Evidence for brain glial activation in chronic pain patients

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Although substantial evidence has established that microglia and astrocytes play a key role in the establishment and maintenance of persistent pain in animal models, the role of glial cells in human pain disorders remains unknown. Here, using the novel technology of integrated positron emission tomography-magnetic resonance imaging and the recently developed radioligand 11C-PBR28, we show increased brain levels of the translocator protein (TSPO), a marker of glial activation, in patients with chronic low back pain. As the Ala147Thr polymorphism in the TSPO gene affects binding affinity for 11C-PBR28, nine patient–control pairs were identified from a larger sample of subjects screened and genotyped, and compared in a matched-pairs design, in which each patient was matched to a TSPO polymorphism-, age- and sex-matched control subject (seven Ala/Ala and two Ala/Thr, five males and four females in each group; median age difference: 1 year; age range: 29–63 for patients and 28–65 for controls). Standardized uptake values normalized to whole brain were significantly higher in patients than controls in multiple brain regions, including thalamus and the putative somatosensory representations of the lumbar spine and leg. The thalamic levels of TSPO were negatively correlated with clinical pain and circulating levels of the proinflammatory cytokine interleukin-6, suggesting that TSPO expression exerts pain-protective/anti-inflammatory effects in humans, as predicted by animal studies. Given the putative role of activated glia in the establishment and maintenance of persistent pain, the present findings offer clinical implications that may serve to guide future studies of the pathophysiology and management of a variety of persistent pain conditions.

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Introduction

Until recently, chronic pain has been thought to arise primarily from neuronal dysfunction within nociceptive pathways of the nervous system (Hains and Waxman, 2006). In the last decade, however, a paradigm shift has occurred in the field of pain neurobiology. Animal studies have clearly demonstrated that microglia and astrocytes in the CNS, as well as neuro-glial interactions, play a key role in the establishment and maintenance of persistent pain (Tsuda et al., 2003; Watkins et al., 2007; Calvo et al., 2012; Ji et al., 2013). Both microglia and astrocytes respond to pathological events in CNS, such as strokes, trauma or neurodegenerative diseases, by undergoing a series of cellular responses collectively known as 'glial activation' (Gehrmann et al., 1995; Pekny et al., 2014). This response includes proliferation, morphological changes, increased or de novo expression of cell surface markers or receptors, and the production of cytokines and other inflammatory mediators. Normally, glial activation is an adaptive defensive mechanism that can contribute to handling acute stress, limiting tissue damage, and restoring homeostasis. However, when malfunctioning (and, in particular when it does not get resolved during the post-acute or early chronic stage after an injury event) (Rolls et al., 2009) glial activation can have deleterious effects, and turn into the primary pathogenic element (Pekny and Pekna, 2014). Several animal studies have now established that glial activation is a key contributing factor in persistent pain. It is well demonstrated that activated microglia and astrocytes can produce cytokines such as tumour necrosis factor alpha (TNFA) and interleukin 1 beta (IL1B), and these cytokines are thought to play an essential role in the pathogenesis of chronic pain (Watkins et al., 2007; Uceyler and Sommer, 2012). It has also been shown that TNFA and IL1B can directly modulate spinal cord synaptic transmission to induce central sensitization and enhance pain states (Kawasaki et al., 2008). Moreover, the intraspinal injection of activated glia produces tactile allodynia, a hallmark of neuropathic pain, in naive rats (Tsuda et al., 2003). Conversely, the injection of drugs inhibiting glial activation can inhibit, delay or reverse pain (Meller et al., 1994; Watkins et al., 1997; Guo et al., 2007; Okada-Ogawa et al., 2009). These observations, alongside other mounting evidence from laboratory models, suggest that chronic pain may result from glialopathy (Ji et al., 2013).

In humans, some observations suggest that activated glia may contribute to the pathophysiology of chronic pain. For instance, post-mortem immunohistochemical studies in the spinal cord of patients with complex regional pain syndrome (Del Valle et al., 2009) and HIV-related neuropathic pain (Shi et al., 2012), as well as CSF sampling in patients with fibromyalgia and chronic low back pain (LBP) (Brisby et al., 1999; Kadetoff et al., 2012), support a role for glia in chronic pain. Despite these observations, however, no study has yet demonstrated in vivo glial activation in humans suffering from chronic pain. Clinically, acknowledging the role of glial activation in pain disorders holds significant promise for the improved diagnostic accuracy of pain disorders. It may also provide a biological basis for the assessment of existing treatments, and the development of novel ones.

