Transgenic Mice Expressing Dominant-negative Activin Receptor IB in Forebrain Neurons Reveal Novel Functions of Activin at Glutamatergic Synapses*

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The transforming growth factor β family member activin is an important regulator of development and tissue repair. It is strongly up-regulated after acute injury to the adult brain, and application of exogenous activin protects neurons in several lesion models. To explore the role of endogenous activin in the normal and acutely damaged brain, we generated transgenic mice expressing a dominant-negative activin receptor IB (dnActRIB) mutant in forebrain neurons. The functionality of the transgene was verified in vivo. Hippocampal neurons from dnActRIB mice were significantly more vulnerable to intracerebroventricular injection of the excitotoxin kainic acid than those from control littermates, indicating a crucial role of endogenous activin in the rescue of neurons from excitotoxic insult. Because dnActRIB is only expressed in neurons, not in glial cells, activin affords protection at least in part through a direct action on endangered neurons. Unexpectedly, the transgenic mice also revealed a prominent novel role of activin in glutamatergic neurotransmission in the intact adult brain. Electrophysiologic examination of excitatory synapses onto CA1 pyramidal cells in hippocampal slices of dnActRIB mice showed a reduced NMDA current response, which was associated with impaired long term potentiation. This is the first demonstration that activin receptor signaling is essential to optimize the performance of neuronal circuits in the mature brain under physiological conditions.

Activins are members of the transforming growth factor β family of proteins, which regulate proliferation and differentiation of various cell types. The predominant activin variants are homo- or heterodimers consisting of βA and/or βB subunits (activin A: 2βA, activin B: 2βB and activin AB: βAβB). Their biological effects are mediated through heteromeric receptor complexes, consisting of type I and type II transmembrane serine/threonine kinase receptors (1). Upon ligand binding, one of the activin type II receptors (ActRII or ActRIIB) dimerizes with a type I receptor (ActRIA, ActRIB, or activin receptor-like kinase 7 (2, 3)), resulting in phosphorylation of the type I receptor by the type II receptor. The subsequently activated serine/threonine kinase of the type I receptor phosphorylates the recruited receptor Smads (Smad2 and Smad3). The latter translocate to the nucleus upon multimerization with Smad4 and modulate as transcription factor complexes the expression of activin target genes (4). In addition, other signaling pathways are also activated by activin receptors, including mitogen activated kinase signaling (1, 4, 5).

Activins are important regulators of development, inflammation, and repair of different tissues and organs (1, 5). In the central nervous system, activin is involved in development, but it may also participate in adaptive and protective mechanisms of the adult brain. Thus, activin βA mRNA is transiently up-regulated after electrical stimuli that induce synaptic long term potentiation (LTP); Refs. 6 and 7) and after brief electroconvulsive seizures that are used to treat certain forms of major depression (8). A much stronger and prolonged induction of activin A expression occurs after acute brain injury (9–11). Since exogenous activin protects cultured neurons (12, 13), these findings suggested that activin might also afford neuroprotection in vivo. Indeed, intracerebroventricular application of activin A rescued neurons after excitotoxic and hypoxic/ischemic insults (10, 14, 15). Nevertheless, the site of action and the function of endogenous activin in the intact and the injured brain remain to be determined. Therefore, we generated transgenic mice expressing a dominant-negative mutant of activin receptor IB (dnActRIB) in forebrain neurons. For this purpose, the complete kinase domain of the receptor was deleted. When expressed in excess in comparison to the endogenous receptor, dnActRIB preferentially forms non-functional heterodimers with type II receptors upon ligand binding, thereby blocking activin receptor signaling (16). Using this strategy, we report here the novel and unexpected finding that activin signaling augments plasticity of excitatory synapses in the adult brain.
while, at the same time, reducing its vulnerability to excitotoxic damage.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—The transgene construct (Fig. 1A) was separated from vector sequences (Fig. 1A), and the purified insert was used for microinjection of fertilized oocytes from B6D2F2 × C57BL/6 mice. Mice were genotyped by PCR, and transgenic animals were backcrossed into the C57BL/6 strain.

Intracerebroventricular Injection of KA, Activin A, or PBS—Mice were anesthetized with ketamine/xylazine and placed into a stereotaxic apparatus. Intracerebroventricular injection of 0.1 μl of KA (1 μg/μl in PBS, pH 7.0) was performed as described by Tretter et al. (9, 10) with permission from the veterinary authorities, Zurich, Switzerland. To verify the functionality of the transgene, 150 ng of activin A in 0.8 μl of PBS or solvent PBS were injected intracerebroventricularly. Ipsilateral hippocampi were removed 2 h later and used for preparation of protein lysates and subsequent Western blotting.

