Genetic enhancement of inflammatory pain by forebrain NR2B overexpression

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***N*-methyl-D-aspartate (NMDA) receptors contribute to many brain functions. We studied the effect of forebrain-targeted overexpression of the NMDA receptor subunit NR2B on the response of mice to tissue injury and inflammation. Transgenic mice exhibited prominent NR2B expression and enhanced NMDA receptor-mediated synaptic responses in two pain-related forebrain areas, the anterior cingulate cortex and insular cortex, but not in the spinal cord. Although transgenic and wild type mice were indistinguishable in tests of acute pain, transgenic mice exhibited enhanced responsiveness to peripheral injection of two inflammatory stimuli, formalin and complete Freund's adjuvant. Genetic modification of forebrain NMDA receptors can therefore influence pain perception, which suggests that forebrain-selective NMDA receptor antagonists, including NR2B-selective agents, may be useful analgesics for persistent pain.**

NMDA receptors, which are expressed at excitatory synapses throughout the central nervous system (CNS), mediate a wide range of brain processes, including the synaptic plasticity associated with memory formation[1], neuronal death in ischemia[2] and central sensitization during persistent pain[3–6]. Functional NMDA receptors contain heteromeric combinations of the NR1 subunit, plus one or more of NR2A–D[7–8], of which the NR2A and NR2B subunits predominate in forebrain structures[9]. NR2A and NR2B subunits confer distinct properties to NMDA receptors; heteromers containing NR1 plus NR2B mediate a current that decays three to four times more slowly than receptors composed of NR1 plus NR2A[9]. At birth, forebrain NMDA receptors are composed almost exclusively of NR1 and NR2B subunits, gradually incorporating more NR2A subunits during postnatal development[10]. This developmental decrease in the forebrain NR2B:NR2A ratio, complete by the third or fourth postnatal week in rodents, parallels a decrease in the duration of NMDA receptor-mediated excitatory postsynaptic currents (EPSCs)[11–13]. This developmental change also parallels a decrease in sensitivity to formalin-induced pain[14].

In transgenic mice with forebrain-targeted NR2B overexpression, this developmental change in NMDA receptor kinetics is reversed[15]. NR2B subunit expression, driven by the α-calcium/calmodulin-dependent protein kinase II (α-CaMKII), is observed extensively in adult transgenic mice throughout the cerebral cortex, striatum, amygdala and hippocampus, but not in the thalamus, brainstem or cerebellum. Presumably by increasing the NR2B:NR2A ratio in NMDA receptor heteromeric complexes, marked alterations occur in the physiology of mature hippocampal synapses, including a prolongation of NMDA receptor-mediated EPSCs and an enhancement of long-term potentiation. Adult NR2B transgenic mice exhibit superior performance on a battery of learning- and memory-related behavioral tasks, compared to wild type adults. Why, then, is the NR2B:NR2A ratio decreased during development, if learning and memory are hindered as a result? We proposed that forebrain-targeted NR2B overexpression would have more broad effects on an animal's behavior than reported previously, including altered responses to tissue injury and inflammation.

Results

First, we examined how forebrain-targeted NR2B overexpression affected NMDA receptor function in two pain-related forebrain areas, the anterior cingulate cortex (ACC) and insular cortex. We prepared brain slices of these areas from adult mice, and we recorded excitatory postsynaptic field potentials (fEPSPs) upon local electrical stimulation[16,17]. In each region, after blockade of AMPA and kainate receptors by CNQX (20 μM), we observed a slow fEPSP (Fig. 1a) that could be entirely and reversibly blocked by the NMDA receptor antagonist AP-5 (100 μM; Fig. 1b). Consistent with the high levels of NR2B transgene expression found throughout the cerebral cortex in transgenic mice[15], these mice, compared to wild type mice, exhibited enhanced NMDA receptor-mediated fEPSPs in both the ACC and insular cortex (Fig. 1c).