In this study we tested the hypothesis that patients with chronic pain demonstrate in vivo activation of brain glia. To assess this hypothesis, we imaged the brain of individuals diagnosed with chronic LBP as well as pain-free healthy volunteers using a Siemens 3 T integrated positron emission tomography/magnetic resonance imaging (PET/MRI) scanner and the recently developed PET radioligand, 11C-PBR28 (Brown et al., 2007; Briard et al., 2008). 11C-PBR28 binds to the translocator protein (18kDa) (TSPO), a protein upregulated in activated microglia and reactive astrocytes in animal models of pain (Hernstadt et al., 2009; Wei et al., 2013), and a putative imaging biomarker of inflammation (Cagnin et al., 2007). In addition, as evidence suggests that proinflammatory cytokines are secreted by glial cells in various animal models of pain (Ji et al., 2013), we assessed blood levels of interleukin 6 (IL6), IL1B and TNFA, and evaluated their association with our imaging findings.

Materials and methods

Study design

The study was conducted at the Athinoula A. Martinos Center for Biomedical Imaging at Massachusetts General Hospital. The protocol was approved by the Institutional Review Board and the Radioactive Drug Research Committee.

Subjects

Nineteen patients diagnosed with chronic LBP for at least 2 years (either with or without radicular pain complaints) and 25 healthy controls with no history of chronic pain were initially screened to participate in the study. Individuals were excluded if they had any PET/MRI contraindications (including pregnancy, metallic implants, claustrophobia), had a history of major medical disorders, or were on benzodiazepines or blood thinners (see Supplementary Table 1 for pain history and demographic information).

The Ala147Thr polymorphism in the TSPO gene predicts binding affinity for 11C-PBR28, with the Ala/Ala, Ala/Thr and Thr/Thr genotypes being associated with high, mixed and low affinity binding, respectively (Owen et al., 2012; Kreisl et al., 2013; Yoder et al., 2013). Thus, all participants

Abbreviations: LBP = low back pain; SUV = standardized uptake value
were tested for this polymorphism and individuals with predicted low-binding affinity (Thr/Thr) were excluded. Given the effect of the TSPO polymorphism on binding affinity for \(^{11}\)C-PBR28, and the fact that the effects of sex and age on ligand binding are unknown, a matched-pairs design was adopted, in which each patient was matched to a TSPO polymorphism-, age- and sex-matched control. Of the 44 subjects initially screened, nine chronic LBP/control matching pairs (with two patients matching the same control) were identified among the initial pool, consisting of seven Ala/Ala and two Ala/Thr pairs, and five male and four female pairs, with a median age difference of 1 year (Supplementary Table 1).

**Screening visit**

All participants considered potentially eligible after an initial phone screening were invited to participate in a 2-h characterization and training visit. In this visit, venous blood was drawn in order to have all participants genotyped for the Ala147Thr TSPO polymorphism, and a urine test was performed to ensure that none of the subjects were on benzodiazepines, or taking illegal drugs. At the end of the visit, all participants completed the Beck Depression Inventory (Turner and Romano, 1984; Geisser et al., 1997) and the Hospital Anxiety and Depression Scale (Zigmond and Snith, 1983). In addition, the patients completed the McGill Pain Questionnaire (MPQ), short form (Melzack, 1987).

**Imaging visit**

On a separate date, eligible participants were invited to participate in an imaging visit. At the beginning of the visit, venous blood was drawn to assess levels of circulating proinflammatory cytokines: IL6, IL1B and TNFA (Supplementary Table 2). These specific proinflammatory cytokines were assessed because they are secreted by glial cells in various animal models of pain (Ji et al., 2013).

Brain imaging was performed with a Siemens PET/MRI scanner (Catana et al., 2008) consisting of a dedicated brain avalanche photodiode-based PET scanner operating in the bore of a 3 T whole-body magnetic resonance scanner equipped with an 8-channel head coil. The use of integrated PET/MRI allowed us to collect structural MRI simultaneously with the PET data. Patients were scanned with \(^{11}\)C-PBR28, a recently developed TSPO radioligand that displays an 80-fold higher in vivo specific binding than the earlier generation TSPO radioligand \(^{11}\)C-(R)-PK11195 (Kreisl et al., 2010). \(^{11}\)C-PBR28 was produced in-house using a procedure modified from the literature (Imaiizumi et al., 2007).