RNase Protection Assay (RPA)—RNA was isolated from hippocampi, and RPAs were performed (9) using a 507-bp probe corresponding to the 5′-end of the murine ActRIB cDNA and some region of the hybrid intron present in the expression vector (Fig. 1A) and a fragment corresponding to nucleotides 566–685 of the murine glyceraldehyde-3-phosphate dehydrogenase cDNA.

In Situ Hybridization—In situ hybridization with a digoxigenin-labeled riboprobe was performed on frozen sections from whole brains as described by Yang et al. (17) using a riboprobe corresponding to sequences of the polyadenylation signal of the vector (Fig. 1A).

Western Blotting—Hippocampi were lysed in 50 mM Tris/ HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 0.5 mM AEBSF, 10 mM NaF, 1 mM Na3VO4, 10 mM sodium pyrophosphate, 20 μM phenylarsinoxide, phosphatase inhibitor mixture I/II (Sigma, Munich, Germany; 1:100). Lysates were analyzed by Western blotting using polyclonal antibodies against the c-Myc epitope, total Smad2 and Smad3 (both from Santa Cruz Biotechnology, Northampton, MA) and were given as mean ± S.E. Pipettes for whole-cell recordings of excitatory postsynaptic currents (EPSCs) were filled with (in mM) 130 potassium gluconate, 3 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 25 NaHCO3, and 10 d-glucose, gassed with 95% O2, 5% CO2, pH 7.4. Field excitatory postsynaptic potential (fEPSP) recordings from stratum radiatum of area CA1 were performed and analyzed by means of a multielectrode system (MED64 in conjunction with Performer 2.0 software, Panasonic, Japan). In the recording chamber, submerged slices were constantly superfused with warmed artificial cerebrospinal fluid (30°C). Before and after tetanization, test and control pathways were stimulated at 0.05 Hz. The stimulus strength was adjusted to obtain ~40% of the maximum fEPSP amplitude under control conditions. LTP was induced by two theta bursts stimuli (TBS) 20 s apart, with each TBS consisting of 15 bursts of 4 pulses at 100 Hz delivered at an interburst interval of 200 ms. fEPSPs slopes were normalized to 100% before TBS and pooled across animals of the same genotype. Data were processed and analyzed using Origin Pro7 software (OriginLab, Northampton, MA) and were given as mean ± S.E. Pipettes for whole-cell recordings of excitatory postsynaptic currents (EPSCs) were filled with (in mM) 130 potassium gluconate, 3 MgCl2, 5 EGTA, 5 HEPES, 2 Na+ -ATP, 0.3 Na-GTP, pH 7.25–7.30. Series resistance (10–20 MΩ) was compensated by 70–85%. EPSCs were recorded in the presence of the GABA_A (γ-aminobutyric acid, type A) receptor antagonist bicuculline (30 μM) at −70 mV, after correcting for liquid junction potentials. Whole-cell recordings were performed at room temperature. Current signals were recorded and analyzed using an Azo-
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RESULTS

Generation of Transgenic Mice Expressing dnActRIB in Hippocampal Neurons—To inhibit activin receptor signaling in hippocampal neurons in vivo, we generated transgenic mice expressing dnActRIB with a carboxyterminal c-Myc epitope under the control of the CaMKII-α promoter and regulatory elements (19) (Fig. 1A). The progeny of seven transgenic founder mice expressed the transgene as determined by RPA using a riboprobe, which can distinguish between transgene-derived and endogenous ActRIB mRNA (Fig. 1B) and data not shown). Levels of total Smad2 were not affected by activin A in wild-type and transgenic mice (Fig. 2A). This result was reproduced in three independent experiments with hippocampal lysates from different animals, and the difference between transgenic and wild-type mice was statistically significant (p = 0.012; Fig. 2B).

Enhanced Vulnerability to KA Excitotoxicity in dnActRIB Mutant Mice—Histological analysis of the brain of the transgenic animals did not reveal any detectable abnormalities in the non-lesioned hippocampus, and neuronal death was not observed as determined by FJB staining, a marker for degenerating neurons (18) (data not shown). To elucidate the neuroprotective potential of endogenous activin, we performed intracerebroventricular kainate (KA) injections, which cause neuronal loss predominantly in the ipsilateral hippocampus (Fig. 2A). In vivo effect of the transgene was detected by Western blotting using an antibody against the c-Myc epitope (Fig. 1C). In situ hybridization with a transgene-specific probe revealed the presence of dnActRIB transcripts in neurons of the cortex, amygdala, and striatum (data not shown).

