cium content of fresh, unstimulated axons (about 70 μmole/kg). Parallel experiments, using arsenazo III or antipyrylazo III as monitors of free calcium, indicated that, with the stimulus parameters used, mean free calcium in the axoplasm does not rise above 2 to 3 μ*M* during loading, although the concentration of free calcium immediately beneath the axolemma may be several times higher [F. J. Brinley, Jr., T. Tiffert, A. Scarpa, L. J. Mullins, *J. Gen. Physiol.* **70**, 355 (1977)].
16. The composition of the injection solution was (m*M*): potassium oxalate, 150; potassium isethionate, 292; K-TES buffer, 5; *p*H 7.3. The solution was diluted about tenfold by diffusion throughout the volume of the axon.
17. Injections were made by using the apparatus described by F. J. Brinley, Jr., and L. J. Mullins [*J. Neurophysiol.* **28**, 526 (1965)] as modified by P. DeWeer.
18. Oxalate is known to penetrate isolated SR vesicles and increase the total calcium uptake by lowering the internal calcium activity [M. Makinose and W. Hasselback, *Biochem. Z.* **343**, 360 (1965)]. Adenosine triphosphate-dependent calcium uptake by similar fractions of neural tissue and other nonmuscle cells has been demonstrated and has been shown to be enhanced by oxalate [L. Moore and I. Pastan, *J. Cell. Physiol.* **91**, 289 (1977); M. P. Blaustein *et al.* (*19*)]. Although oxalate is a mitochondrial poison, the axons in this experiment were exposed to it for only about 20 minutes, and ATP is depleted in squid axons poisoned with CN^- only after 1½ to 2 hours [J. C. Caldwell, *J. Physiol. (London)* **152**, 545 (1960)].
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23. Transmission micrographs were taken with a Philips 400 electron microscope, and scanning transmission micrographs and x-ray analyses were done on a Hitachi H-500 electron microscope equipped with a Kevex energy-dispersive x-ray spectrometer.
24. The axon was prepared as described for *Aplysia* neurons [M. Henkart, *Science* **188**, 155 (1975)].
25. Measurements were made by cutting out and weighing the various structures in electron micrographs.
26. In calcium-loaded axons not injected with oxalate the dense deposit did not fill the lumen of the ER cisterns as uniformly.
27. Oxalate may increase the quantity of calcium sequestered by the ER (*18*), but since our observations with the analytical microscope were only roughly quantitative we have not yet evaluated this possibility.
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Tricyclic Antidepressants: Long-Term Treatment Increases Responsivity of Rat Forebrain Neurons to Serotonin

Abstract. *Long-term treatment of rats with clinically effective tricyclic antidepressant drugs induced a selective increase in the inhibitory response of forebrain neurons to serotonin applied by microiontophoresis. Long-term administration of some related drugs which lack antidepressant efficacy failed to induce such a change. The enhanced response to serotonin induced by the clinically active tricyclic drugs took 1 to 2 weeks to develop, a time course which correlates with the delayed onset of therapeutic effects in humans.*

Glowinski and Axelrod (*1*) demonstrated in 1964 that the tricyclic antidepressant (TCA) drug imipramine blocks norepinephrine uptake into synaptic terminals in the central nervous system. Subsequently, other TCA drugs have been shown to be potent blocking agents of norepinephrine or serotonin. Since reuptake is believed to be a major mechanism for termination of neurotransmitter action in monoaminergic systems, it has often been assumed that this pharmacological property accounts for the clinical efficacy of these drugs. However, uptake inhibition can take place within minutes (*2*), whereas the clinical response requires a minimum of 8 days (*3*). Moreover, some clinically active TCA drugs such as iprindole (*4, 5*) are not efficient blocking agents of amine uptake. Conversely, some drugs such as cocaine (*6*) and FG-4963 (*7*) are potent blocking agents of amine uptake but are not effective antidepressants. Furthermore, there is little correlation between clinical response and degree of amine uptake blockade in humans (*8*). Finally, despite their rather similar intrinsic antidepressant effects, tertiary and secondary amine TCA drugs have differential effects on psychomotor activity (*9*) and on amine uptake [for example, tertiary amine TCA drugs preferentially block serotonin uptake, whereas secondary TCA drugs preferentially block norepinephrine uptake (*2, 10*)].