At the beginning of the imaging visit, we performed a series of magnetic resonance scans, including a multi-echo MPRAGE volume (repetition time/echo time 1/2 echo time 2/ echo time 3/ echo time 4 = 2530/1.64/3.5/6.36/7.22 ms, flip angle = 7°, voxel size = 1 mm isotropic) for the purpose of anatomical localization, spatial normalization of the imaging data, as well as generation of attenuation correction maps (Izquierdo-Garcia et al., 2014). The radioligand was then injected as an intravenous bolus, with a median administered dose (interquartile range) of 11.2 (0.3) mCi for patients with chronic LB and 11.2 (0.6) mCi for controls, and a median specific activity at time of injection of 2.7 (0.8) mCi/nmol for chronic LB and 2.0 (1.2) mCi/nmol for controls (both values not significantly different across groups). PET data were acquired over the course of 90 min for all but two subjects (see below) and stored in list-mode format. During the imaging visit, all subjects except one rated their level of pain using a verbal 0–100 numerical ratings scale (pain: 0 = ‘no pain’, 100 = ‘most intense pain tolerable’). The remaining subject (a healthy volunteer) was scanned under a separate protocol, which did not call for ratings of pain before and after the functional runs (but was otherwise identical in the procedures and parameters of the imaging visit).

**Data analysis**

Using in-house software, standardized uptake values (SUV; i.e. mean radioactivity/injected dose/weight) were computed for each subject. SUVs have been previously used as a measure of TSPO expression in both humans (Hirvonen et al., 2012; Fujita et al., 2013) and rodents (Shao et al., 2013). SUVs were computed voxel-wise from the 60–90 min post-injection PET data, except for two subjects (one patient and one control) for whom SUVs were computed from 72–89 min and 60–86 min, respectively, because of unavailability of the full 60–90 min frame. The two subjects matching these subjects with incomplete data were reconstructed with identical frame onset and duration for the group analyses, to ensure that differences in the PET framing scheme would not affect our results. To maximize accuracy in the reconstruction, SUVs were generated in a two-step iterative process. First, a preliminary SUV image was created for each subject using an attenuation correction map (mu-map) computed from the MPRAGE in its native space (Izquierdo-Garcia et al., 2014). To account for motion that may have occurred between the time the MPRAGE and the 60–90 min PET data were acquired, a new mu-map was created from the MPRAGE registered to this temporary SUV map. This registration was performed using spmregister, a tool from the FreeSurfer suite (http://surfer.nmr.mgh.harvard.edu) (Dale et al., 1999) that performs a rigid-body registration of a functional volume to its relative FreeSurfer anatomical volume with Normalised Mutual Information using spm_coreg from the SPM suite (http://www.fil.ion.ucl.ac.uk/spm/) (Friston, 2003). A final SUV map was created using this new mu-map, now well in register with the 60–90 min PET data. SUV maps were then normalized to MNI space using non-linear registration (FNIRT, from the FSL suite; FMRI’s Software Library, version 4.1.9, www.fmrib.ox.ac.uk/fsl/) (Smith et al., 2004). Spatially-normalized SUV images were then spatially smoothed (full-width at half-maximum = 8 mm) to improve signal-to-noise ratio, and intensity-normalized to a mean of 1 (SUVRs) in order to account for global signal differences across subjects, such as those introduced by differences in the Ala147Thr polymorphism, which affects global binding affinity.

