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Reversal of Bone Loss in Mice by Nongenotropic Signaling of Sex Steroids

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We show that sex steroids protect the adult murine skeleton through a mechanism that is distinct from that used to preserve the mass and function of reproductive organs. The classical genotropic actions of sex steroid receptors are dispensable for their bone protective effects, but essential for their effects on reproductive tissues. A synthetic ligand (4-estren-3α,17β-diol) that reproduces the nongenotropic effects of sex steroids, without affecting classical transcription, increases bone mass and strength in ovariectomized females above the level of the estrogen-replete state and is at least as effective as dihydrotestosterone in orchidectomized males, without affecting reproductive organs. Such ligands merit investigation as potential therapeutic alternatives to hormone replacement for osteoporosis of bone mass in both women and men.

Estrogens and androgens exert many biological effects that cannot be explained by interactions of their receptors with DNA (*1*, *2*). Heretofore, there has been no evidence that such "nongenotropic" actions of sex steroids (*3*, *4*) are of biological relevance in vivo. We have recently elucidated potent anti-apoptotic effects of estrogens and androgens on murine osteoblasts and osteocytes (*5*). These effects are due to stimulation of the Src/Shc/ERK and repression of the JNK signaling cascades via a nongenotropic action of the classical sex steroid receptors, leading to downstream modulation of the activity of transcription factors such as Elk-1, C/EBPβ (NF-IL6), CREB, and c-Fos/c-Jun (*6*). Unlike classical effects of sex steroids on reproductive tissues, all these actions are non–sex-specific, require only the ligand-binding domain of the receptor, and are eliminated by nuclear targeting. Moreover, these nongenotropic actions can be faithfully reproduced by 4-estren-3α,17β-diol, a synthetic compound referred to hereafter as estren, which has no classical transcriptional activity. Conversely, 1,2,5-tris(4-hydroxylphenyl)-4-propylpyrazole, referred to hereafter as pyrazole, has potent transcriptional activity but minimal (if any) effects on ERK or JNK kinases.

We compared the effects of estren to those of 17β-estradiol (E_2) or dihydrotestosterone (DHT) on bone and reproductive tissues of adult Swiss Webster mice (*7*). E_2, DHT, and estren, but not pyrazole, not only attenuated osteoblast apoptosis but also stimulated osteoclast apoptosis, with identical potency in primary cultures of cells from female or male mice, which is consistent with a non–sex-specific mechanism of action (Fig. 1A). Furthermore, irrespective of the sex of the mouse, a replacement dose of either E_2 or DHT, or administration of estren to gonadectomized females or males, prevented gonadectomy-induced osteoblast apoptosis in the lumbar vertebrae (Fig. 1B).

For the in vivo studies, we used a ~300-fold higher dose of estren, as compared to E_2, on the basis of its lower binding affinity for the estrogen receptor (ER) (fig. S1). In spite of this, estren, unlike E_2, did not stimulate estrogen response element–mediated transcription of the C3 gene in the uterus, in agreement with its in vitro properties (*5*) (Fig. 1C). None of the compounds affected body weight. Estren was at least as effective as estradiol in preserving global and spinal bone mineral density (BMD) in females (Fig. 1D and fig. S2). In addition, ovariectomized (OVX) mice receiving estren exhibited greater BMD change in the hindlimb, not only compared with the OVX mice receiving E_2 replacement but also compared with the estrogen-replete sham controls, suggesting an anabolic effect (that is, the addition of new bone) at this site of predominantly cortical bone. Estren also appeared to be at least as effective as DHT replacement in orchidecto-

Table 1. Increased trabecular and cortical width, osteoblast number, and serum osteocalcin after treatment with estren. Histomorphometric analysis of L1 to L4 vertebrae from 6-month-old females and serum osteocalcin levels from the 6- and 8-month-old females are shown. Data are means ± SD.