To verify the dominant-negative effect of the transgene in vivo, activin A or vehicle PBS were injected intracerebroventricularly, and the phosphorylation of Smad2 in the ipsilateral hippocampus was monitored by Western blotting 2 h after injection. A basal level of Smad2 phosphorylation was already observed in non-injected or vehicle-injected hippocampi (Fig. 2A), and this signal was predominantly attributed to hippocampal neurons as demonstrated by immunohistochemistry (Fig. 2C).

patch 200 amplifier in conjunction with a Digidata 1200 interface and pClamp 9 software (all from Axon Instruments). Data were expressed as means ± S.E. Statistical comparisons were performed using Student's t test. All drugs were from Sigma.
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body against cleaved caspase-3 (Fig. 3I). Staining with this antibody correlated with the pattern of FJB staining in the same area (Fig. 3K), although cleaved caspase-3 positive cells comprise only a part of the population of the FJB positive cells. The area of FJB-positive hippocampal cells was measured ipsilaterally and contralaterally in a rectangular area of 1381.5 × 1094.6 μm (Fig. 3, E–H), and every third section through the posterior-anterior extent of the hippocampi was analyzed. Therefore, the data represent an unbiased sampling of FJB-positive cells within the hippocampus. The number of FJB-positive cells in the ipsilateral hippocampi was significantly higher in transgenic (tg) mice of both lines compared with wild-type (wt) littermates (L1-tg, n = 93 sections from 10 mice; L1-wt, n = 100 sections from 10 mice; Pips = 0.0015 (Fig. 3L); L3-tg, n = 60 sections from 6 mice; L3-wt, n = 60 sections from 6 mice, Pips < 0.0001) (Fig. 3M). Furthermore, the extent of cell death in the contralateral hippocampus, the so-called “distant damage” produced by hyperactivity of the commissural fibers connecting the hippocampi was elevated, although the difference was only statistically significant in one mouse line (L3-tg, n = 60 sections from 6 mice; L3-wt, n = 60 sections from 6 mice; Pcon = 0.0004 (Fig. 3M); L1-tg, n = 80 sections from 8 mice; L1-wt, n = 90 sections from 9 mice; Pcon = 0.3697) (Fig. 3L). Since intracerebroventricular KA up-regulates activin βA mRNA also in the contralateral hippocampus (9), the results suggest that disruption of activin signaling renders hippocampal neurons more vulnerable to both local and distant excitotoxic injury.

The NMDA Receptor-mediated Component of Glutamatergic Neurotransmission Is Reduced in Hippocampi from dnActRIB Mutant Mice—Are neurons from dnActRIB mutant hippocampi more vulnerable to excitotoxic damage, because they respond more sensitively to glutamate? To explore this possibility, we recorded EPSCs from visually identified CA1 pyramidal cells in hippocampal slices. To partially relieve the Mg2+ block of NMDA receptors, extracellular Mg2+ was lowered to 0.3 mM (4.0 mM Ca2+). D-2-amino-5-phosphonovaleric acid (50 μM) and CNQX (10 μM) served to isolate the AMPA and the NMDA receptor-mediated component of the EPSC, respectively. Whereas the input-output relationship of the overall EPSC was not appreciably altered in mutant hippocampi, the NMDA component was significantly reduced (Fig. 4, A and B; wt, n = 9 slices from 3 mice; L1-dn, n = 9 from 3 mice; L3-dn, n = 8 from 2 mice). The histogram of Fig. 4C shows that the relative contribution of the NMDA current to the overall EPSC (12.9 ± 1.3% in pyramidal cells of normal hippocampi) decreased to 8.1 ± 1.3% and 8.1 ± 0.6% in pyramidal cells of L1 and L3 mutant hippocampi, respectively. By contrast, the time course of NMDA currents did not vary between the different groups (decay time constants: wt, 22.8 ± 2.9 ms, n = 9; L1-dn, 22.3 ± 3.3 ms, n = 9; L3-dn, 23.3 ± 5.3 ms, n = 8, p > 0.9). These data suggest that dnActRIB is unlikely to influence considerably NMDA receptor 2 (NR2) subunit expression, since changes in NR2 subunit composition would be expected to alter the kinetics of NMDA currents (21). A comparison of the expression of NR1, NR2A, and NR2B subunits by means of quantitative real-time RT-PCR lends further support to this notion. Using hippocampal total RNA from wild-type and transgenic mice, we failed to detect a significant change in the expression levels of any of the above subunits (Fig. 5). Furthermore, protein levels of NR2A were not altered as demonstrated by Western blotting of total hippocampal lysates from adult wild-type and transgenic mice (data not shown).