Although the subjects scanned were carefully selected from a larger sample size to match for genetic and demographic factors, the size of our final sample was relatively small. For this reason, imaging and behavioural group comparisons were performed using non-parametric testing. As glial activation has been postulated to spread transsynaptically from the site of an injury, following the lesioned neural pathways (Banati, 2003), we hypothesized that in patients with chronic LBP glial activation would be detected in early pain processing regions, as they are most proximal to the pathological sites in the back (e.g. spinal cord/nerve roots). Thus, a thalamus-targeted region of interest analysis was first performed to test this regionally-specific
Results

Higher brain TSPO levels in patients with chronic LBP

In the thalamic region of interest analysis, $^{11}$C-PBR28 SUVs, normalized to whole brain, (SUVRs) were significantly higher in patients with chronic LBP than controls (left thalamus: $P \leq 0.01$; right thalamus: $P \leq 0.05$; Fig. 1A). The voxel-wise distribution of thalamic SUVRs (Fig. 1B) revealed that in control subjects non-zero median voxel counts were observed only below values of 1.4, whereas in patients with chronic LBP a substantial number of voxels demonstrated values higher than 1.4 in both hemispheres (medians: 57.5 and 64 in the left and right thalamus, respectively).

The examination of individual thalamic SUVRs (Fig. 1C) shows that, strikingly, each patient exhibited higher SUVRs than his/her age-sex- and TSPO genotype-matched control in the thalamus. In all patients, the areas of maximal TSPO levels were consistently localized in dorsomedial subregions of the thalamus, as illustrated by the 3D rendering (Fig. 1C).

In the whole-brain voxel-wise analyses, SUVRs were significantly higher in thalamus, pre- and postcentral gyri and paracentral lobule (Fig. 2 and Supplementary Table 3). The peak group difference was observed in left thalamus, consistent with the mediodorsal nucleus. There were no brain regions for which the healthy controls showed statistically higher SUVRs than the patients with chronic LBP. When examined at the exploratory threshold of $P < 0.01$ uncorrected for multiple comparisons (Supplementary Fig. 1), additional regions demonstrated higher SUVs in chronic LBP than controls, including insulae, middle cingulate cortex, ventromedial prefrontal cortex, posterior cingulate cortex, supplementary motor area and basal ganglia. Moreover, excluding the two patients on opioids (and their matching controls), yielded similar results (Supplementary Table 4). Thus, while exposure to opioids may also lead to a glial reaction (Watkins et al., 2009), our results do not seem to be confounded by opioid intake.

**TSPO expression as a protective mechanism?**

In the patients, $^{11}$C-PBR28 imaging metrics, corrected for genotype, were negatively associated with pain outcome measures (Fig. 3A–C) and circulating levels of IL6 (Fig. 3D). First, the number of thalamic voxels with a SUV value $> 1.4$ was negatively associated with pain levels at the time of scan and the total score on the McGill Pain Questionnaire ($P < 0.05$), but not with IL6 or TNFA levels ($P$-values $> 0.3$). Second, the average SUVRs extracted from the thalamic cluster statistically significant in the voxel-wise analysis were negatively associated with levels of IL6 ($P < 0.05$), but not with TNFA levels ($P = 0.38$). The association of thalamic $^{11}$C-PBR28 SUVR with the McGill Pain Questionnaire total scores was negative, but did not reach statistical significance ($P = 0.11$). No association was observed between this imaging metric and pain levels during the scan ($P = 0.25$). In all subjects, IL1B levels were below the detection threshold, and therefore the association between imaging and IL1B data could not be assessed.

**Discussion**

Our study demonstrates the occurrence of glial activation, as measured by an increase in $^{11}$C-PBR28 binding, in the brain of patients with chronic pain. Increased tracer...
binding was observed most prominently in the thalamus, and with remarkable across-subject consistency (Fig. 1C).

Within the primary somatosensory and motor cortices (S1/M1) the SUVRs were higher in the putative sensorimotor representations of the lumbar spine [in the postcentral gyrus; Boendermaker et al. (2014), and leg (in the paracentral lobule); Loggia et al. (2012)]. This spatial pattern is consistent with the majority of patients suffering from pain in the lower back and leg(s), and is suggestive of somatotopically organized glial activation in S1/M1 (which in turn is also consistent with the observation that spinal glial activation generally follows somatotopic boundaries after unilateral spared nerve injury in rats) (Beggs and Salter, 2007).