In short-term, single-cell recording studies, Bradshaw *et al.* (*11*) reported that TCA drugs applied iontophoretically could rapidly modify the response of cortical neurons to serotonin and norepinephrine; however, these effects were not specific for the monoamines. Short-term binding studies have shown that some TCA drugs interfere with the binding of ^{3}H-labeled lysergic acid diethylamide (LSD) to serotonin receptors (*12*). However, the effect of long-term systemic administration of TCA drugs on serotonin receptors has, to our knowledge, never been studied. We report here that the responsiveness of postsynaptic neurons in rat forebrain to serotonin, but not norepinephrine, is increased by the long-term administration of TCA drugs.

Male albino rats (Charles River, 200 to 250 g) were given daily intraperitoneal injections of the hydrochloride salts of iprindole (2.5 mg/kg; Wyeth), desipramine (5 mg/kg; Ciba-Geigy), imipramine (5 mg/kg; Ciba-Geigy), chlorimipramine HCl (5 mg/kg; Ciba-Geigy), amitriptyline (5 mg/kg; Merck Sharp & Dohme), FG-4963 (5 mg/kg; Ferrosan), fluoxetine (10 mg/kg; Lilly), or chlorpromazine (10 mg/kg; Smith Kline & French). All these doses fall within the range of daily doses used clinically (*3*). The drugs were administered for various lengths of time ranging from 1 to 14 days.

A *cerveau isolé* unanesthetized preparation was used for iontophoretic experiments in both untreated controls and drug-treated rats. The experiments were performed 24 hours after the last drug injection. Single-cell recording and iontophoresis were carried out in two representative forebrain areas: the ventral nucleus of the lateral geniculate body (VLG), which receives a dense input of serotonin-containing nerves (*13*), and the CA_3 region of the dorsal hippocampus (pyramidal cells), which is innervated by both ascending serotonin (*14*) and norepinephrine projections (*15*).

A conventional iontophoretic technique was used (*16*). The following substances were tested: serotonin creatinine sulfate, 0.04*M*, *p*H 3.6 (Regis); γ-aminobutyric acid (GABA), 0.05*M* in 0.05*M* NaCl, *p*H 4 (Calbiochem); norepinephrine bitartrate, 0.1*M*, *p*H 4 (Regis); and LSD bitartrate 0.001*M* in 0.1*M* NaCl, *p*H 4 (PHS-NIDA). The LSD was tested only in the VLG and norepinephrine only in the hippocampus. One side channel containing a 4*M* NaCl solution was used as a current-balancing channel. The central channel, used for recording unitary activity, contained a 2*M* NaCl solution saturated with Fast Green. Fast Green was deposited at the bottom of each electrode track and enabled us to verify histologically the locations of the recording sites (*17*). The time of onset of the effect of iontophoretically applied substances is the most sensitive index of receptor responsiveness (*18*). Accordingly, the sensitivity of the neurons to iontophoretic application of these substances was estimated from the charge (C_{50}) required to obtain a 50 percent decrease in the rate of spontaneous firing (*19*).

Twenty-four hours after a 14-day treatment with TCA drugs both VLG

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and hippocampus neurons exhibited an increased response to the depressant effect of iontophoretically applied serotonin (Figs. 1 and 2). All five TCA drugs that were tested induced this increase regardless of known presynaptic effects: desipramine, a potent blocking agent of norepinephrine reuptake; chlorimipramine, a potent blocking agent of serotonin reuptake; amitriptyline and imipramine, which affect the uptake of both amines (*1, 2, 10*); and iprindole, which is devoid of such presynaptic actions (*4, 5*). The response of VLG neurons to GABA or of hippocampal neurons to norepinephrine and GABA was not altered, indicating specificity for serotonin of this sensitization by TCA drugs (Fig. 2).

Fluoxetine, a selective blocking agent of serotonin reuptake (*20*), and FG-4963, a potent but less selective blocking agent of serotonin reuptake (*21*), were administered for the same 14-day period. Unexpectedly, both failed to modify the response of VLG neurons to sertonin (Fig. 2). Chlorpromazine, a tricyclic antipsychotic devoid of specific intrinsic antidepressant activity (*22*) despite some blocking action on norepinephrine reuptake (*23*), also failed to alter sensitivity of these neurons to serotonin after 14 days of drug treatment (Fig. 2). A significant decrease of the sensitivity to GABA was found in chlorpromazine-treated animals (Fig. 2). This effect may be of interest in relation to the potent inhibitory action of chlorpromazine on GABA reuptake (*24*).

