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Peripherally restricted acute phase response to a viral mimic alters hippocampal gene expression

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Abstract

We have previously shown that peripherally restricted acute phase response (APR) elicited by intraperitoneal (i.p.) injection of a viral mimic, polyinosinic-polycytidylic acid (PIC), renders the brain hypersusceptible to excitotoxic insult as seen from profoundly exacerbated kainic acid (KA)-induced seizures. In the present study, we found that this hypersusceptibility was protracted for up to 72 h. RT-PCR profiling of hippocampal gene expression revealed rapid upregulation of 23 genes encoding cytokines, chemokines and chemokine receptors generally within 6 h after PIC challenge. The expression of most of these genes decreased by 24 h. However, two chemokine genes, i.e., *Ccl19* and *Cxcl13* genes, as well as two chemokine receptor genes, *Ccr1* and *Ccr7*, remained upregulated for 72 h suggesting their possible involvement in the induction and sustenance of seizure hypersusceptibility. Also, 12 genes encoding proteins related to glutamatergic and GABAergic neurotransmission featured initial upregulation or downregulation followed by gradual normalization. The upregulation of the *Gabrr3* gene remained upregulated at 72 h, congruent with its plausible role in the hypersusceptible phenotype. Moreover, the expression of ten microRNAs (miRs) was rapidly affected by PIC challenge, but their levels generally exhibited oscillating profiles over the time course of seizure hypersusceptibility. These results indicate that protracted seizure susceptibility following peripheral APR is associated with a robust polygenic response in the hippocampus.

Keywords

Polyinosinic-polycytidylic acid; Seizures; Inflammation; Neurotransmission; Cytokines; MicroRNA

Introduction

The acute phase response (APR) is the first line of defense against viral infection and is mediated by innate immune cells that recognize molecular signatures of viral replication. dsRNA is an intermediate generated by most viruses during their replication cycle (Jacobs and Langland 1996; Weber et al. 2006). Mammalian cells have several receptors that detect

the presence of extra- and intracellular dsRNA, i.e., Toll-like receptor 3 (TLR3), retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation-associated protein 5 (MDA-5) and protein kinase R (PKR) (Berke et al. 2013). The ligation of these receptors leads to the production of type I interferons and other inflammatory cytokines with antiviral activity (Muller et al. 1994). Although the primary role of APR is to combat infections, the cytokines released into the circulation have significant effects on the brain. Thus, intraperitoneal injection of a synthetic dsRNA, polyinosinic-polycytidylic acid (PIC), in mice induces behavioral symptoms collectively referred to as “sickness behavior” (Muller et al. 1994; Guha-Thakurta and Majde 1997; Cunningham et al. 2007) that are congruent with behavioral effects of peripheral viral infections in humans (Loftis et al. 2008; Huckans et al. 2009; Nelligan et al. 2008). The PIC-induced symptoms peak at 6 h after the injection and subside by 48 h (Cunningham et al. 2007). The induction of sickness behavior is concomitant with transient upregulation of genes encoding IL-1 β , IL-6, TNF α and IFN β in the hippocampus and hypo-thalamus (Cunningham et al. 2007). We have confirmed the expression of these cerebral genes and shown that their upregulation is global rather than regional, as it is featured in all parts of the brain, i.e., the forebrain, cerebellum and brain stem (Konat et al. 2009). We have further shown that the brain also features upregulation of a plethora of chemokines and their receptors (Fil et al. 2011). Generally, the upregulation of respective mRNAs peaks between 3 and 6 h following the PIC challenge and reaches from several- to several thousand-fold over control. After 24 h, the expression of most of the genes returns to the baseline levels. We also demonstrated that APR is restricted to the peritoneal cavity as PIC does not enter the blood circulation. Moreover, the cerebral inflammatory response can be induced in naïve mice by a passive transfer of blood from PIC-challenged mice indicating that the response is mediated by circulating inflammatory factors.

