animal responses to the sarin insult*

We observed increased mRNA transcription levels in both seizing and non-seizing animals for five genes in at least four brain regions (Table 2). Of these, Ier2 is an immediate early gene, which plays a largely non-specific role as an earlier response to cellular stress. Btg2 and Ilf3 are transcription factors implicated in the control of neuronal differentiation and the expression of interleukins such as Il-2 (Zhu et al., 2010), respectively. Ilf3, together with Dusp1 and Zfp36, is associated with regulation of the inflammatory response. By phosphorylating and negatively regulating JNK and p38 MAPK, Dusp1 regulates innate immunity and can profoundly affect the outcome of inflammatory challenges (Abraham and Clark, 2006). The protein of Zfp36, tristetrapolin, promotes the degradation of pro-inflammatory cytokines Tnf-α and Il-6 (Carballo et al., 1998; Zhao et al., 2011), which are known to be up-regulated in seizing rats (Spradling et al., 2011a). We also observed a statistically significant increase in mRNA transcription levels for Fos in select brain regions of both seizing and non-seizing rats, with the latter showing levels of less intensity. The up-regulation of the immediate early gene Fos is seen following a convulsion-inducing dose of the OP nerve agent soman (Denoyer et al., 1992; Zimmer et al., 1997). Taken together, the commonality of the above DEGs in both groups at 0.25 h points to shared components of a physiological defense response mounted against the sarin exposure.

3.1.1. *Differential activation of MAPK sub-pathways distinguished the seizing and non-seizing responses*

Among the shared pathways that were activated in both groups (Fig. 2), we hypothesized that the details in the activation pattern of the MAPK signaling pathway carried important implications for the downstream inflammatory responses (Herlaar and Brown, 1999; Kyriakis and Avruch, 2001). First, there was more extensive activation of the MAPK signaling pathway at 0.25 h in the seizing rats, with five brain regions affected, as opposed to two in the non-seizing group. This activation also persisted at later time points, in spite of the countermeasures given to the seizing group. Second, we found that the activation of particular sub-pathways of the MAPK signaling pathway differentiated the seizing from the non-seizing animals exposed to sarin.

When we mapped all the genes up-regulated in the MAPK signaling pathways, the number of genes involved in the classical MAPK signaling and ERK5 MAPK signaling was similar. Although both sub-pathways lead to proliferation and differentiation (Abraham and Clark, 2006; Nishimoto and Nishida, 2006), the number of up-regulated genes associated with JNK and p38 MAPK signaling increased for seizing rats up until 3 h and remained at an elevated number at 24 h, while the number of JNK and p38 MAPK signaling genes for non-seizing rats remained unaltered (Fig. 3).

The already increased levels of Il-1β at 0.25 h observed in seizing rats (Fig. 4b), and notably absent in the non-seizing group, serve as the potential trigger of the JNK and p38 MAPK signaling sub-pathway in OP-induced seizures (Chapman et al., 2006; Dhote et al., 2007; Johnson and Kan, 2010; Svensson et al., 2001, 2005; Williams et al., 2003). Further evidence of the role of Il-1β is its significantly higher and longer-lasting levels of expression in rats experiencing longer seizures (Chapman et al., 2006), and its involvement in long-term brain damage after OP intoxication (Svensson et al., 2001). Importantly, animals that do not exhibit seizures following soman intoxication did not over-express Il-1β (Svensson et al., 2005).

The activation of potentially harmful inflammation through specific MAPK pathways in the seizing animals is likely an initial event that, if unabated, triggers apoptosis and sustains persistent seizures (Abraham and Clark, 2006; Baille

et al., 2005; Herlaar and Brown, 1999; McLeod et al., 1983; Zhang and Liu, 2002). On the other hand, in the non-seizing animal, mechanisms that are able to control or inhibit the inflammatory response would constitute innate neuroprotective mechanisms. Evidence for the specific activation and suppression of such processes is discussed next.