Parameter	Sham	OVX	OVX + E_2	OVX + estren
Cortical width (μm)	117 ± 13	107 ± 10	108 ± 10	138 ± 28*‡
Bone area per tissue area (%)	23 ± 6	18 ± 3	15 ± 3†	20 ± 5
Trabecular width (μm)	59 ± 7*	48 ± 4	41 ± 6†	56 ± 7‡
Osteoid perimeter per bone perimeter (%)	19.3 ± 5.3*	25.3 ± 5.1	4.3 ± 2.9*†	12.3 ± 2*†‡
Osteoblast number per bone perimeter (*N*/mm)	14.4 ± 4	20.3 ± 6.9	3.6 ± 2.8*†	10.9 ± 3*‡
Osteoclast number per bone perimeter (*N*/mm)	2.3 ± 0.4	3.4 ± 1.6	0.9 ± 0.3*†	1.8 ± 0.6*
Bone formation rate per bone area (%/day)	0.457 ± 0.262	0.915 ± 0.475	0.080 ± 0.048*	0.158 ± 0.108*
Serum osteocalcin (ng/ml)	126 ± 24*	150 ± 31	81 ± 14*	157 ± 19*†‡

*$P < 0.05$ versus OVX. †$P < 0.05$ versus sham. ‡$P < 0.05$ versus OVX + E_2.

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mized (ORX) mice, because BMD values in the spine of the ORX + estren group, but not the ORX + DHT group, were significantly higher than in the untreated ORX group (fig. S2). Furthermore, compression strength in both femora and vertebrae of the estren-treated female mice was greater than in mice receiving E_2 (Fig. 2, A and B). In the male mice, however, estren and DHT were equally