In contrast, the spinal cord dorsal horn exhibited no change in NMDA receptor-mediated synaptic transmission. Intracellular recordings were done from dorsal horn neurons in slices from adult mice, and EPSPs were evoked by dorsal root stimulation. Slow AP-5-sensitive EPSPs, isolated in the presence of CNQX (20 μM; Fig. 2a), had similar slopes in wild type and transgenic slices

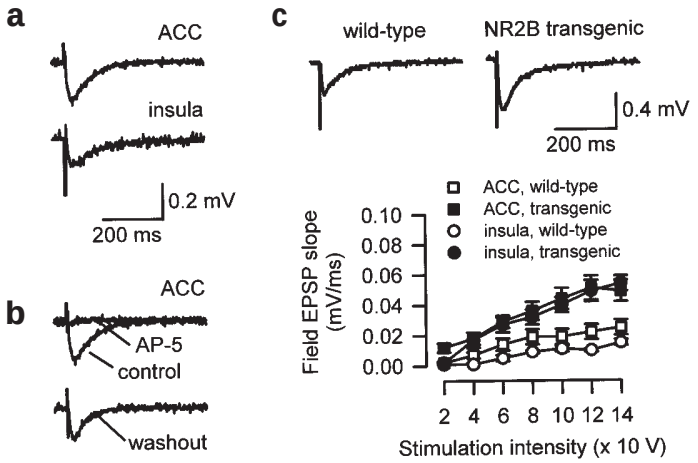


Fig. 1. Forebrain-targeted NR2B overexpression enhanced NMDA receptor-mediated synaptic responses in the ACC and insular cortex. (**a**) Traces of NMDA receptor-mediated fEPSPs recorded from the ACC and insular cortex in the presence of 20 μM CNQX. (**b**) Bath application of 100 μM AP-5 completely (top) and reversibly (bottom) blocked NMDA receptor-mediated fEPSPs in the ACC. Similar results were found in insular cortex (data not shown). (**c**) The input (stimulation intensity, 200-μs duration)–output (fEPSP slope) relationship in the ACC (wild type, $n = 8$; transgenic, $n = 12$) and insular cortex (wild-type, $n = 6$; transgenic, $n = 9$) reveals enhanced NMDA receptor-mediated responses in transgenic relative to wild-type mice ($p < 0.001$). Above, examples of fEPSP traces recorded from the ACC in wild-type and transgenic animals. We also integrated fEPSPs in the ACC and found an increased area under the curve for transgenic (33.2 ± 4.3 mV•ms) relative to wild-type mice (19.1 ± 3.3 mV•ms, $p < 0.05$). Similar results were found in the insular cortex (transgenic, 33.1 ± 6.1 mV•ms versus wild type, 17.0 ± 2.7 mV•ms; $p < 0.05$).

(Fig. 2b–d). Resting neuronal membrane potentials were not different in wild type (–68.7 ± 2.7 mV, $n = 27$) and transgenic (–69.7 ± 3.0 mV, $n = 26$) slices. To enhance sensitivity to possible changes in NMDA receptor-mediated EPSPs, we injected current through the recording electrode to depolarize neurons and relieve any voltage-dependent Mg^{2+} blockade. EPSP slopes were similar in wild type and transgenic mice at 0 mV (wild type, 1.6 ± 0.4 mV/ms, $n = 5$; transgenic, 1.4 ± 0.3 mV/ms, $n = 18$) and +20 mV (wild type, 1.6 ± 0.4 mV/ms, $n = 6$; transgenic, 1.3 ± 0.3 mV/ms, $n = 16$). Thus, we found no evidence that spinal NMDA receptor function was altered by forebrain-targeted NR2B overexpression.

Because electrophysiological experiments are sensitive to alterations in NMDA receptor function only in the sampled neurons, we did *in situ* hybridization with a probe for NR2B, to detect possible changes in the expression of this NMDA receptor subunit in a subpopulation of spinal neurons. We found no detectable NR2B expression in the dorsal horn of wild type or transgenic mice (Fig. 3). In contrast, NR2B expression was significantly enhanced in two forebrain structures, the ACC and insular cortex (Fig. 3), consistent with a previous report[15].