The reduction of the NMDA component was also observed in experiments in which we used bath application of either AMPA (1 μM) or NMDA (10 μM) to mimic the massive rise in extracellular glutamate that is a hallmark of acute excitotoxicity (Fig. 4D). Under these conditions, extra-
synaptic glutamate receptors are also activated. In cells of both mutant lines, AMPA responses at more negative potentials were smaller than those in cells from normal hippocampus, but only in L1-dn neurons, this decrease reached statistical significance (Fig. 4E: WT, n = 9 slices from 3 mice; L1-dn, n = 7 slices from 2 mice; L3-dn, n = 8 slices from 3 mice). Since the AMPA receptor-mediated component of the EPSC was not reduced in a similar fashion, it is tempting to speculate that the decreased current responses to AMPA superfusion resulted mainly from an altered expression and/or function of extrasynaptic AMPA receptors. NMDA currents displayed the characteristic increase with membrane depolarization, which was, however, drastically diminished in mutant hippocampi of both lines (Fig. 4F: WT, n = 7 slices from 2 mice; L1-dn, n = 7 slices from 2 mice; L3-dn, n = 8 slices from 3 mice).

**Synaptic Plasticity Is Impaired in Hippocampi from dnActRIB Mutant Mice**—Since NMDA receptor activation is essential for the induction of LTP in the CA1 region, we wondered whether the decrease of NMDA responses bears...
significance on the extent of synaptic plasticity in hippocampi of mutant mice. Using a MED64 multielectrode stimulation and recording system, two independent afferent pathways were electrically stimulated to evoke fEPSPs in stratum radiatum of area CA1 (Fig. 6A). During control stimulation, fEPSP slopes did not differ significantly between hippocampi from wild-type and transgenic mice (L1-wt: \(111 \pm 9 \mu V/ms, n = 8\) versus L1-dn: \(86 \pm 10 \mu V/ms, n = 10\), \(p > 0.10\); L3-wt: \(86 \pm 6 \mu V/ms, n = 18\) versus L3-dn: \(95 \pm 5 \mu V/ms, n = 15\), \(p > 0.23\)). Also, paired-pulse stimulation (50-ms interstimulus interval) yielded virtually identical ratios of facilitation (L1-wt: \(1.51 \pm 0.03\), \(n = 16\) versus L1-dn: \(1.47 \pm 0.03\), \(n = 20\), \(p > 0.5\); L3-wt: \(1.33 \pm 0.03\), \(n = 14\) versus L3-dn: \(1.34 \pm 0.05\), \(n = 22\), \(p > 0.5\)). Following theta burst stimulation, hippocampi from both mutant lines showed significantly impaired LTP compared with controls (Fig. 6B: L1-wt, \(n = 8\) slices from 3 mice; L1-dn, \(n = 10\) slices from 4 mice; Fig. 6C: L3-wt, \(n = 18\) slices from 6 mice; L3-dn, \(n = 15\) slices from 4 mice). Whereas in L1-wt hippocampi, the mean fEPSP slope at 25–30 min post-TBS attained 165 ± 9% of control, L1-dn hippocampi displayed an increase of fEPSP slope to only 135 ± 8% of control (\(p = 0.033\)). In recordings from L3 hippocampi, in which extracellular Mg\(^{2+}\) was lowered to 1 mM, the corresponding values were 179 ± 7% for the wt-hippocampi and 155 ± 5% for the dn-hippocampi (\(p = 0.012\)). These data indicate that the decrease of the NMDA component observed in whole-cell
recordings from mutant hippocampi is sufficient to compromise synaptic plasticity.

**DISCUSSION**

To gain insight into the role of endogenous activin in the normal and injured hippocampus, activin receptor signaling was disrupted in hippocampal neurons. ActRIB was mutated, since it specifically binds to ActRII-activin ligand-receptor complexes (22), and since it is expressed in hippocampal neurons (23). Therefore, it can inhibit, in a dominant-negative manner, activin signaling via all activin type I receptors (16). Since expression of the transgene was hardly detectable in cultured hippocampal neurons (data not shown), we confirmed the activity of this mutant and its specificity for activin versus transforming growth factor β in cultured keratinocytes (24). Most importantly, the dominant-negative effect of the transgene was verified in vivo after intracerebroventricular injection of activin A. In these experiments, activin increased the levels of phosphorylated Smad2 in the hippocampus of wild-type mice but not of transgenic mice. The CaMKII-α promoter was chosen, because it drives expression of transgenes in forebrain neurons but not in glial cells. Since appreciable activity of this promoter is not observed before the second postnatal week, the transgene is unlikely to interfere with prenatal and early postnatal development (20). Although our study focuses on the role of activin signaling in the hippocampus, it is worth noting that, by using the CaMKII-α promoter, the transgene is also expressed in other forebrain regions such as cortex, amygdala, and striatum. Consistent with this notion, in situ hybridization indeed revealed the presence of dnActRIB transcripts in these regions. Thus it is well conceivable that the disruption of activin signaling will also bear significance on the normal operation of other brain circuits. Further experiments should help to determine whether activin regulates glutamatergic neurotransmission and excitotoxicity in other forebrain structures in a fashion similar to that reported here from the hippocampus.