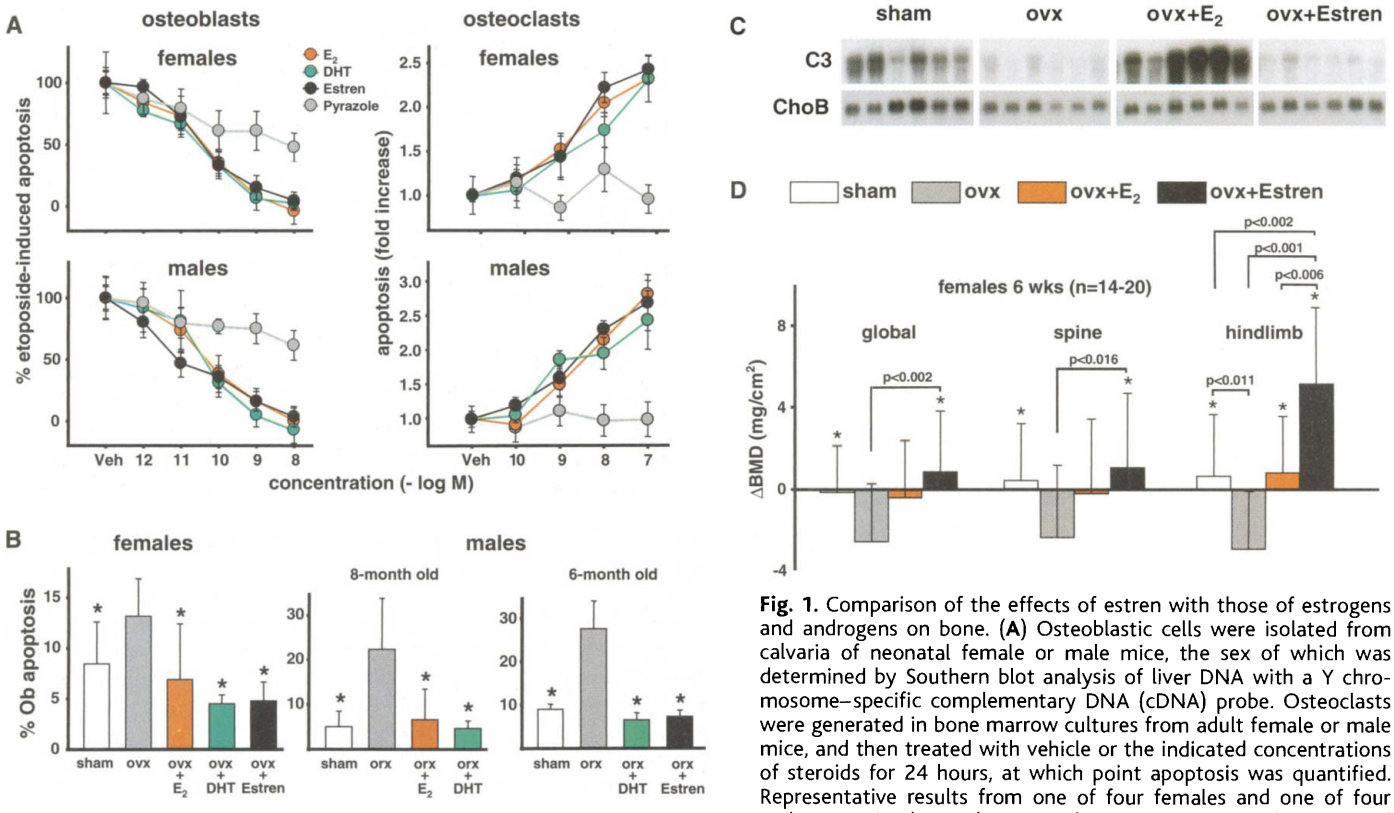

Fig. 1. Comparison of the effects of estren with those of estrogens and androgens on bone. (**A**) Osteoblastic cells were isolated from calvaria of neonatal female or male mice, the sex of which was determined by Southern blot analysis of liver DNA with a Y chromosome–specific complementary DNA (cDNA) probe. Osteoclasts were generated in bone marrow cultures from adult female or male mice, and then treated with vehicle or the indicated concentrations of steroids for 24 hours, at which point apoptosis was quantified. Representative results from one of four females and one of four males examined are shown. Each point represents the mean of triplicate determinations ± SD. *$P < 0.05$ versus vehicle, by analysis of variance (ANOVA). [(B) to (D)] Six- or 8-month old Swiss Webster mice ($n = 8$ to 10 per group) were sham-operated on, ovariectomized (OVX), or orchidectomized (ORX). The OVX and ORX animals were then left untreated or were implanted immediately with 60-day slow-release pellets containing E_2 (0.025 mg), DHT (10 mg), or estren (7.6 mg). (**B**) Six weeks later, osteoblast (Ob) apoptosis was determined in sections of L1 through L4 vertebrae from females and males; bars indicate means ± SD. *$P < 0.05$ versus OVX or ORX. (**C**) mRNAs of C3 and ChoB, a housekeeping gene control, were quantified by Northern blot analysis of total RNA isolated from uteri; each lane represents one mouse. (**D**) Global, spine, and hindlimb BMD were determined 1 to 3 days before and 6 weeks after surgery. Data represent pooled values of the change (ΔBMD) from the two experiments with the 6- and 8-month-old mice. Bars indicate means ± SD. *$P < 0.05$ versus OVX by Dunnett's procedure; P values obtained by all pairwise comparisons (Bonferroni's method) are also depicted.