3.2. *Anti-inflammatory processes specifically activated in non-seizing rats*

We found groups of genes common to the non-seizing group that were either up- or down-regulated in at least three brain regions at the earliest time point of 0.25 h, but not differentially expressed among the seizing rats. Tables 3 and 4 show the biological processes and tentative mechanism associated with these genes.

3.2.1. *Up-regulation of genes in the non-seizing response*

We found increased expression of Ttr and Homer1 in the non-seizing group, but not in the seizing rats at the earliest time point. These genes have been linked to anti-inflammatory or anti-apoptotic processes in a number of previous studies: Ttr was found to inhibit monocyte and endothelial cell Il-1 production (Borish et al., 1992), and cerebrospinal fluid Ttr contributes to controlled neuronal cell death, edema, and inflammation (Santos et al., 2010). Similarly, overexpression of Homer1a (the short variant of Homer1) blocked Tnf-α/cycloheximide-induced apoptotic cell death (Luo et al., 2012). Homer1a is rapidly and selectively up-regulated in spinal cord neurons after peripheral inflammation in an N-methyl-D-aspartate (NMDA) receptor-dependent manner, strongly attenuating calcium mobilization as well as MAPK activation induced by glutamate receptors (Tappe et al., 2006).

3.2.2. *Down-regulation of genes in the non-seizing response*

We found decreased expression of Ephx2, Retsat, Nqo2, and Zeb2 in at least three brain regions in non-seizing rats (but not in seizing rats) at 0.25 h. Ephx2 has been linked to inflammation via both drug inhibition and knockout experiments. Inhibitors of soluble epoxide hydrolase 2 (sEH, the protein from Ephx2) decreased plasma levels of pro-inflammatory cytokines and nitric oxide metabolites (Schmelzer et al., 2005). Similarly, the neuroprotection provided by estradiol against ischemic brain injury is partially explained by sEH suppression (Koerner et al., 2008); inhibition of sEH enhances the anti-inflammatory effects of aspirin (Liu et al., 2010), and inhibition of sEH reduces renal inflammation and injury in diabetic wild-type mice (Elmarakby et al., 2011). Knockout experiments show that *Ephx2*$^{-/-}$ mice each exhibited a significant attenuation of endotoxin-induced activation of nuclear factor (NF)-κB signaling, cellular adhesion molecules, chemokine and cytokine expression, and neutrophil infiltration into the lungs (Deng et al., 2011).

The lowered expression of Retsat, Nqo2, and Zeb2 can also be linked to the mitigation of inflammation and oxidative stress. Specifically reducing the expression of Retsat strongly protects cells from oxidative stress (Nagaoka-Yasuda et al., 2007). The loss of Nqo2, together with Nqo1, leads to altered intracellular redox status, decreased expression and activation of NF-κB, and alteration of chemokine levels (Iskander et al., 2006). Similarly, drug inhibition of Nqo2 could prevent oxidative damage of membrane structures, proteins, and nucleic acids (Kadnikov et al., 2014). Lastly, Zeb2 was shown in a *Zeb2*$^{+/-}$ mice knockout experiment to decrease sensitivity to inflammatory pain (Pradier et al., 2014). Thus, the decreased expressions of Ephx2, Retsat, Nqo2, and Zeb2 were compatible with an active response aimed at mitigating inflammatory stress.

3.2.3. *Opposing regulation of genes in non-seizing versus seizing animals*

A third group of genes comprises those that have significant opposite high/low or low/high expression levels that differentiate the seizing and non-seizing groups. We observed a number of such pairs specifically located in the amygdala and the thalamus at the 0.25-h time point (Table 5). In the amygdala, a brain region important for initiation and control of seizures (Aroniadou-Anderjaska et al., 2009), 1) Avp was down-regulated in non-seizing rats and up-regulated in seizing rats, and 2) Prim1 was up-regulated in non-seizing rats and down-regulated in seizing rats. The state of these genes in the seizing group has been independently linked to seizures (Avp) (Iwanaga et al., 2011) and extensive apoptosis (Prim1) (Yamaguchi et al., 2008).