Furthermore, we have found that this robust, albeit generally transient, genomic response to PIC-induced APR renders the brain hypersusceptible to excitotoxic insult (Kirschman et al. 2011). In this study, mice were i.p. injected with PIC and after 2 days challenged with kainic acid (KA). The PIC pretreatment profoundly increased both the intensity and duration of KA-induced seizures as compared to naïve animals challenged with KA alone. This finding indicates that the brain inflammatory response instigated by peripheral APR induces protracted remodeling of neural circuits. This finding also supports a causative role of peripheral viral infections in the increased seizure propensity inferred from epidemiological studies (Tellez-Zenteno et al. 2005). Possible mechanisms of APR-induced seizure hypersusceptibility may entail increased neuronal excitability and/or decreased neuronal inhibition instigated by inflammatory mediators. Inflammation-induced changes in the expression of neurotransmitter receptors (Guo et al. 2002; Harre et al. 2008; Galic et al. 2012) support such mechanisms. Moreover, several microRNAs (miRs), important regulators of gene expression at the neuroimmune interface (Soreq and Wolf 2011), have been implicated in the pathology of seizures (Jimenez-Mateos et al. 2011; Hu et al. 2011; Aronica et al. 2010; Liu et al. 2010), suggesting their role as upstream determinants of PIC-induced seizure hypersusceptibility.

The present study was undertaken to gain insight into the genetic mechanisms underlying seizure hypersusceptibility following PIC-induced APR. We focused on the hippocampus

because this structure is the primary region of ictal onset instigated by KA administration (Ben-Ari and Cossart 2000). We profiled the expression of several inflammatory, neurotransmission-related as well as miR genes during the period of seizure hypersusceptibility.

Materials and methods

Animals

Eight-week old C57BL/6 J mice obtained from Charles River Laboratories (Wilmington, MA) were housed under 12-h light/dark conditions (lights on at 6 am) and fed ad libitum. Peripheral APR was induced by a single intraperitoneal (i.p.) injection of 12 mg/kg of PIC (Invivogen, San Diego, CA) in 100 μ l of saline. Saline injected mice served as controls. All procedures were approved by the West Virginia University Animal Care and Use Committee and conducted in compliance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Open-field test

Locomotor activity was assessed using the automated activity monitoring system PAS-Open field (41 cm $\times$ 41 cm $\times$ 38 cm; San Diego Instruments, San Diego, CA). The chambers were equipped with a 16 $\times$ 16 array of infrared photo-beams to measure horizontal (XY) movement and an additional frame of 16 beams to monitor rearing. Locomotor activity was recorded for 15 min.

Evaluation of seizure susceptibility

At different time points (1–7 days) following PIC challenge, mice received subcutaneous injections of 20 mg/kg of KA (Sigma Chemical Co., St. Louis, MO) in saline. Saline injected mice served as controls. Seizure severity was scored by blinded observers in 5 min intervals as described previously (Kirschman et al. 2011). Cumulative seizure scores were assessed as the summation of all scores over the 2 h observation period.

Blood cytokine measurement

Mice were deeply anesthetized with 65 mg/ml of pentobarbital (Fatal Plus, Vortech Pharmaceutical, Dearborn, MI) administered i.p. and sacrificed by pneumothorax. Blood was quickly collected by heart puncture and citrated. IFN β was measured using the VeriKine Mouse Interferon Beta ELISA kit (PBL Interferon Source, Piscataway, NJ) per manufacturer's instructions. IL-6 levels were measured using the Milliplex MAP Mouse Cytokine/Chemokine panel (Millipore, Billerica, MA) per manufacturer's instructions and analyzed using a Luminex 200 System (Luminex, Austin, TX).

qRT-PCR

Mice were anesthetized and sacrificed as described above, and transaortically perfused with saline. Brains were removed from the skull and hippocampi were dissected out. The tissue was immediately homogenized in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), and RNA was isolated per manufacturer's protocol. For quantitation of

mRNA, cDNA was synthesized using SuperScript III First-strand Synthesis kit (Invitrogen, Carlsbad, CA) and quantified using RT² SYBRGreen (Qiagen, Valencia, CA). For miR quantitation, cDNA was synthesized using NCode VILO miRNA cDNA Synthesis kit (Invitrogen, Carlsbad, CA) and quantified using EXPRESS SYBR GreenER miRNA qRT-PCR kit (Invitrogen, Carlsbad, CA). qRT-PCR was performed in an ABI7500 Real-Time PCR system (Applied Biosystems, Foster City, CA). Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA or U6 snRNA were used as internal controls for mRNAs and miRs, respectively. The Ct method was used for quantitation. Specific primer sequences are available upon request.

Statistical analysis

Data were analyzed by ANOVA and expressed as means $\pm$ SD. Statistical comparisons between groups were performed using Student's *t* test. Differences between groups were considered significant at *p* < 0.05.