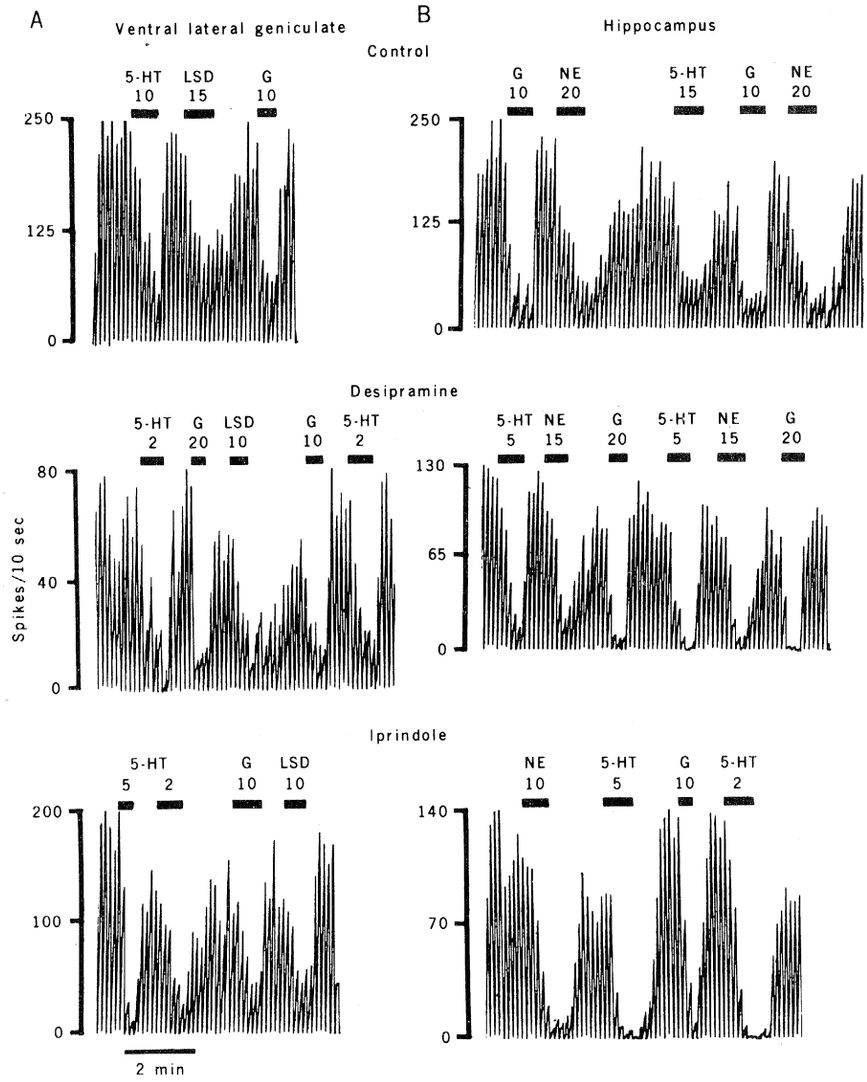

Fig. 1. Integrated rate histograms of action potentials recorded extracellularly from (A) ventral lateral geniculate and (B) hippocampal CA_3 neurons in untreated control rats and in rats injected intraperitoneally with desipramine (5 mg/kg daily) or iprindole (2.5 mg/kg daily) for 14 days. Solid lines indicate duration of iontophoretic applications of serotonin (*5-HT*), lysergic acid diethylamide (*LSD*), γ-aminobutyric acid (*G*), and norepinephrine (*NE*). The iontophoretic currents are indicated in nanoamperes. For all drugs, a retention current, 8 nA, was passed between applications. The number of spikes per 10 seconds is indicated in ordinate. The time base applies for all traces.