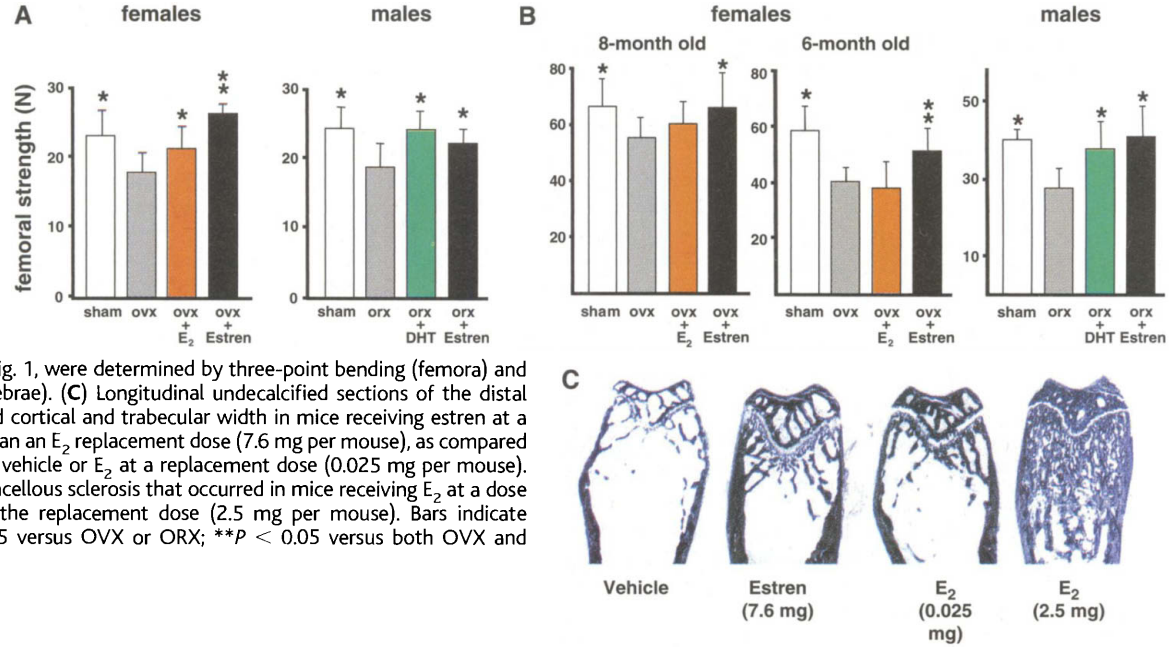

Fig. 2. Increased femoral and vertebral strength and preservation of the marrow cavity in mice treated with estren. Bone strength at the (**A**) midshaft of the femora of 6-month-old females and males, and at the (**B**) fifth lumbar vertebrae of 8- and 6-month-old females and 6-month-old males, of the experiments described in Fig. 1, were determined by three-point bending (femora) and compression tests (vertebrae). (**C**) Longitudinal undecalcified sections of the distal femur. There is increased cortical and trabecular width in mice receiving estren at a dose 300 times higher than an E_2 replacement dose (7.6 mg per mouse), as compared to the animals receiving vehicle or E_2 at a replacement dose (0.025 mg per mouse). By contrast, note the cancellous sclerosis that occurred in mice receiving E_2 at a dose 100 times higher than the replacement dose (2.5 mg per mouse). Bars indicate means ± SD. *$P < 0.05$ versus OVX or ORX; **$P < 0.05$ versus both OVX and OVX + E_2.

effective. Estren had no adverse effects on the marrow cavity, in contrast to a pharmacologic dose of E_2 (100 times the replacement dose for mice), which caused closure of the bone marrow cavity (*8*) (Fig. 2C).

Histomorphometric analysis of the lumbar vertebrae (L1 through L4) showed that mice receiving estren had significantly greater cortical and trabecular width as compared to OVX mice treated with E_2: 27.8 and 33.9%, respectively (Table 1). The number of osteoblasts on the trabeculae of the estren-treated mice was greater (319%) than that in the E_2-treated group; and consistent with this, the unmineralized matrix produced by osteoblasts (the osteoid perimeter) was also greater by 270%. The rate of bone formation and osteoclast number were suppressed by either E_2 or estren as compared to the OVX group. These findings confirm the adequacy of the E_2 replacement dose in suppressing the ovariectomy-induced increase in bone turnover and support the BMD findings. In line with the BMD data and the higher osteoblast number in the estren-treated mice, serum osteocalcin, which is a biochemical index of osteoblast number, was significantly higher in two separate experiments, not only compared to OVX + E_2 mice but also to the estrogen-replete sham controls. Also consistent with the similar effects of DHT and estren on BMD in the male, the two compounds had indistinguishable effects on histomorphometric parameters and osteocalcin (table S1).