Results

Verification of sickness behavior

PIC-induced sickness behavior strongly suppresses the burrowing activity of mice (Cunningham et al. 2007; Konat et al. 2009). Although at nadir (6 h post-injection), the burrowing activity of PIC-challenged animals drops below 10 % of the respective controls, the test is rather cumbersome and lengthy (2 h). Cunningham et al. also found suppression of locomotor activity using the open field test, albeit to a much lesser extent than the burrowing activity. In concordance with this study, we observed the locomotion of mice to be reduced by approximately 30 and 60% at 3 and 6 h after PIC injection, respectively (Fig. 1). However, we found that the rearing activity was suppressed equally to the burrowing activity as it dropped by 70 and 96 % at 3 and 6 h, respectively. Consequently, the rearing test, which lasts only 15 min, provides a convenient, highly sensitive method to verify successful induction of sickness behavior.

Duration of seizure hypersusceptibility

We have previously demonstrated that PIC challenge strongly enhances the susceptibility of mice to KA-induced seizures (Kirschman et al. 2011). However, this feature was analyzed only 48 h after PIC injection. Therefore, we determined the duration of this hypersusceptible phenotype to provide a time frame for the subsequent genetic analysis. As seen from Fig. 2, the seizure response measured as cumulative seizure scores was highest 1 day post-PIC, reaching nearly 3-fold over saline injected controls. Although the hypersusceptibility gradually decreased, it was still significant at 2 and 3 days post-PIC. By days 4 and 7, the response of the PIC challenged animals was indistinguishable from that of controls.

Cytokine surge

Intraperitoneal PIC injection induces the synthesis of IFN β , IL-6, IL-1 β and TNF α that rapidly reach the circulation as seen from the surge of these inflammatory cytokines in the blood (Cunningham et al. 2007). Their levels peak sharply at 3 h post-injection and then quickly decline. We confirmed these kinetics for IFN β and IL-6 as shown in Fig. 3. Thus,

both cytokines reached the highest levels 3 h after PIC challenge, and decreased rapidly thereafter, reaching baseline levels at 12 h post-injection. The maximal blood concentrations of IFN β and IL-6 were 33.5 and 21.5 ng/ml, respectively, which is in concordance with the values observed by Cunningham et al. (2007).

Expression of hippocampal genes

Inflammatory genes—Based on our previous studies (Konat et al. 2009; Fil et al. 2011), we selected 23 inflammation-related genes comprising cytokines, chemokines and chemokine receptors, and profiled their expression in the hippocampus following PIC challenge. The blood cytokine surge was associated with a rapid upregulation of cytokine (Fig. 4) and chemokine (Fig. 5) genes, buttressing the cause-effect relationship between the circulating cytokines and the genetic response of the hippocampal cells. However, differences in the expression pattern of the genes were evident. The expression of the *Il6*, *IFNb*, *Cxcl17*, *Ccl4*, *Cxcl1*, *Cxcl2*, *Cxcl9*, *Cxcl10* and *Cxcl11* genes peaked between 3 and 6 h after PIC injection and dwindled rapidly thereafter. The *Tnfa*, *Il1b*, *Ccl7*, *Ccl12* and *Ccl2* genes featured an extended timeframe of upregulation with high levels of their mRNA remaining at 24 h. The expression of the *Ccl19*, *Cxcl13* and *Ccl5* genes actually peaked at 24 h. Most of the genes, except the *Ifnb*, *Il1b*, *Cxcl17*, *Cxcl1*, and *Cxcl2* genes, were significantly upregulated even at 72 h. The *Ccl9* gene exhibited a unique expression profile, peaking early at 3 h and then peaking again from 48 to 72 h. Also, the extent of upregulation varied greatly among the genes. The cytokine genes were upregulated by approximately 2- to 32-fold over control with the *Il6* gene being the most and the *Il1b* gene being the least upregulated. These results corroborate a previous study of the response of hippocampal cytokine genes to PIC challenge (Cunningham et al. 2007). Among the chemokine genes, *Cxcl11*, *Cxcl10*, *Cxcl9* and *Cxcl1* featured the highest upregulation by more than a thousand-fold over control. The *Cxcl2*, *Ccl12* and *Ccl2* genes were upregulated up to several hundred-fold, whereas several ten-fold upregulations were observed for the *Ccl7*, *Ccl4* and *Ccl5* genes. The *Cxcl13*, *Ccl19*, *Cxcl17* and *Ccl9* genes were upregulated by less than ten-fold.

Also, five genes encoding chemokine receptors were significantly upregulated several fold over the baseline by PIC challenge (Fig. 6). The *Ccr1*, *Ccr6* and *Ccr7* gene expression peaked at 3 h, whereas the expression of the *Cxcr2* and *Cxcr5* genes was delayed and peaked at 9 h. By 72 h after PIC challenge, the *Ccr1* and *Ccr7* genes featured approximately 2-fold upregulation, while expression of the *Cxcr2* and *Cxcr5* genes dwindled to the baseline levels. In contrast, the *Ccr6* gene featured downregulation beginning at 48 h and dipped to over 2-fold below control level at 72 h.