Next, we asked whether NR2B overexpression affected acute nociception. No significant difference in tail-flick response latency was observed (wild type, 6.64 ± 0.35 s, $n = 7$; transgenic, 6.16 ± 0.31 s; $n = 9$), indicating that spinal nociceptive transmission was not significantly altered in transgenic mice. Likewise, wild type and transgenic mice were indistinguishable in response latencies to noxious hot and cold stimuli. (Response latencies after placement on a hot or cold plate were, in wild type mice, 31.8 ± 2.3 s at 50°C, 17.7 ± 1.6 s at 52.5°C, 11.3 ± 1.5 s at 55°C and 20.6 ± 2.9 s at 0°C; in transgenic mice, 31.3 ± 3.0 s at 50°C, 19.4 ± 2.1 s at 52.5°C, 12.8 ± 1.8 s at 55°C and 19.3 ± 3.1 s at 0°C; $n = 6$–12 mice per condition.)

We then examined the responses of transgenic and wild type mice to a more prolonged noxious stimulus, peripheral formalin injection, a common model for tissue injury and inflammation[18,19]. First, we examined the pattern of formalin-induced neuronal activation in wild type mice by probing expression of the immediate-early gene product c-Fos. Although the physiological link between induction of c-Fos expression and nociceptive transmission is unclear, neuronal c-Fos expression is widely used as a correlative indicator for neuronal activity induced by noxious stimuli, including formalin injection in particular[20–22]. In adult wild type mice, subcutaneous injection of formalin into the dorsum of a hind paw induced increased c-Fos expression, relative to saline-injected controls, in various CNS regions related to nociceptive transmission and modulation. Among forebrain areas, formalin injection induced prominent c-Fos staining in the ACC

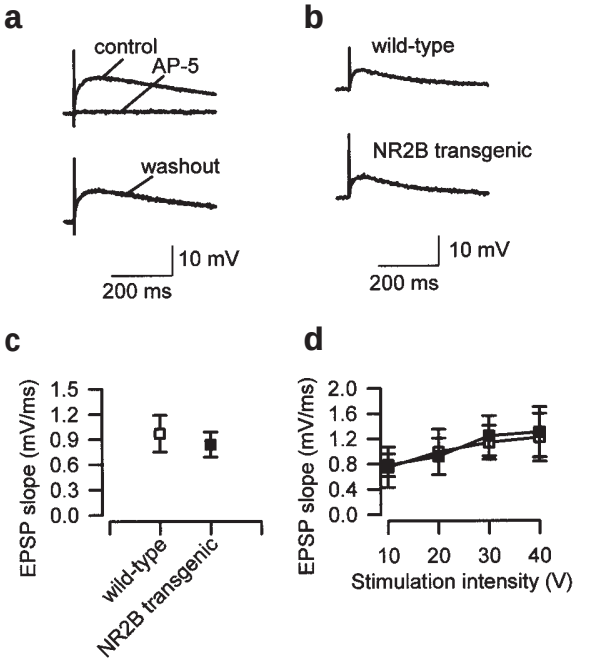


Fig. 2. NMDA receptor-mediated synaptic responses in the spinal cord were not affected by forebrain-targeted NR2B overexpression. (**a**) Traces of EPSPs recorded in the presence of 20 μM CNQX, after bath application of 100 μM AP-5 (top) and after washout (bottom). (**b**) Examples of EPSP traces recorded from dorsal horn neurons in wild-type and transgenic animals. (**c**) Summarized data of NMDA receptor-mediated responses to a single stimulation intensity (10 V) in spinal dorsal horn neurons (□, wild-type, $n = 27$; ■, transgenic, $n = 26$). (**d**) In some experiments, synaptic responses were compared at three stimulation intensities (□, wild-type, $n = 8$–14; ■, transgenic, $n = 17$–22), revealing no significant difference between wild-type and transgenic mice.