In the last decade, animal research has led to the increased recognition of the importance of glial cells (such as microglia and astrocytes), and their interaction with neuronal cells, in the pathogenesis of pain conditions (Tsuda et al., 2003; Watkins et al., 2007; Calvo et al., 2012; Ji et al., 2013). For instance, nerve injury induces a profound activation and proliferation of spinal microglia (Liu et al., 2000; Beggs and Salter, 2007; Echeverry et al., 2008; Beggs et al., 2012; Calvo and Bennett, 2012) and the upregulation of a variety of receptors in these cells, such as the adenosine triphosphate (ATP) receptor P2RX4 (also known as P2X4) (Tsuda et al., 2003) and the chemokine receptor CX3CR1 (Verge et al., 2004), which induce hyperalgesia. Activated microglial cells produce inflammatory mediators, including proinflammatory cytokines and brain-derived neurotrophic factor (BDNF; Coull et al., 2005; Ji and Suter, 2007) that activate or sensitize nociceptive neurons. The intrathecal injection of activated microglia produces tactile allodynia in naive rats, suggesting that microglial activation is sufficient to induce pain sensitization (Tsuda et al., 2003). Finally, pharmacological inhibition of microglial activation prevents or delays neuropathic pain (Raghavendra et al., 2003; Ledeboer et al., 2005). Similar to microglia, astrocytes have been shown to have a role in the induction and maintenance of pain sensitization (Ji et al., 2006; Ren and Dubner, 2010; Ji et al., 2013). For instance, in trigeminal models of inflammatory hyperalgesia (Guo et al., 2007) or following trigeminal nerve injury (Okada-Ogawa et al., 2009), astrocytes exhibit hypertrophy, and express enzymes, such as nitric oxide

Figure 1 Evidence for glial activation in the thalamus of chronic LBP patients. (A) Boxplots are presented for the mean $^{11}$C-PBR28 SUVRs extracted for all 10 patients with chronic LBP and nine control subjects from the thalamic regions of interest.
Figure 2  Whole-brain voxel-wise analyses. (A) Median SUVR map from healthy controls ($n=9$) and patients with chronic LBP ($n=10$) are presented. Matched-pairs tests (nine versus nine) revealed significantly higher TSPO levels in patients, in thalamus, pre- and postcentral gyri, and paracentral lobule ($P<0.05$ corrected for multiple comparisons; permutation testing, 10,000 permutations). As two patients were matching the same controls, the analyses were performed first using the patient best matching the control. A second analysis was performed using the other patient, limiting our search to significant clusters from the first analysis, with identical results. (B) Boxplots for each of the four regions demonstrating statistically higher SUVRs in patients are shown for illustrative purposes. postc. = postcentral; g. = gyrus; parac. lob. = postcentral lobule; prec. = precentral.
synthase (Meller et al., 1994), as well as inflammatory mediators, such as the proinflammatory cytokine IL1B (Guo et al., 2007) and chemokines such as CXCL2 (Chen et al., 2014), that contribute to hyperalgesia and allodynia. Thermal and mechanical hyperalgesia are inhibited or attenuated by the injection of agents that disrupt either astroglial function (such as fluorocitrate, a glial metabolic inhibitor) (Meller et al., 1994; Watkins et al., 1997; Guo et al., 2007; Okada-Ogawa et al., 2009), or the action of glial products (such as IL1 receptor antagonists) (Watkins et al., 1997). Taken together, these studies demonstrate that microglia and astrocytes play an important role in the pathogenesis of persistent pain in animals.