Neurotransmission genes—Seizure hypersusceptibility can result from increased neural excitability, decreased neural inhibition, or both. The most direct effectors of these potential changes in excitability are the neurotransmitter receptors. Therefore, we screened the expression of genes encoding all glutamatergic and GABAergic neurotransmitter receptors and found nine genes to be significantly altered by PIC challenge (Fig. 7). These genes were: the kainate receptor gene *Grik3*, the AMPA receptor gene *Gria4*, the metabotropic glutamate receptor genes *Grm1*, *Grm6*, and *Grm7*, and the GABA_A receptor subunit genes *Gabrq*, *Gabre*, *Gabrr2*, and *Gabrr3*. The *Grik3*, *Gria4*, *Grm6*, *Gabrq*, *Gabrr2*

and *Gabrr3* genes showed initial several-fold upregulation coincident with the blood cytokine surge that peaked 3–6 h after PIC injection. While the expression of five of these genes gradually returned to the baseline level, the upregulation of the *Gabrr3* gene was protracted and its mRNA level at 72 h was approximately 2-fold over control. The *Grm6* gene featured moderate upregulation after 48 h, reaching approximately 1.5-fold over the control level at 72 h. In contrast, the *Grm7*, *Grm1* and *Gabre* displayed initial downregulation by approximately 2-fold, followed by normalization.

In addition, we found the expression of three genes whose products are involved in synaptic buffering of glutamate and potassium to be significantly altered by PIC challenge. As shown in Fig. 7, the lactate dehydrogenase A gene (*Ldha*) was rapidly upregulated at 3 h post-PIC injection and gradually returned to the baseline level. The expression of the glutamate dehydrogenase gene (*Gldh*) also peaked at 3 h but featured subsequent downregulation below control level at 24 h. Its expression gradually returned to the baseline by 72 h. The expression of the *Kcnj10* gene encoding the inward rectifying potassium channel Kir4.1 was not affected during the blood cytokine surge, but featured a gradual downregulation thereafter, reaching a nadir at 48 h and returning to the baseline level at 72 h (Fig. 7).

miR genes—miRs are important regulators of gene expression. Of a particular interest here were miRs that modulate both neural and immune functions (Soreq and Wolf 2011) and miRs associated with seizure pathology (Jimenez-Mateos et al. 2011; Hu et al. 2011; Liu et al. 2010; Aronica et al. 2010). We screened expression of these miRs in the hippocampus following PIC challenge and identified ten species whose expression underwent significant changes (Fig. 8). Generally, the levels of these miRs changed in an oscillating manner. During the first 9 h after PIC challenge, the levels of miR-128-3p, miR-509-5p, miR-28a-5p, miR-138-5p and miR-466i-5p were initially increased and then decreased below control, whereas the levels of miR-188-5p, miR-302a-5p and miR-221-3p were initially downregulated. At 24 h, the levels either returned to baseline (miR-28a-5p, miR-138-5p, miR-128-3p, miR-509-5p, miR-188-5p and miR-302a-5p) or underwent secondary upregulation (miR-466i-5p and miR-221-3p). Two species, miR-132-3p and miR-181a-5p, were not changed during the blood cytokine surge but were upregulated at 24 h. At 72 h, the expression of miR-302a-5p was downregulated by approximately 2-fold below the baseline. miR-28a-5p and miR-138-5p were slightly downregulated, while miR-466i-5p, miR-221-3p and miR-128-3p were slightly upregulated.

Discussion

The present study extends our previous finding that peripheral APR induced by a viral mimic, PIC, increases the susceptibility to KA-induced excitotoxicity (Kirschman et al. 2011). Here, we have demonstrated that the window of increased susceptibility lasts up to 3 days after PIC challenge (Fig. 2). This is in contrast to a previous report on hypersusceptibility induced by i.p. injection of LPS (Sayyah et al. 2003). In that model of bacterial infection/sepsis, seizure hypersusceptibility was limited to the initial 24 h. Several factors may contribute to the observed differences in the duration of the hypersusceptible phenotype induced by PIC vs. LPS. For example, LPS rapidly enters the circulation (Lenczowski et al. 1997; Romanovsky et al. 2000) and thus, the brain is exposed