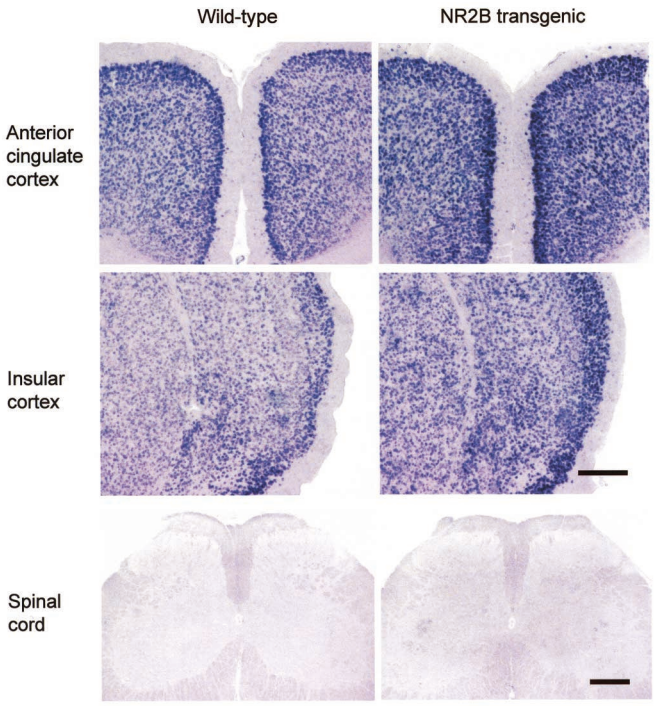
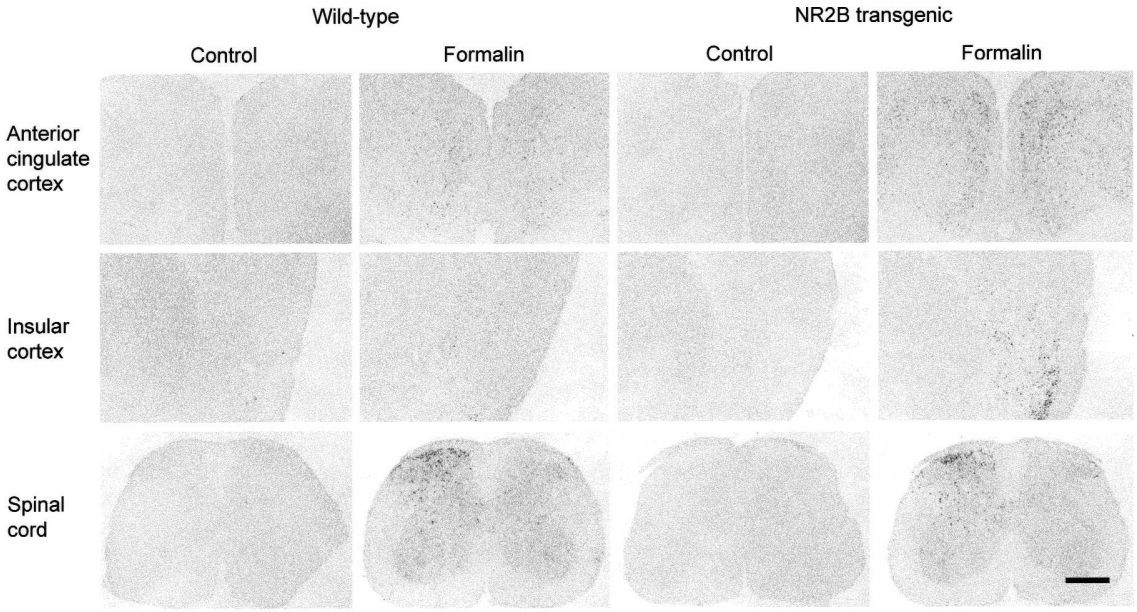


Fig. 3. Enhanced NR2B expression in the ACC and insular cortex but not the spinal dorsal horn in NR2B transgenic mice. Expression of NR2B was examined by *in situ* hybridization in wild-type and NR2B transgenic mice. Whereas the two forebrain regions exhibited enhanced NR2B expression in transgenic compared to wild-type mice, no upregulation of NR2B expression was seen in the spinal cord dorsal horn. Scale bars, 300 μm.