Lysergic acid diethylamide is a weak serotonin agonist at postsynaptic sites in the forebrain (*16, 25*). The mean C_{50} value for LSD in animals treated with TCA drugs ($5.04 \pm 0.65 \times 10^{-6}$; $N = 19$) was significantly lower ($P < .001$) than the C_{50} for the animals treated with the non-TCA drugs, FG-4963, fluoxetine, and chlorpromazine ($12.5 \pm 1.05 \times 10^{-6}$; $N = 29$).

To assess the time course of the development of this sensitization of serotonin by TCA drugs, we also conducted the iontophoretic experiments on day 2 (24 hours after a single dose) and on days 4 to 7. The sensitivity to serotonin was not significantly modified on day 2 (that is, after a single dose of the TCA drugs); on days 4 to 7 the C_{50} values were intermediate between those of days 2 and 15 (Fig. 2). At both times the sensitivity of the two neuronal populations to GABA remained unchanged; the response of hippocampal neurons to norepinephrine was not modified signicantly although some C_{50} values tended to be elevated.

Our data show that the long-term administration of five clinically effective TCA drugs induced a sensitization of postsynaptic serotonin receptors in two populations of neurons receiving a serotonin input. The specificity of their action is indicated by the lack of a significant change in sensitivity in response to GABA or norepinephrine. Since FG-4963 and chlorpromazine [fluoxetine has been tested only in normal subjects (*26*)] are not effective antidepressants (*7, 22*), their failure to induce sensitization to serotonin in the present study suggests that the sensitizing effect of TCA drugs may be related to clinical efficacy in depression. Furthermore, several days of treatment with TCA drugs are required before a consistent sensitization is detectable. This time course parallels the delayed clinical effectiveness of these drugs (*3*).

A possible investigator bias in our study cannot be dismissed. However, the failure of long-term treatment with fluoxetine and FG-4963 to enhance responsiveness to serotonin was unexpected because both drugs are blocking agents of potent serotonin reuptake. Furthermore, in view of Green's report of an increased "serotonin syndrome" after long-term treatment with chlorpromazine (*27*), an enhanced responsiveness to serotonin would have been expected. Nevertheless a blind procedure would be of value in comparing the effect of long-term treatments with TCA drugs and

with saline on responsiveness to serotonin.

The failure of chlorpromazine to sensitize forebrain serotonin receptors may seem at variance with Green's report (*27*). However, the serotonin syndrome largely originates in the brainstem and spinal cord (*28*), and the serotonin receptors in the forebrain areas may have different properties. This is further suggested by the failure of long-term treatment with desipramine to affect the serotonin syndrome (*29*).

The finding that iprindole is as effective as the other TCA drugs in inducing a sensitization of forebrain postsynaptic serotonin receptors suggests that this phenomenon is not related to presynaptic amine uptake mechanisms. This is further indicated by the increased responsiveness to LSD, which is not a substrate for the high-affinity serotonin uptake process (*30*). Thus, the increased responsiveness to serotonin induced by TCA drugs may reflect an increased sensitivity of postsynaptic serotonin receptor mechanisms.

Vetulani *et al.* (*31*) reported a decreased responsiveness of the norepinephrine-sensitive adenosine 3′-5′-monophosphate (cyclic AMP) generating systems of limbic forebrain in rats treated for at least 4 weeks with 10 mg of desipramine or iprindole per kilogram per day (*31*). This finding has been confirmed by two other groups with desipramine (*32*), but the specificity of the effect has been questioned by Schultz (*33*) who obtained a similar change in cortical slices after a 6-day treatment with either chlorpromazine or imipramine (20 mg/kg daily). Although we did not observe any consistent changes in responses to iontophoretically applied norepinephrine, it is possible that a longer treatment period or higher doses of TCA drugs than used in the present study would have produced a change. Alternatively, changes in amine-sensitive adenylate cyclase may not be directly reflected in physiological tests of receptor sensitivity. Kanof and Greengard (*34*) proposed that the clinical efficacy of TCA drugs could be related to their ability to block brain histamine-sensitive adenylate cyclase. The relation between their findings and our results is unclear, since chlorpromazine is a potent blocking agent in their system whereas it fails to induce a sensitization of forebrain serotonin receptors in our model.