simultaneously to peripherally-generated inflammatory factors and the LPS itself. On the contrary, PIC does not reach the circulation (Fil et al. 2011) and therefore, elicits the cerebral response solely through blood-borne inflammatory mediators. Moreover, the composition of the blood-borne mediators induced by PIC vs. LPS challenge may differ. For example, $\text{IFN}\gamma$ is produced in response to LPS (Gibb et al. 2008; Finney et al. 2012) but not in response to PIC (Gandhi et al. 2007). Regardless of the mechanisms, our finding has important clinical implications because it indicates that viral infections may increase the risk of ictal attacks even several days after the active phase of infection has subsided. This should be particularly relevant to populations of vulnerable individuals, e.g., epileptics and post-stroke victims, or individuals exposed to seizure-inducing conditions such as heat stroke or exhaustion.

The augmentation of neuronal excitability is likely induced by inflammatory mediators generated in the peritoneal cavity that reach the brain via circulation. There are several possible mechanisms (Quan and Banks 2007). For instance, the blood-borne inflammatory mediators per se may be transported through the blood-brain barrier (BBB) or circumventricular organs (CVO). Alternatively, the mediators may activate BBB and/or CVO cells, resulting in the secretion of secondary mediators that are released into the brain parenchyma. Four major inflammatory cytokines, $\text{IFN}\beta$, IL-6, IL-1 β and $\text{TNF}\alpha$, surge in the blood, reaching peak concentrations at 3 h after PIC injection and then rapidly diminish (Cunningham et al. 2007; Fig. 3). $\text{IFN}\beta$ and IL-6 reach the highest concentrations and thus, could be plausible triggers of the hypersusceptible phenotype. However, we found that systemic injection of $\text{IFN}\beta$ and IL-6 at respective concentrations failed to induce seizure hypersusceptibility (results not shown). This result is reminiscent of the previous observation that $\text{IFN}\beta$ injection does not elicit the fatigue phenotype observed with PIC challenge (Matsumoto et al. 2008). Therefore, other inflammatory mediators are likely requisite for the induction of seizure hypersusceptibility and fatigue response. Finally, it should also be considered that the peripheral inflammatory signals can be conveyed by vagal afferents and activate the expression of cytokine genes within the brain (Marquette et al. 2003).

The cytokine surge is coincident with the upregulation of genes encoding the same cytokines in the hippocampus (Cunningham et al. 2007; Fig. 4). The brain cells including neurons, microglia and astrocytes express receptors for these cytokines (McCusker and Kelley 2013). Ligation of these receptors with either peripherally-generated or brain-generated cytokines can lead to upregulated expression of the same or different cytokines. These, in turn, can further amplify the response through positive feedback loops. Such loops can also upregulate the expression of a slew of other inflammatory mediators as exemplified by chemokines (Fig. 5) and chemokine receptors (Fig. 6). Altogether, this neuroinflammatory response creates an intricate network of autocrine/paracrine and intracellular signaling pathways that may affect neuronal networks. For example, IL-1 β and $\text{TNF}\alpha$ have an excitatory effect on hippocampal neurons by increasing ceramide synthesis within neurons and by the ensuing NMDA-mediated calcium influx (Viviani et al. 2007; Wheeler et al. 2009). IL-1 β also inhibits glutamate reuptake via the downregulation of GLT-1 expression in astrocytes (Prow and Irani 2008), which may further enhance excitability of neuronal circuits. Also, the injection of IL-1 β into the hippocampus has been shown to increase the

severity of limbic seizures (Vezzani et al. 2002). The protracted upregulation of the *Tnfa* and *Il1b* genes following PIC challenge (Fig. 4) further strengthens their putative role in the induction and sustenance of seizure hypersusceptibility. Although IL-6 and IFN β seem not to directly affect neuronal networks, they amplify the effects of IL-1 β and TNF α (McCusker and Kelley 2013). Consistent with this amplifying role, overexpression of IL-6 results in severe neurologic impairment including seizures (Campbell et al. 1993).