(Figs. 4 and 5), lateral septal nucleus, secondary motor cortex, some nuclei in the amygdaloid complex (medial, basolateral and cortical nuclei), piriform cortex, retrosplenial cortex, several midline thalamic nuclei (lateral habenular, paraventricular, mediodorsal, centromedial, paracentral and anterodorsal nuclei) and various hypothalamic nuclei (paraventricular, periventricular, supraoptic and dorsomedial nuclei). Less prominent but significant c-Fos expression was observed in the somatosensory cortex (S1, S2 and hindlimb areas; Fig. 5), the hippocampal CA1 (Fig. 5) and CA3 subfields, insular cortex (Figs. 4 and 5), accumbens nucleus and lateral preoptic area. A small amount of formalin-induced c-Fos expression was observed in other thalamic nuclei, including the lateral posterior nucleus, posterior group, ventral lateral, ventral posterolateral, ventral posteromedial and ventral medial nuclei. No c-Fos expression was observed in the caudate-putamen nucleus. In the midbrain, formalin treatment augmented c-Fos expression within all subareas of the periaqueductal gray (PAG; Fig. 5), dorsal raphe, interpeduncular nucleus, mesencephalic reticular formation, superficial gray layer of the superior colliculus and inferior colliculus. In the brainstem, c-Fos expression was also observed in the locus coeruleus, parabrachial nucleus and rostroventral medulla. Abundant c-Fos expression was observed in the dorsal horn of lumbar spinal cord, particularly in superficial laminae and the deep neck of the dorsal horn ipsilateral to the injected hind paw, with much smaller (but nonetheless evident) expression contralaterally (Figs. 4 and 5).

To determine whether forebrain-targeted NR2B overexpression affected formalin-induced c-Fos expression, we examined the

Fig. 4. Enhanced forebrain c-Fos expression following formalin injection in NR2B transgenic mice. Immunohistochemical staining for c-Fos is illustrated in the ACC, insular cortex and spinal cord in wild-type and NR2B transgenic mice 2 h after hind paw injection of saline or formalin (wild type, saline injected, $n = 4$ mice; wild type, formalin injected, $n = 6$; transgenic, saline, $n = 4$; transgenic, formalin, $n = 4$). Although the two forebrain regions exhibited enhanced c-Fos staining after formalin injection in transgenic compared to wild-type mice, the pattern of c-Fos expression in the spinal cord was not changed by forebrain-targeted NR2B overexpression. Scale bar, 300 μm.



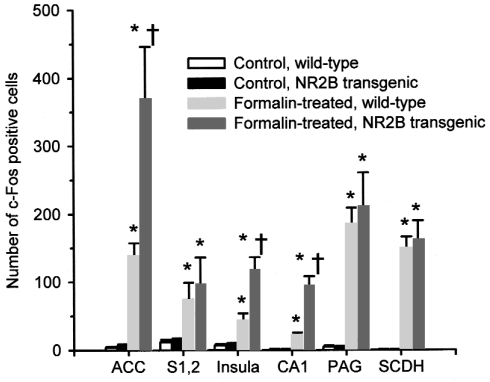


Fig. 5. Enhanced forebrain c-Fos expression following formalin injection in NR2B transgenic mice. Numbers of c-Fos positive cells in the anterior cingulate cortex (ACC), the S1 and S2 regions of somatosensory cortex (S1,2), insular cortex, the CA1 subfield of the hippocampus, the periaqueductal gray (PAG) and spinal cord laminae I–VI (SCDH) are illustrated in wild-type and NR2B transgenic mice with hind paw injection of saline (control; 4 wild-type, 6 transgenic) or formalin (4 wild type, 4 transgenic). Cells were counted contralateral to the injected hind paw except in the spinal cord, where cells were counted ipsilaterally. *Significant difference from wild type and transgenic controls; †significant difference from wild type, formalin-treated mice.

pattern of c-Fos immunoreactivity induced in NR2B transgenic mice after formalin injection in experiments done in parallel to those described above. In saline-injected control animals, baseline c-Fos expression was indistinguishable between wild type and transgenic mice (Figs. 4 and 5). After formalin treatment, the most prominent expression occurred in the ACC, insular cortex and hippocampal CA1 subfield, all of which exhibited significantly more c-Fos immunoreactivity in transgenic than wild type formalin-treated mice (Figs. 4 and 5). Similar results were noted in the lateral septal nucleus, hippocampal CA3 subfield and accumbens nucleus. No significant differences in c-Fos staining between transgenic and wild type mice after formalin injection were found in somatosensory cortex (Fig. 5), amygdala, thalamus or hypothalamus. Formalin-induced c-Fos expression was likewise unaffected by forebrain-targeted NR2B expression in the midbrain, brainstem and spinal cord (Figs. 4 and 5). The brain areas in which significantly greater c-Fos expression was observed in transgenic than in wild type mice were areas in which NR2B overexpression was present[15] (Figs. 1–3). Moreover, these areas of greater c-Fos expression encompassed some parts of the limbic system and other forebrain areas important for the central processing of pain information, including, in particular, the ACC and insular cortex[23,24].