Our study demonstrates that activation of activin receptor signaling by endogenous ligands is capable of rescuing hippocampal neurons from excitotoxic cell death. The responsible ligand is most likely activin A, since the βB chain is not

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5 M. R. Müller and S. Werner, unpublished data.

**FIGURE 6. LTP is impaired in hippocampi from mutant mice.** A, using a multielectrode system, TBS delivered to experimental pathway produced input-specific LTP in area CA1. Insets depict individual fEPSP recordings taken at the like-numbered time points. B and C, compared with hippocampi from wt mice, LTP was significantly reduced in both transgenic lines.
expressed in the normal or lesioned hippocampus (9). A contribution of bone morphogenetic protein 3 (BMP3) to the observed phenotype cannot be fully excluded, since it also binds to activin receptors (25) and since we found a weak expression of BMP3 in normal and injured hippocampus, using real-time RT-PCR (data not shown). However, a contribution of BMP3 to the enhanced vulnerability of the hippocampus of our transgenic mice seems unlikely, since BMP3 is an inhibitor of activin receptor signaling (25) and since activin was shown to be neuroprotective (see above). Enhanced vulnerability to KA toxicity was observed in both transgenic mouse lines, demonstrating that the effect is not due to integration of the transgene into a host gene, which is important for neuroprotection. Unexpectedly, we found a stronger vulnerability in the transgenic line, which expressed lower levels of dnActRIB. Although the transgene was expressed in forebrain neurons in both mouse lines as determined by in situ hybridization, minor differences in the temporal and spatial expression of the transgene, which are often observed with the CamKII-α promoter (20), may account for the difference in vulnerability.

Given the selective disruption of activin receptor signaling in neurons, but not in glial cells, neuroprotection by activin receptor ligands is obviously afforded at least in part through a direct action on endangered neurons. Since fibroblast growth factor 2, whose neuroprotective potential is closely linked to the induction of activin A (10), exerts its beneficial action in part by down-regulating NMDA receptors (26), we wonder whether the enhanced vulnerability of dnActRIB mutant mice might be attributable to abnormal NMDA responses. To our surprise, however, we found that under physiological conditions (synaptic stimulation) as well as under pathological conditions (glutamate overflow), the NMDA current was significantly depressed in pyramidal cells of mutant hippocampi, whereas the AMPA current was not or less affected. Thus we discard the hypothesis that the heightened vulnerability of mutant mice to excitotoxic injury results from an overactivity of glutamate receptors. Whereas the neuroprotective mechanisms of activin have thus to await further study, our findings reveal a prominent new function of activin in the normal adult brain that bears particular relevance to synaptic plasticity. The NMDA receptor deficiency that we observed in mature CA1 pyramidal cells of mutant hippocampi should not represent a developmental disturbance, because the transgene is expressed under the control of the CaMKII-α promoter. We rather propose that activin receptor signaling in adult hippocampus is required to augment NMDA responses, thereby endowing the synapse with its full range of plasticity. Thus genetic or acquired defects of the activin system are likely to impact on synaptic performance in the learning brain as well as on neuronal survival during injury. In the future, it will be important to identify the intracellular signaling pathways, which mediate the effect of activin on forebrain neurons. Since activin was shown to signal via Smad2 in the hippocampus (this study), an involvement of this pathway seems likely. In addition, a role of mitogen-activated kinase signaling should be considered, since activation of ERK1/2 by activin was found to be responsible for the activin-induced expression of tyrosine hydroxylase in mouse E14 primary striatal cell cultures and in a hippocampal neuronal cell line (27).

Our results reveal novel functions of activin at glutamatergic synapses. Thus, in addition to its endocrine functions and its role in inflammation and repair (1, 5), activin seems to be an important modulator of neuronal activity. This finding is likely to be of clinical interest, since application of exogenous activin or stimulation of activin receptors should not only provide protection of endangered neurons but might also emerge as a novel therapeutic approach to improve cognitive impairments.

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