A body of evidence also implicates several chemokines upregulated in the hippocampus by PIC challenge (Fig. 5) in the induction of neuronal hyperexcitability. Thus, genes encoding ligands of the CXCR3 chemokine receptor, i.e., CXCL9, CXCL10 and CXCL11, featured the highest upregulation. Neurons are the primary cell types that express CXCR3, and its ligation potentially enhances electrical activity of hippocampal neurons (Nelson and Gruol 2004). In addition, CXCR3 ligation alters the expression of several GABA and glutamate receptors (Cho et al. 2009). The genes encoding CXCL1 and CXCL2 chemokines also featured robustly upregulated expression following PIC challenge (Fig. 5). Moreover, the gene encoding their receptor, CXCR2, was also highly upregulated (Fig. 6). Signaling through CXCR2 has been shown to increase neuronal excitability, potentially through the association of CXCR2 with GluR1 AMPA receptors (Lax et al. 2002; Wang et al. 2008). CCL2, CCL4 and CCR7 are elevated in brain tissue from epilepsy patients, as well as in animal models (Fabene et al. 2010; Lehtimäki et al. 2003; Liimatainen et al. 2013; Vezzani et al. 2008; Vezzani et al. 2002; Hung et al. 2013). In concordance with this, we found the *Ccl2* and *Ccr7* genes to feature a prolonged upregulation following PIC challenge (Figs. 5 and 6). Furthermore, CCL2 and CCL4 seem to be crucial for epileptogenesis (Fabene et al. 2010; Kan et al. 2012). Altogether, the above data strongly implicate the role of cytokine and chemokine gene upregulation in PIC-induced seizure hypersusceptibility.

Although some of the genes discussed above displayed sharply transient upregulation, one has to be cognizant that transient expression of the mRNA does not necessarily translate to transient expression of the cognate protein. For example, the *Cxcl1* and *Cxcl2* mRNA peaked at 6 h but returned to the baseline at 9 h after PIC challenge (Fig. 5). However, the protein synthesized within this 9-h period may persist much longer. This argument also applies to the genes featuring protracted elevation of their mRNA. For example, the *Ccl5* mRNA peaked at 24 h (Fig. 5), and albeit its levels gradually dropped, the cognate protein, CCL5, may have peaked later and been present at high concentrations during the whole period of hypersusceptibility, i.e., up to 72 h. Finally, it should also be considered that even a short-term upregulation of an inflammatory gene may contribute to the hypersusceptible phenotype through the activation of downstream pathways. These mechanisms will be addressed in future studies.

We have previously shown a quantitative variability in the response of inflammatory genes to PIC challenge among the major subdivisions of the brain, the forebrain, brain stem and cerebellum (Konat et al. 2009; Fil et al. 2011). In general, the cerebellum featured the highest upregulation of these genes. For example, the maximal upregulation of the *Il1b*, *Il6* and *Cxcl11* genes in the cerebellum was approximately four-fold higher than the respective values for the forebrain. The comparison of the present results from the isolated hippocampus (Figs. 4 and 5) to the whole forebrain (Konat et al. 2009; Fil et al. 2011)

reveals further regional heterogeneity. Thus, among the 23 hippocampal genes studied three were significantly less upregulated and four were significantly more upregulated in comparison to the whole forebrain (Table 1). In the most extreme case, the peak upregulation of the *Ifnb* gene was 50 times lower in the hippocampus than in the whole forebrain. On the other hand, the *Cxcl11* gene was nine-fold more robustly upregulated in the hippocampus in comparison with the whole forebrain. Clearly, the hippocampal cells feature a highly specific pattern of genetic response to PIC challenge vs. the average forebrain cell. This finding warrants further experimental inquiry into the responsiveness of other discrete brain structures that may provide a basis for various behavioral traits associated with immune-to-brain communication.

Prevailing theories behind seizure development and epileptogenesis converge upon changes in neuronal excitation and inhibition. These mechanisms strongly hinge on neurotransmitter receptors, where excitatory, glutamatergic neurotransmission is increased and inhibitory, GABAergic transmission is diminished (Casillas-Espinosa et al. 2012; Gonzalez 2013; Werner and Covenas 2011). Consequently, in addition to the effects of the cytokines/chemokines discussed hitherto, peripheral inflammatory signals may alter the balance between excitatory and inhibitory neurotransmission by changing the expression of neurotransmitter receptor genes. In support of this notion, we found altered expression of a number of hippocampal genes encoding neurotransmitter receptors (Fig. 7) in the same time frame as the blood cytokine surge (Fig. 3) and the rapid upregulation of inflammatory genes (Figs. 4, 5, 6). Although the significance of these changes must be verified at the protein level and through functional analysis, tentative correlations can be inferred from the changes in the mRNA levels. For example, the upregulation of the *Grik3* gene (Fig. 7) may contribute to the hypersusceptible phenotype, as long-lasting kainate receptor-mediated events have been associated with sustained, rhythmic firing in a rodent model of temporal lobe epilepsy (Artinian et al. 2011). The GABA_A- ϵ subunit, encoded by the *Gabre* gene, is associated with increased spontaneous channel activity (Bollan et al. 2008), and therefore, the prolonged downregulation of the *Gabre* gene following PIC challenge (Fig. 7) may increase hyperexcitability by impeding spontaneous inhibitory currents. Also, the GRM7 receptor negatively regulates GABAergic inhibition (Casillas-Espinosa et al. 2012), and knockout of the *Grm7* gene results in increased susceptibility to seizures (Sansig et al. 2001). Thus, the downregulation of the *Grm7* gene induced by PIC challenge (Fig. 7) is consistent with the gene's contribution to the hypersusceptible phenotype. However, two metabotropic glutamate receptor genes revealed unpredicted changes. Thus, while the GRM1 receptor tends to be pro-epileptic (Ure et al. 2006), the *Grm1* gene was downregulated following PIC challenge (Fig. 7). Likewise, the upregulation of the *Grm6* gene (Fig. 7) is incongruent with the function of GRM6 receptor that negatively regulates glutamate release, and therefore is protective against seizures (Ure et al. 2006).