We next asked whether forebrain-targeted NR2B overexpression affected the behavioral responses of mice to tissue injury and inflammation. We used two models: peripheral injection of formalin, and peripheral injection of complete Freund's adjuvant (CFA). In the first set of experiments, formalin was injected subcutaneously into the dorsal side of a hind paw. Mice responded by licking the injected paw, and this behavior was typically concentrated in two distinct phases: phase one (0–10 min) and phase two (10–55 min). We continued to observe mice until 120 min after injection, for two reasons. First, in previous studies (ref. 25 and unpublished data), we showed that mice continue to exhibit behavioral responses during the 55–120 min period (n = 50 mice, mean total response time 159 ± 11 s), which we have called 'phase three.' Second, NMDA receptor-mediated, activity-dependent synaptic plasticity at central synapses can last for hours after induction. Thus, if forebrain-targeted NR2B overexpression alters the behavioral responses of mice to formalin injection, then it is reasonable that these alterations might be observed during phase three.

Within the first 55 min after hind paw formalin injection, behavioral responses were similar between wild type and transgenic mice (Fig. 6a and b). However, transgenic mice exhibited more pronounced phase-three responses compared to wild type

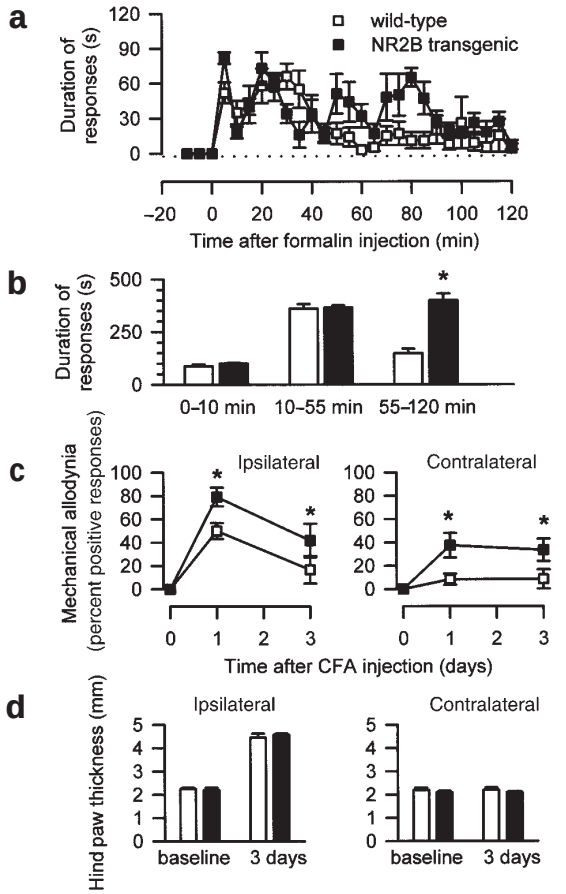


Fig. 6. Enhanced behavioral responses to formalin or CFA injection in NR2B transgenic mice. (**a**) The number of seconds during which wild-type (□, n = 16) and NR2B transgenic mice (■, n = 9) were engaged in nociceptive behavioral responses to hind paw formalin injection were plotted in 5-min intervals. Among nine NR2B transgenic mice, six of them were from transgenic line 1, and three were from line 2. Because no significant difference was found between them, the data were pooled. (**b**) Results from (**a**) were grouped into three phases (see Results). *Significant difference from wild-type mice. (**c**) The responses of animals to a mechanical stimulus (a 0.4-mN von Frey fiber applied to the dorsum of a hind paw) that elicited no responses before dorsal hind paw CFA injection were recorded 1 and 3 days after injection. The data were plotted as percent positive responses to stimulation of the ipsilateral or contralateral hind paw (relative to side of injection) for wild-type (□, n = 4) and NR2B transgenic (■, n = 4) mice. *Significant difference between wild-type and transgenic mice in the indicated conditions. (**d**) Hind paw edema was measured with a fine caliper in wild-type and NR2B transgenic mice (white bars, wild-type, n = 4; black bars, NR2B transgenic, n = 4). No significant difference was found between wild-type and transgenic mice.