In contrast to E_2 or DHT, estren had no effect on the uterine or seminal vesicle weight of the gonadectomized mice (Fig. 3, A and B). The lack of an effect of estren on reproductive tissues was confirmed by histologic analysis of the uterus (Fig. 3D and fig. S3). Lastly, unlike E_2 or pyrazole, estren did not stimulate MCF-7 cell proliferation (Fig. 3C). None of the three ligands affected the proliferation of the ER-negative MDA-MB-231 cells (*9*).

In summary, we show here that the skeletal effects of sex steroids can be dissociated from their reproductive effects. Similar to bone, several other nonreproductive target tissues of sex steroids display relatively low receptor levels, relaxed sex specificity, and activation of mitogen-activated protein (MAP) kinases by a nongenotropic mechanism of receptor action (*10*). It is therefore possible that the dissociation of nonreproductive from reproductive effects of sex steroids by activation of nongenotropic signals may extend to other tissues as well (*11*–*13*). Inactivation of both the genotropic and nongenotropic function of the glucocorticoid receptor causes lethality in mice, whereas elimination of the transcriptional activity of this receptor does not (*14*). We therefore propose that mechanism-specific ligands of the ERs or the AR [as opposed to tissue-specific ligands (SERMs) or classic estrogens or androgens], and perhaps mechanism-specific ligands of other nuclear receptors, represent a novel class of pharmacotherapeutics.

The potent effects of estren on hindlimb BMD, the mechanical strength of both the axial and apendicular skeleton, and the increase in serum osteocalcin suggest that this compound has the potential to cause positive focal balance between formation and resorption and continuous gain in bone mass, thereby rebuilding a normal skeleton. How can elimination of the genotropic effects of sex steroids lead to this advantageous skeletal profile? In contrast to our findings that activation of MAP kinases by an extranuclear function of the ERα suppresses AP-1 activity (*6*), others have shown that E_2-activated ERα stimulates AP-1 activity by a genotropic mechanism (*15*). Hence, the response of a target cell to sex steroids may be determined by the balance between nongenotropic and genotropic actions. Consequently, at the molecular level, the effects of estren on bone could very well be due to the absence of a counterregulatory effect on AP-1. According to this hypothetical scenario, a greater suppression of c-Jun could lead to decreased transcription of the Wnt antagonist Dickkopf, thereby unleashing Wnt signaling, which is a potent bone anabolic stimulus (*16*, *17*).

Changes in the birth and life-span of osteoclasts and osteoblasts resulting from estrogen deficiency at menopause are responsible for one of the most common metabolic bone diseases of the modern era: postmenopausal osteoporosis (*18*). Prevention of this disease is the best justified rationale (and the only approved U.S. Food and Drug Administration indication) for prolonging estrogen replacement therapy for several decades after menopause. On the basis of the efficacy of estrogen replacement therapy in the prevention of osteoporosis and the assumption that the effects of estrogens on reproductive and nonreproductive tissues result from similar mechanisms of receptor action, replacement therapy with estrogens has been given during the past 60 years to millions of postmenopausal women in order to prevent the adverse effects of estrogen deficiency in repro-