LPS challenge has been shown to alter expression of the *Grik1* and *Grik2* genes in the spinal cord (Guo et al. 2002), as well as the genes encoding NMDA receptors in the hippocampus (Harre et al. 2008). We did not observe changes in these genes following PIC challenge suggesting a divergence in the cerebral effects of APR induced by bacterial vs. viral

inflammagens. This is consistent with the induction of the inflammatory genes discussed earlier.

In addition to neurotransmitter imbalances, dysfunction in the metabolic coupling between neurons and astrocytes may be causative of hyperexcitation, seizure spread and neurotoxicity (Seifert and Steinhauser 2013). PIC challenge upregulated the *Ldha* and downregulated the *Gldh* genes (Fig. 7) that encode two key metabolic enzymes involved in glutamate recycling. Such enzymatic changes are expected to enhance the accumulation of extracellular glutamate resulting in hyperexcitation. Our results are concordant with previous studies that found the same changes in epilepsy patients and animal kindling models (Erakovic et al. 2001; Malthankar-Phatak et al. 2006). Moreover, another astrocytic gene, the *Kcnj10* gene that encodes potassium channel Kir4.1 featured a transient downregulation (Fig. 7). Reduced expression of Kir4.1 has been found in patients with congenital epilepsy (Bockenbauer et al. 2009), and conditional knockout of *Kcnj10* in mice results in the development of stress-induced seizures through increased synaptic potentiation (Djukic et al. 2007).

As in the case of inflammatory genes (see above), the expression of mRNAs encoding neurotransmission-related genes does not necessarily reflect their expression at the protein level. miRs are important regulators of mRNA translation, and thus, may further modulate protein production from the upregulated or downregulated mRNAs. In addition, miRs could alter protein production even in the absence of measurable changes in the levels of preexisting cognate mRNAs through translational regulation (Petersen et al. 2006). Consequently, miRs may provide another regulatory layer in the development of hyperexcitability. In support of this notion, we found changes in the expression of several hippocampal miRs instigated by PIC challenge (Fig. 8). Although it is hard to interpret the role of these miRs in the development of hyperexcitability, other studies provide some clues. For example, miR-132 and miR-138 are associated with changes in synaptic spine morphology and excitability. Specifically, the upregulation of miR-132-3p and downregulation of miR-138-5p observed in our study has been shown to result in larger, stubby spines and an increase in mEPSC frequency (Edbauer et al. 2010; Siegel et al. 2009). Alterations in other miRs have been associated with changes in cell migration, proliferation and differentiation, the features involved in the development of hippocampal hyperexcitability (Parent et al. 1997). Thus, the upregulation of miR-128 has been associated with increased cell number and neurite length (Guidi et al. 2010), as well as with enhanced mobility through the downregulation of reelin and doublecortin (Evangelisti et al. 2009). Doublecortin seems to be critical for normal hippocampal excitability, as doublecortin knockout mice exhibit spontaneous seizures that originate in the hippocampus (Kerjan et al. 2009). Furthermore, miR-302a-5p negatively regulates the CXCR4 pathway (Fareh et al. 2012) that is important in cell migration in the hippocampus (Bagri et al. 2002). Therefore, the downregulation of miR-302a in response to PIC challenge might result in the upregulation of CXCR4 leading to hyperexcitability. Finally, a recent microarray study identified several miRs, including miR-132-3p, miR-138-5p, miR-181a and miR-221-3p analyzed in this study, that are differentially expressed following pilocarpine-induced status epilepticus (Risbud and Porter 2013).