Although a plethora of animal studies has demonstrated that glial cells are involved in the establishment and maintenance of persistent pain, no study has previously demonstrated in vivo glial activation in humans suffering from chronic pain. Our observations have several potential implications. Firstly, they provide a rationale for exploring the role of glia as therapeutic target for chronic pain. In animals, drugs that reduce glial activation (e.g. propentofylline and minocycline) have been found to potently inhibit proinflammatory cytokines, thereby suppressing the development of neuropathic pain (Mika, 2008; Leblanc et al., 2011). Importantly, some of these molecules are already FDA approved to treat human conditions of different aetiologies and testing for new, chronic pain-related indications would therefore be immediately possible. Two recent clinical trials (8- and 12-weeks long, respectively) have indicated that low-dose naltrexone (LDN) may have a clinically beneficial impact on fibromyalgia pain (Younger and Mackey, 2009; Younger et al., 2013). As LDN is thought to produce anti-inflammatory effects mainly by antagonizing the activity of glial cells (Mattioli et al., 2010), these studies suggest that glial modulators may be beneficial for fibromyalgia and perhaps other subgroups of chronic pain patients. On the other hand, it should be noted that some clinical trials have had negative results. A recent clinical trial assessing the efficacy of propentophylline to reduce pain in post-herpetic neuralgia was negative (Landry et al., 2012). Another study suggested that perioperative minocycline administration did not improve persistent pain after lumbar discectomy.
(Martinez et al., 2013). However, methodological concerns with these studies (Watkins et al., 2012) limit the significance of these negative study outcomes. In particular, the duration of trial design was unusually short in both studies. In the former study, propentofylline was administered for 4 weeks, and in the latter minocycline was administered peri-operatively for only 8 days, in both cases a significantly shorter duration than 12 weeks, as is more typically adopted in clinical trials. Moreover, as studies have shown, the rapid microglial response is paralleled by a delayed but steadily increasing astrocytic component (Kuhlmann and Guilarte, 2000; Chen et al., 2004; Chen and Guilarte, 2006; Martin et al., 2010; Liu et al., 2014). Human post-mortem data seem to corroborate this phase-dependent glial contribution; in acute multiple sclerosis lesions, microglia and macrophages represent most TSPO + cells, whereas astrocytes are the dominant TSPO + cells in chronic, silent lesions (Cosenza-Nashat et al., 2009). Because our patients suffered from years of pain, it is plausible that astrocytes provided a significant contribution to the increased PET signal observed in our data.

Although TSPO is a marker of activated glia, a phenomenon thought to be responsible for the amplification of pain signals within the CNS (Ji et al., 2013), TSPO expression itself has been shown in animals to exert inhibitory effects on neuroinflammation (Wei et al., 2013; Bae et al., 2014; Wang et al., 2014), and to promote recovery from neuropathic pain (Wei et al., 2013), likely through the stimulation of steroidogenesis (Batarseh and Papadopoulos, 2010; Wei et al., 2013). In fact, studies suggest that one of the functions of TSPO in activated glia is to limit the magnitude of inflammatory responses after their initiation (Wang et al., 2014). Our observation that [11C]PBR28 SUVRs negatively correlate with levels of the circulating proinflammatory cytokine IL6 and pain further corroborate the hypothesis that TSPO expression has anti-inflammatory and pain-protective effects. The negative correlations found in our study therefore support the contention that TSPO ligands may be a novel therapeutic target for the treatment of pathological pain (Wei et al., 2013), as previously suggested for a variety of conditions (Rupprecht et al., 2009, 2010). As TSPO expression is upregulated in activated glia and was found to negatively correlate with peripheral cytokines and pain in our study, low levels of TSPO might be interpreted differently between groups. For example, in healthy volunteers, low levels could simply reflect low levels of glial activation. On the other hand, patients with chronic pain exhibiting lower TSPO levels may have impairments in TSPO expression in activated glia, and therefore in their ability to limit the glial responses after their initiation. Clearly, additional studies are required to elucidate the relationship between [11C]PBR28 and peripheral markers of inflammation, particularly as peripheral...
cytokine levels are often found to be decoupled from those within the CNS (Bromander et al., 2012).

Future studies, including some currently already underway in our laboratory, will need to determine whether different pain populations present differences in the spatial distribution of glial activation. The discovery of ‘glial signatures’ of chronic pain states might lead to the identification of objective imaging markers that could (i) complement the patient’s subjective assessment and other measures (e.g. quantitative sensory testing) to guide clinical practice; and (ii) reduce the patient heterogeneity which has traditionally led to poor signal-to-noise ratios in most clinical drug evaluation studies (Gomez-Mancilla et al., 2005).

Finally, as glial cells respond to very subtle changes in their microenvironment that even precede pathological changes that are detectable histologically (de Vries et al., 2006; Cagnin et al., 2007), glial activation might be an early marker of the alterations that have been shown to occur in the brains of chronic pain patients (Tracey and Bushnell, 2009). This might allow early identification of individuals at risk of transitioning from acute to chronic pain, thus optimizing treatment strategies.

In sum, our findings demonstrate a role of glia in human pain disorders, support the role of the assessment of glial activation and TSPO expression in selective brain areas as an imaging marker and potential treatment target for chronic pain disorders in humans.

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Supplementary material

Supplementary material is available at Brain online.

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