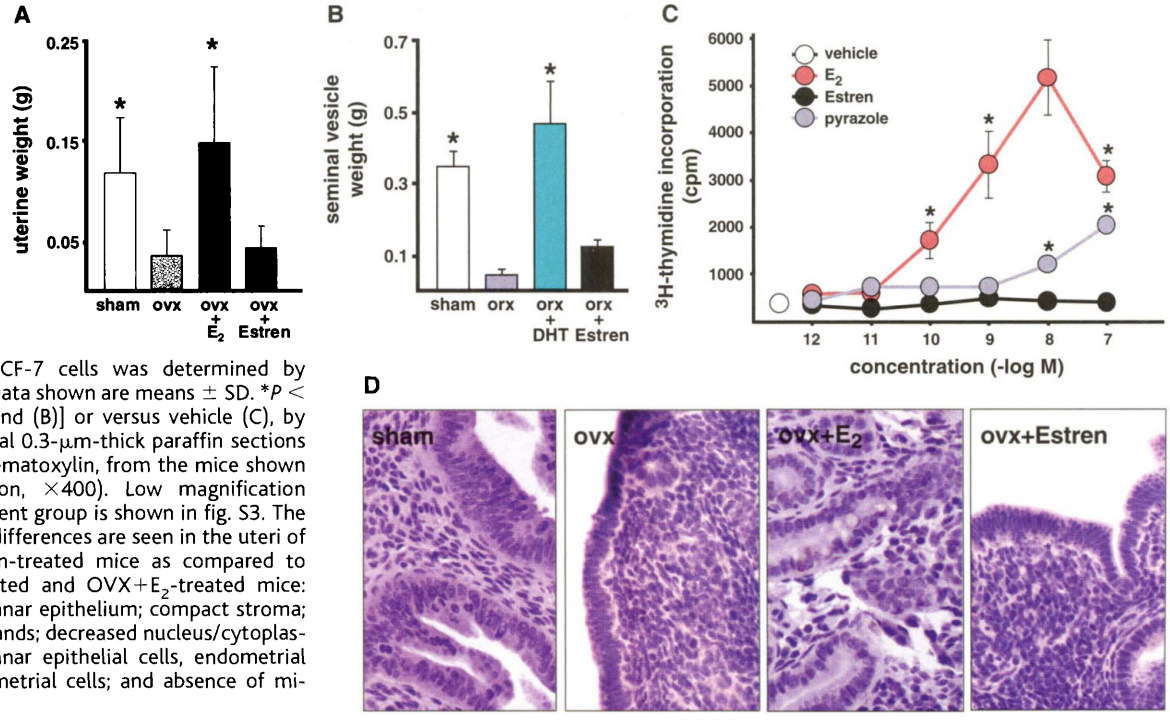

Fig. 3. Lack of an effect of estren on female and male reproductive tissues or breast cancer cells. The wet uterine (**A**) or seminal vesicle (**B**) weight of female and male mice described in Fig. 1 is shown. The data in (A) represent pooled values from the two experiments with the 6- and 8-month-old females. (**C**) Proliferation of MCF-7 cells was determined by [^{3}H]thymidine uptake. Data shown are means ± SD. *$P <$ 0.05 versus OVX [(A) and (B)] or versus vehicle (C), by ANOVA. (**D**) Longitudinal 0.3-μm-thick paraffin sections of uteri, stained with hematoxylin, from the mice shown in Fig. 1 (magnification, ×400). Low magnification (×100) for each treatment group is shown in fig. S3. The following morphologic differences are seen in the uteri of OVX- and OVX+estren-treated mice as compared to uteri from sham-operated and OVX+E_2-treated mice: thin and atrophic columnar epithelium; compact stroma; decreased number of glands; decreased nucleus/cytoplasmic ratio in the columnar epithelial cells, endometrial stroma cells, and myometrial cells; and absence of mitotic activity.

ductive and nonreproductive tissues alike. Deficiency of androgens (and probably estrogens) in males, due to castration or a decline in production with old age, is a major factor in the development of osteoporosis in men as well (*19*, *20*).