mice (n = 6; Fig. 6a and b). Similar results were obtained from a second line of transgenic mice (n = 3). No obvious difference in the degree of hind paw edema was found between wild type and transgenic mice (data not shown). When a lower formalin concentration was used (1%, 10 μl), no difference between the two groups of mice was observed at any time point (wild type, n = 3, phase 1, 56 ± 7 s; phase 2, 217 ± 9 s; phase 3, 99 ± 22 s; NR2B transgenic, n = 3, phase 1, 68 ± 25 s; phase 2, 231 ± 37 s; phase 3, 121 ± 18 s). These results suggest that forebrain-targeted NR2B overexpression selectively enhanced delayed behavioral responses to hind paw formalin injection, and that this enhancement required a threshold dose.

Finally, we tested the responses of wild type and transgenic mice to hind paw injection of CFA. Application of a 0.4 mN von Frey fiber to the dorsum of a hind paw elicited no response in untreated mice, but at one and three days after CFA injection, mice responded to stimulation of either the injected (ipsilateral) or, to a lesser extent, the contralateral hind paw by hind paw withdrawal. This mechanical allodynia, or display of a withdrawal response to a previously non-noxious mechanical stimulus, was significantly enhanced in NR2B transgenic mice (Fig. 6c). We also observed similar changes in hind paw edema in wild type and NR2B transgenic mice (Fig. 6d).

Discussion

Our findings demonstrate that genetic manipulation of forebrain NMDA receptors could enhance persistent or chronic behavioral responses to tissue injury and inflammation, without affecting acute nociception. The finding that forebrain-targeted NR2B overexpression seemed to enhance phase-three responses selectively in the formalin test suggests that forebrain NMDA receptor-mediated phenomena may represent a mechanistic basis for these delayed behavioral responses. Taken together with the observation that transgenic mice also experienced enhanced mechanical allodynia one and three days after CFA injection, these data are consistent with the hypothesis that forebrain NMDA receptor activation is responsible, at least in part, for a prolonged nociceptive reaction to tissue injury and inflammation.

Although our study does not identify the specific brain region(s) responsible for the altered behavior of transgenic mice, the observed phenotype is most likely explained by changes in NMDA receptor physiology within the forebrain, because α-CaMKII promoter-driven overexpression of NR2B is expected to be restricted to the forebrains of mice[15]. Moreover, we present anatomical, electrophysiological and behavioral evidence in support of this conclusion. Anatomical evidence was the enhanced expression of NR2B in the ACC and insular cortex, but not in the spinal cord, as detected by *in situ* hybridization. Also, we found enhanced formalin-induced c-Fos expression in the ACC, insular cortex and other forebrain areas, but not in the midbrain, brainstem or spinal cord of transgenic mice. Our electrophysiological evidence was the enhanced NMDA receptor-mediated synaptic transmission in the ACC and insular cortex but not in the spinal cord dorsal horn of transgenic mice. Our behavioral evidence was that phase two formalin-induced behavioral responses, which are thought to depend on spinal NMDA receptors[19,26], were not affected in transgenic mice. Taking together these four independent lines of evidence, the enhanced responsiveness of transgenic mice to inflammatory stimuli is most likely explained by changes in NMDA receptor composition in the forebrain alone.

Our results suggest that a genetic manipulation conferring enhanced cognitive abilities may also provide unintended traits, such as increased susceptibility to persistent pain. We do not know why the forebrain NR2B:NR2A ratio undergoes a developmental reduction. However, we note that although pain perception normally is a protective for an organism, it may, in some cases, be considered maladaptive, particularly when pain from a previous injury persists and cannot be avoided. Our data show that behavioral responses to inflammatory pain—not just learning and memory—were affected by enhanced forebrain expression of NR2B. Such a blunt manipulation likely affects an animal's physiology in many ways. More selective regional manipulation of NR2B receptor expression (for example, targeting the CA1 subfield of the hippocampus exclusively) may help to avoid such unintended side effects.