Although not the site of seizure origin, the thalamus regulates other structures involved in seizures (Miller and Ferrendelli, 1990) and supports the propagation and synchronization of limbic seizures (Chang et al., 2013). Furthermore, pretreatment of the thalamus can protect against seizures originating from the piriform cortex (Cassidy and Gale, 1998). In the study by Spradling et al. (2011b), the thalamus was identified as a brain region with a unique molecular response to nerve agent-induced seizures (Spradling et al., 2011a).

At 0.25 h, we found only five down-regulated genes in the thalamus of the seizing rats, whereas all of the genes were up-regulated in the non-seizing rats (Table 5). Two of these genes – Gja1 and Ppp3r1 – are known to be involved in the inflammation pathway. Consistent with the pro-inflammatory phenotype of the seizing animals, decreased Gja1 expression has been linked to the presence of the inflammatory cytokines Tnf-α and interferon-γ (Zhang et al., 2013), and to the inflammatory attack on central nervous system white matter (Brand-Schieber et al., 2005). Similarly, down-regulation of Ppp3r1 is associated with traumatic brain injury (Wu et al., 2013), and its pro-inflammatory activity is inversely related to p38MAPK-diP (Bodega et al., 2007). Furthermore, pretreatment with Ppp3r1 in cardiomyocytes reduces apoptosis in response to hypoxia (Guo et al., 2012).

3.3. *Hypothesis on the role of anti-inflammatory processes in non-seizing animals*

The microarray analyses of the two groups of sarin-exposed rats suggested that non-seizing rats were active in suppressing inflammation related to sarin poisoning. As discussed above and summarized in Tables 3–5, the appropriate up- or down-regulation of the genes unique to non-seizing rats was generally beneficial in protecting cells via anti-inflammatory or anti-apoptotic processes. In addition, specific brain regions in non-seizing rats suppressed defense and immune responses, while seizing rats activated them (Fig. 6). The transcriptional evidence of activation of these mechanisms in non-seizing animals and lack thereof in the seizing animals indicates an initial active

response that was able to suppress downstream activation of the traditional three-stage process associate with OP poisoning. Differential activation of the MAPK inflammatory pathways in the two groups was linked to downstream activation of apoptosis in the seizing group, but not in the non-seizing group. While it is established that seizure causes inflammation, the intimate connection between the two processes has also led to the hypothesis that inflammation may cause and contribute to seizures (Vezzani et al., 2011), a hypothesis that is also supported by experimental results (Marchi et al., 2014; Shimada et al., 2014; Vitaliti et al., 2014).

The response of non-seizing rats to sarin exposure included the differential regulation of the MAPK signaling pathway, which served to reduce a harmful inflammatory response. Thus, up-regulation of Ttr and Dusp1 served to inhibit Il-1 and Tnf-α, the latter being known triggers of the pro-apoptotic JNK and p38 MAPK signaling sub-pathways (Abraham and Clark, 2006; Borish et al., 1992), which are related to seizure activity (Yang et al., 1997). Similarly, the preferential up-regulation of Homer1 could be linked to attenuation of MAPK activation (Tappe et al., 2006). Hence, it is interesting to hypothesize that the transcriptional activity associated with the regulation of anti-inflammatory genes may in fact be part of an active and sufficient response to protect against the onset of seizures.