The benefits of sex steroid replacement during late postreproductive life derive primarily from the actions of sex steroids on nonreproductive tissues, whereas its side effects result from actions on reproductive tissues. The favorable effects of estren on bone and its lack of effect on reproductive tissues indicate that mechanism-specific ligands may offer advantages over estrogens or SERMs (*21*, *22*) in the setting of hormone replacement therapy. Growing concern about the efficacy and safety of existing hormone replacement therapies (*23*, *24*) makes these new ideas timely.

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Treatment of Ischemic Brain Damage by Perturbing NMDA Receptor–PSD-95 Protein Interactions

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***N*-methyl-D-aspartate receptors (NMDARs) mediate ischemic brain damage but also mediate essential neuronal excitation. To treat stroke without blocking NMDARs, we transduced neurons with peptides that disrupted the interaction of NMDARs with the postsynaptic density protein PSD-95. This procedure dissociated NMDARs from downstream neurotoxic signaling without blocking synaptic activity or calcium influx. The peptides, when applied either before or 1 hour after an insult, protected cultured neurons from excitotoxicity, reduced focal ischemic brain damage in rats, and improved their neurological function. This approach circumvents the negative consequences associated with blocking NMDARs and may constitute a practical stroke therapy.**

Protein-protein interactions govern signals involved in cell growth, differentiation, and intercellular communication through dynamic associations between modular protein domains and their cognate binding partners (*1*). At excitatory synapses of central neurons, ionotropic glutamate receptors are organized into multiprotein signaling complexes within the postsynaptic density (PSD) (*2*). A prominent organizing protein is PSD-95 (*3*), which couples the NMDAR to intracellular proteins and signaling enzymes (*2*, *4*). Through its second PDZ domain (PDZ2), PSD-95 binds the COOH-terminus tSXV motif of NMDAR NR2 subunits as well as neuronal nitric oxide synthase (nNOS) (*3*, *4*). This binding couples NMDAR activity to the production of nitric oxide (NO), a signaling molecule that mediates NMDAR-dependent excitotoxicity (*5*). NMDAR activity is unaffected by genetically disrupting PSD-95 in vivo (*6*) or by suppressing its expression in vitro (*7*). Nonetheless, PSD-95 deletion dissociates NMDAR activity from NO production and suppresses excitotoxicity (*7*).

Although NMDARs mediate ischemic brain injury (*8*), blocking them is deleterious to animals and humans (*9*–*11*). Targeting PSD-95 protein therefore represents an alternative therapeutic approach for diseases that involve excitotoxicity. Because mutation or suppression of PSD-95 is therapeutically impractical, we hypothesized that perturbing its interaction with NMDARs could suppress excitotoxicity and ischemic brain damage. This might be achieved by the intracellular introduction of peptides that bind to either the NR2 or the PDZ2 interaction domains (Fig. 1A). To bind PDZ2 domains, we constructed a peptide comprising the nine COOH-terminal residues of NR2B (Lys-Leu-Ser-Ser-Ile-Glu-Ser-Asp-Val; NR2B9c) (*3*). To bind NR2 subunits, we used residues 65 to 248 of PSD-95, encoding the first and second PDZ domains (PDZ1-2). NR2B9c and PDZ1-2 were rendered cell-permeant by fusing each to the cell-membrane transduction domain of the human immunodeficiency virus–type 1 (HIV-1) Tat protein (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg) (*12*) to obtain a 20–amino acid peptide (Tat-NR2B9c) and the fusion protein pTat-PDZ1-2, respectively (*13*).

First, we determined whether Tat-NR2B9c would transduce into neurons. The fluorophore dansyl chloride was conjugated to Tat-NR2B9c and to a control peptide comprising HIV-1 Tat residues 38 to 48 (Lys-Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys; Tat38-48) outside the transduction domain (*14*). These were bath-applied to cultured cortical neurons and their fluorescence was visualized by confocal microscopy. Neurons treated with Tat-NR2B9c-dansyl (10 μM) exhibited fluorescence in their cytoplasm and processes, indicating intracellular peptide uptake (Fig. 1B, left), whereas cultures treated

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