The model proposed here of TCA drug-induced increases in serotonin receptor sensitivity could prove to have a heuristic value since it is time-related to the clinical effect. Beyond the usefulness of a new pharmacological model, our findings could have some implication for the monoamine hypothesis of depression since they point to the serotonin system as a target common to all types of TCA drugs. This is consistent with the reports of Shopsin *et al.* (*35*), that parachlorophenylalanine, a serotonin-synthesis inhibitor (*36*), reversed drug-induced remission in depressed patients. However, parachlorophenylalanine does not seem to have such a "depression-inducing" effect in psychiatrically normal individuals (*37*). Moreover, direct evidence of a presynaptic defect in the serotonin system of depressives is still lacking. Thus, the possibility should be considered that subjects with higher vulnerability to depression could have defective mechanisms modulating the sensitivity of postsynaptic serotonin receptors. This possibility is consistent with the hypothesis that neuronal receptor sensitivity changes may underlie the pathophysiology of affective illness (*38*).

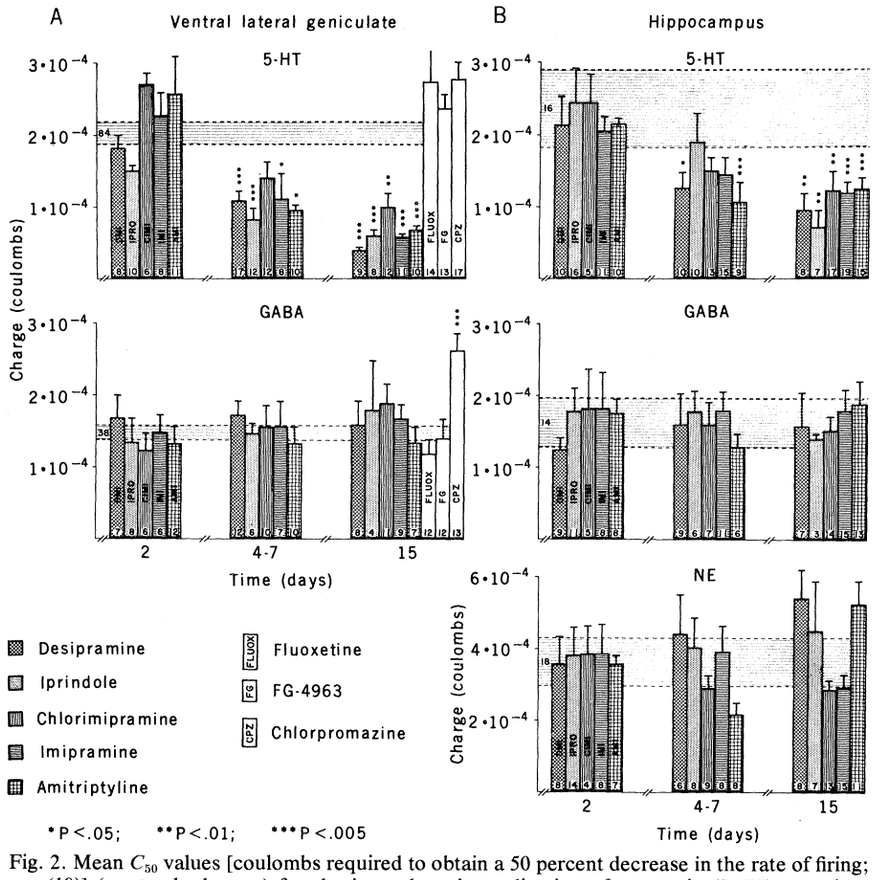

Fig. 2. Mean C_{50} values [coulombs required to obtain a 50 percent decrease in the rate of firing; see (*19*)] (± standard error) for the iontophoretic application of serotonin (*5-HT*), γ-aminobutyric acid (*GABA*), and norepinephrine (*NE*) to ventral lateral geniculate and hippocampal neurons in male albino rats treated with daily intraperitoneal injections of desipramine, chlorimipramine, imipramine, amitriptyline, and FG-4963 (5 mg/kg), iprindole (2.5 mg/kg), fluoxetine, and chlorpromazine (10 mg/kg) for 1 day, 3 to 6 days, or 14 days. The iontophoretic experiments were performed 24 hours after the last injection. The shaded areas represent the control C_{50} values (± standard error) obtained in untreated animals. Analysis of variance was used to compare the mean C_{50} values of treated and control animals. Four to six rats were used in each treatment group. The number of cells tested iontophoretically with each substance is indicated for each group.