Our study implicates a molecular mechanism by which forebrain activity could modulate behavioral responses to inflammatory pain. In addition to the well characterized contributions of spinal or brainstem NMDA receptors, forebrain NMDA receptors may be important and unique in the response of animals to persistent pain. Because NR2B expression is restricted to forebrain neurons in wild type adults[9], NR2B-selective NMDA antagonists, by preferentially targeting forebrain neurons, may prove beneficial in the treatment of chronic pain in humans.

Methods

Transgenic mice. Both NR2B transgenic and littermate wild type mice were provided by Y. P. Tang and colleagues at Princeton University[15]. Adult male mice weighing 15–29 g were used for all experiments, and 'wild type,' as used throughout this manuscript, refers to littermate mice not segregating the NR2B transgene. Experimental protocols were approved by the Animal Studies Committee at Washington University.

Electrophysiology. Mice were anesthetized with 1–2% halothane, and brain or spinal cord was isolated. Recordings from cortex were done as described[16]. Briefly, transverse cortical slices were maintained in an interface chamber at 28°C, where they were subfused with artificial cerebrospinal fluid (ACSF) consisting of 124 mM NaCl, 4.4 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.0 mM Na_2HPO_4 and 10 mM glucose, bubbled with 95% O_2 and 5% CO_2. Stimulation with a bipolar tungsten electrode in layer V evoked extracellular field potentials in layer II/III. For recordings from spinal cord, transverse spinal slices with attached dorsal roots were maintained in ACSF at 34°C. Intracellular microelectrode recordings were made from dorsal horn laminae I/II neurons, and dorsal root stimulation evoked synaptic responses that were acquired and analyzed with the Axoclamp 2B amplifier and pCLAMP software (Axon Instruments, Foster City, California). In all experiments, bicuculline methiodide (10 μM) and strychnine hydrochloride (1 μM) were added to the perfusion solution.

Anatomical analysis. *In situ* hybridization experiments were done as described[27] using an NR2B plasmid (gift from J.E. Huettner). For immunocytochemistry, mice were anesthetized with 3–4% halothane at 120 min following formalin or saline injection, perfused with fixative, and processed for immunostaining as described[16] using an anti-c-Fos rabbit antibody (1:20000; Oncogene Science, Uniondale, New York). Anatomical terminology is based on the atlas of Franklin and Paxinos[28]. The rostrocaudal levels corresponded to 0.98 to 0.5 mm (ACC), 0.7 to –1.22 mm (the S1 and S2 regions of somatosensory cortex), 1.10 to 0.5 mm (insular cortex), –1.70 to –2.18 mm (the CA1 subfield of the hippocampus), and –4.24 to –4.72 mm (the periaqueductal gray), relative to bregma. Spinal sections analyzed were from L4–5.

Behavioral experiments. To test acute pain responses, the latency of response to heating of the tail (tail-flick test) or to placement on a hot (50–55°C) or cold (0°C) plate was measured as described[29–30]. To test inflammatory pain, formalin (5%, 10 μl) or complete Freund's adjuvant (CFA, 50%, 10 μl; Sigma, St. Louis, Missouri) was injected subcutaneously into the dorsal side of a hind paw. For formalin, the total time spent licking or biting the injected hind paw was recorded during each

five-minute interval, for two hours. For CFA, mechanical sensitivity was assessed with a set of von Frey filaments (Stoelting, Wood Dale, Illinois) using the up-down protocol[31]. Positive responses to application of a filament to a hind paw included prolonged hind paw withdrawal followed by licking or scratching. In untreated wild type (n = 4) or transgenic mice (n = 4), the 0.4 mN (No. 2.44) filament, representing 50% of the threshold force, never produced responses, and was used to detect mechanical allodynia in CFA-injected mice. Hind paw edema was evaluated by measuring dorsal–ventral hind paw thickness with a fine caliper at three days after CFA injection.

Data analysis. Results were expressed as mean ± s.e.m. Statistical comparisons were made with one- or two-way analysis of variance (ANOVA) with the *post-hoc* Scheffe F-test in immunocytochemical experiments, or the Student-Newmann-Keuls test in behavioral experiments, to identify significant differences. In all cases, $p < 0.05$ was considered significant.

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