In conclusion, we have demonstrated that the APR in the peritoneal cavity alters the expression of a multitude of hippocampal genes encoding inflammatory proteins, neurotransmission-related proteins and miRs. This robust polygenic response of hippocampal cells indicates an extensive genetic reprogramming that is likely to underscore the protracted hypersusceptible phenotype. The differentially expressed genes identified here are likely only a subset of genes that are altered by peripheral APR. For instance, we have previously found that 79 out of 280 genes related to inflammation, signaling and stress/toxicity pathways featured differential expression in the cerebellum following PIC challenge (Konat and Borysiewicz 2009). Altogether, our results warrant a comprehensive investigation of the extent of genomic reprogramming in the hippocampus induced by peripheral APR through whole genome expression analysis.

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Fig. 1.

The suppression of locomotor and rearing behavior by PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC and after 3 or 6 h the locomotor and rearing activities were evaluated (for details see Materials and Methods). Saline injected mice served as controls (0 h). Bars represent means $\pm$ S.D. from 6 to 10 animals. Values significantly different from controls are indicated by asterisks. * p 0.05; ** p 0.01

Fig. 2.

Time course of seizure susceptibility following PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC. At different time points after PIC challenge, the animals were s.c. injected with 20 mg/kg of KA and seizure severity was evaluated in 5 min increments for 2 h and expressed as cumulative seizure scores (for details see Materials and Methods). Mice injected with saline instead of PIC served as controls. *Bars* represent means $\pm$ S.D. from 3 to 7 animals. Values significantly different from controls are indicated by *asterisks* ($p < 0.05$)

Fig. 3.

Blood cytokine surge induced by PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC and the levels of IFNβ and IL-6 in the blood plasma were determined at various time points as indicated. Data represent averages from 2 animals

Fig. 4.

The expression of cytokine genes in the hippocampus following PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC and the levels of selected cytokine mRNAs were determined in the hippocampi by qRT-PCR at different time points as indicated. Data represent means $\pm$ S.D. from 3 to 8 animals. Values significantly different from baseline levels (0 h) are indicated by asterisks ($p < 0.05$)

Fig. 5.

The expression of chemokine genes in the hippocampus following PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC and the levels of selected chemokine mRNAs were determined in the hippocampi by qRT-PCR at different time points as indicated. Data represent means $\pm$ S.D. from 3 to 8 animals. Values significantly different from baseline levels (0 h) are indicated by asterisks ($p < 0.05$)

Fig. 6.

The expression of chemokine receptor genes following PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC and the levels of selected chemokine receptor mRNAs were determined in the hippocampi by qRT-PCR at different time points as indicated. Data represent means $\pm$ S.D. from 3 to 8 animals. Values significantly different from baseline levels (0 h) are indicated by asterisks ($p < 0.05$)

Fig. 7.

The expression of neurotransmission-related genes following PIC challenge. Mice were i.p. injected with 12 mg/kg of PIC. At different time points, mRNA levels of selected neurotransmitter receptors and proteins involved in synaptic buffering of glutamate and potassium were determined in the hippocampi by qRT-PCR. Data represent means $\pm$ S.D. from 3 to 6 animals. Values significantly different from baseline levels (0 h) are indicated by asterisks ($p < 0.05$)

Fig. 8. The expression of hippocampal miRNAs following PIC challenge. Mice were i.p. injected with 12 mg/kg PIC and the levels of selected miRNAs were determined in the hippocampi by qRT-PCR at different time points as indicated. Data represent means $\pm$ S.D. from 3 to 8 animals. Values significantly different from baseline levels (0 h) are indicated by asterisks ($p < 0.05$)

Table 1

Maximal upregulation of selected cytokine and chemokine genes in the forebrain vs. hippocampus in response to PIC challenge

Gene	Hippocampus (H)	Forebrain (F) [#]	H/F
<i>Ifnb</i>	4.05±1.14	201.55±37.00 [*]	0.02
<i>Ccl5</i>	20.03±3.88	132.60±30.01 [*]	0.15
<i>Ccl2</i>	107.94±28.26	215.05±51.11 [*]	0.50
<i>Cxcl11</i>	6547.68±692.19	720.88±101.16 [*]	9.1
<i>Ccl12</i>	154.19±13.80	20.50±7.01 [*]	7.5
<i>Cxcl10</i>	3213.78±458.29	1112.07±93.22 [*]	2.9
<i>Cxcl1</i>	1192.98±285.75	718.50±111.02 [*]	1.7

[#] data from Konat et al. (2009) and Fil et al. (2011);

^{*}
p 0.05