The observed "opposing" effects of separate sets of genes that exhibited opposite up/down or down/up transcriptional levels among the seizing/non-seizing groups could potentially be exploited for therapeutic purposes. Pretreatments using down-regulated genes linked to the seizing phenotypes have shown a protective effect against inflammation and injury (Guo et al., 2012). Similarly, pretreatment using NMDA was shown to reduce the incidence of seizures up to 50% (Boeck et al., 2004) by modulating the MAPK pathway response (de Araújo Herculano et al., 2011). Treatment or pre-conditioning with gene products or drugs that affect these proteins could channel and strengthen the brain's protective physiological response against otherwise lethal sarin challenges.

4. Experimental procedure

4.1. *Data collection and processing*

In our analyses, we used the publicly available gene expression microarray dataset from rat studies of sarin exposure (Spradling et al., 2011a). In summary, male Sprague–Dawley rats were subcutaneously challenged with $1 \times LD_{50}$ sarin (108 μg/kg) and, accordingly, approximately 50% of the animals challenged with sarin produced seizure activity following exposure. Seizing rats were treated with atropine sulfate (to block excess ACh from the muscarinic receptors) and 2-PAM (to reactivate non-aged AChE) one minute after seizure onset, and with the anticonvulsant diazepam 30 min later. Matched control animals received an equivalent volume of vehicle (saline) and countermeasure drugs corresponding to the injections administered to seizing rats. Drug treatments were not given to the remaining sarin-exposed rats that did not develop seizures or to their matched controls. Fig. 1a illustrates the four groups of rats (sarin-exposed seizing rats, sarin-exposed non-seizing rats, saline control for seizing rats, saline control for non-seizing rats) used in the microarray analyses. Three to four animals from each group of rats were euthanized at time points 0.25, 1, 3, 6, and 24 h, and five different brain regions (amygdala, hippocampus, piriform cortex, septum, and thalamus) were immediately collected at each time point for microarray hybridization (Spradling et al., 2011a).

We downloaded the raw gene expression microarray datasets with series accession number GSE28435 from the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/) in January 2014. Each microarray corresponds to gene transcription changes in the brain regions of the rats as caused by sarin exposure versus control samples. We performed background correction, quantile normalization, and summarization using the robust multi-array average method implemented in the BioConductor R-language suite of bioinformatics tools (Irizarry et al., 2003). With genefilter, a BioConductor package, we carried out non-specific filtering of the genes. Probe sets without Entrez ID or with low variances across all microarrays based on inter-quartile range were removed, and the number of replicates for each microarray that had a "Present" call was determined for each probe set. Only probe sets for which at least 25% of the conditions had "Present" calls for all replicates within a condition were retained for further analysis. With the remaining genes, we computed $\log_2$ expression values for treatment and control as averages over replicates. After determining the average intensity between the replicates, we calculated the $\log_2$ fold changes (FC) for each gene as the difference between treatment and corresponding control RMA expression levels (log ratios).

4.2. *Identifying genes relevant to seizing and non-seizing rats*

We used the rank product – a non-parametric, permutation based method – to identify differentially expressed genes (Breitling et al., 2004). In our analyses, we separately identified significantly activated (up-regulated) and suppressed (down-regulated) genes using an FDR-corrected p-value of 0.05 as the statistical significance cutoff. We considered up- and down-regulated genes separately, as biological processes are characterized by interacting, co-regulated proteins (Yu et al., 2011). We carried out the rank product analyses separately for each combination of brain region and time point in either seizing or non-seizing rats. In addition, we used R and Perl to analyze the DEGs and FCs of the genes.

4.3. *Pathway enrichment analyses*

To identify molecular pathways that were significantly regulated at each combination of brain region and time point in seizing and non-seizing rats, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/home.jsp) tool to perform Gene Ontology (GO) analysis and KEGG pathway enrichment analysis (Huang et al., 2009). We considered GO terms and KEGG pathways below a p-value of 0.05 to be significantly enriched. We used the DEGs identified from Section 4.2 as inputs for the

pathway enrichment analysis. Then, we separately used the up-regulated genes and down-regulated genes (unless otherwise noted) in this set and carried out the pathway enrichment analysis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2015.05.034.

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