Note added in proof: Since submitting this report, one of us (C.de M.) ran a blind comparison of the sensitivity of hippocampal pyramidal cells to serotonin, GABA, and norepinephrine in rats given daily intraperitoneal injections of iprindole (2.5 mg/kg) or saline (0.5 ml) on a long-term basis. The group given iprindole showed an enhanced responsiveness to serotonin ($P < .02$), whereas the responses to GABA and norepinephrine remained unchanged.

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Cellular Analysis of Long-Term Habituation of the Gill-Withdrawal Reflex of *Aplysia californica*

Abstract. *Long-term habituation training in* Aplysia californica *produces a profound depression in the efficacy of synaptic transmission between mechanoreceptor neurons and gill motor neurons. This depression persists for more than 3 weeks. Thus a critical synaptic site for plasticity underlying long-term habituation is the same as that for short-term habituation. For this simple form of learning, short- and long-term memory share a common locus and aspects of a common mechanism: synaptic depression.*

An unresolved question in the study of learning is the relation between short- and long-term memory. Some behavioral and pharmacological studies suggest that short- and long-term memory are two different processes having different loci and different neural mechanisms (*1*). Other studies suggest that memory consists of a single trace which changes in character with time and with processing [for a review, see (*2*)]. This question can be directly approached by examining the loci and cellular mechanisms of both short- and long-term memory in a given behavioral system and by determining the interrelation between them. To accomplish this end, we have studied the retention of habituation of the gill-withdrawal reflex in *Aplysia californica*; this reflex involves both short- and long-term memory in habituation training (*3, 4*).

The neural circuit of the reflex to stimuli of weak and moderate intensity is well understood in terms of individual nerve cells and their interconnections (*5, 6*). We have found that in this reflex, short- and long-term habituation share a common locus: the synapses made by the sensory neurons on their central target cells. Moreover, both the short-term and long-term memory involve aspects of a common mechanism: homosynaptic

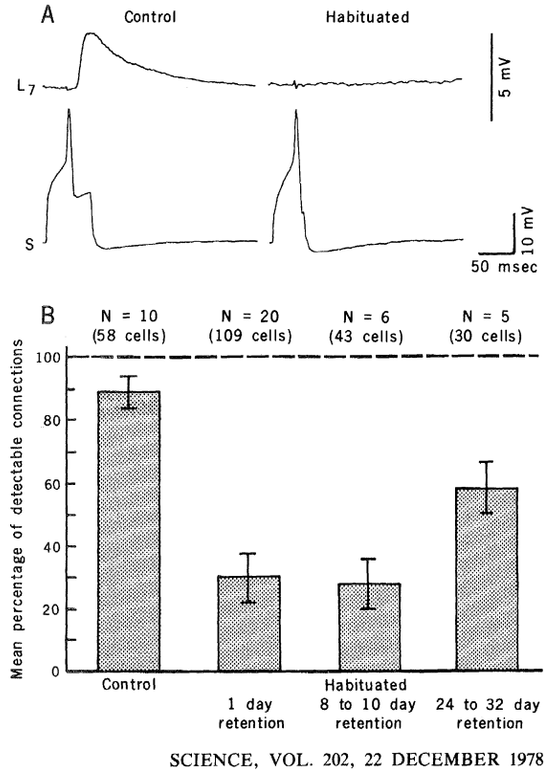

Fig. 1. Detectable and nondetectable connections. (A) Detectable EPSP from a control abdominal ganglion, and a nondetectable EPSP from an experimental animal that received long-term habituation training and whose synaptic connections were examined 24 hours after the last training session. The sensory neuron (S) was depolarized intracellularly to trigger a single action potential and to evoke a monosynaptic EPSP in the gill motor neuron L7. (B) Summary of the first experiment in which the ratio of the number of detectable EPSP's over the total number of connections sampled was determined in control animals ($N = 10$) and in habituated animals 1 day ($N = 20$), 8 to 10 days ($N = 6$), and 24 to 32 days ($N = 5$) after their last training session. Each animal contributed one ratio, and the graph indicates the mean percentage (± standard error) of detectable connections.

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