the sulfated fibrinopeptides. The Tyr sulfate residue in gastrin II and in hirudin is also preceded by several acidic amino acids.

The biological actions of CCK in mammalian systems include the stimulation of pancreatic enzyme secretion, gall bladder contraction, and possible CNS involvement in the mechanism of satiety (*13, 16*). Nonsulfated CCK's are 250 to 350 times less effective in stimulating amylase secretion and gall bladder contraction than the sulfated peptide (*13, 17*), demonstrating the importance of the sulfate moiety. Although the primary gastrointestinal function of gastrin in mammals is to induce gastric secretion, it also stimulates smooth muscle contraction, increases blood circulation and water secretion in the stomach and intestine, and stimulates pancreatic secretion. The sulfate ester is not required in gastrin to elicit gastric acid secretion (*18, 19*). Contraction of visceral muscle promoted by LSK in the cockroach hindgut and its attendant stimulation of hemolymph (blood) circulation (*20*) are analogous to gastrin-induced motility and increased blood circulation in mammalian intestines. It is unknown whether LSK is also involved in the secretion of digestive enzymes and regulation of water balance or satiety in the cockroach, other insects, or even other invertebrates.

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5. Operating conditions for columns on Waters ALC-100 HPLC system (columns i-v): (i) Waters μ-Bondapak phenyl, 4.6 mm by 30 cm (Waters Associates, Milford, MA). Solvent A, 0.1% trifluoroacetic acid (TFA) in water; Solvent B, 25% acetonitrile in 0.1% aqueous TFA. Conditions: 100% A for 8 minutes, then linear gradient to 100% B over 1 hour; flow rate, 1.5 ml per minute; detector, 2.0 absorption units at full scale (AUFS) at 214 nm. (ii) Rainin Microsorb C1, 4.6 mm by 25 cm (Rainin Instrument Co., Woburn, MA). Solvents and conditions: same as μBondapak phenyl except final condition was 50% B. (iii) Techsphere 3 C18, 4.6 mm by 15 cm (Phenomenex, Rancho Palos Verdes, CA). Solvents A and B: same as μBondapak phenyl. Conditions: 50% A for 8 minutes, then linear gradient to 100% B over 40 minutes; flow rate, 1.0 ml per minute; detector, 0.5 AUFS at 214 nm. (iv) Waters I-125 Protein-Pac, 7.8 mm by 30 cm. Solvent A, 95% acetonitrile made to 0.01% TFA. Solvent B, 75% acetonitrile made to 0.01% TFA. Conditions: 100% A for 8 minutes, then linear gradient to 100% B over 40 minutes; flow rate, 1.5 ml per minute; detector, 0.2 AUFS at 214 nm. (v) Waters Novapak C18. Solvent A, 95% 1 m*M* sodium acetate, *p*H 5.25; 5% acetonitrile, Solvent B, acetonitrile and water (3:2). Conditions: 100% A, then manufacturer's program 5 initiated upon injection to 80% B over 24 minutes; flow rate, 1 ml per minute; detector, 0.05 AUFS at 269 nm. (vi) Vydac C18 (Phenomenex), 1 cm by 25 cm, on Beckman 332 HPLC system. Solvent A, 0.1*N* ammonium acetate, *p*H 6.5. Solvent B, 80% acetonitrile, 20% 0.1*N* ammonium acetate, *p*H 6.5. Conditions: 12 to 20% B over 90 minutes, then 21 to 80% B over 2 minutes and held at 80% B for 10 minutes; flow rate, 2 ml per minute; detector, 1.0 AUFS at 280 nm.
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9. Solid phase synthesis of the peptide amide and peptide acid utilized *p*-methylbenzhydrylamine and Merrifield resins, respectively, on a Beckman 990 peptide synthesizer. Derivatized amino acids were purchased from Protein Research Foundation, Osaka, Japan. Amino acid side chain groups were protected as follows: His and Arg, tosyl; Tyr, 2,6-dichlorobenzyl ether; Asp, cyclohexyl ester; Glu, benzyl ester. The protected peptide-resins were treated with mixture of 1.5 ml of anisole, 0.25 ml of methylethyl sulfide, and 10 ml of hydrogen fluoride per gram at 0°C for 1 hour to cleave the peptide from the resin anchor and free it of side-chain protecting groups (*7*).
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Neurons Containing NADPH-Diaphorase Are Selectively Resistant to Quinolinate Toxicity

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Exposure of cultures of cortical cells from mouse to either of the endogenous excitatory neurotoxins quinolinate or glutamate resulted in widespread neuronal destruction; but only in the cultures exposed to quinolinate, an *N*-methyl-D-aspartate agonist, was there a striking preservation of the subpopulation of neurons containing the enzyme nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d). Further investigation revealed that neurons containing NADPH-d were also resistant to the toxicity of *N*-methyl-D-aspartate itself but were selectively vulnerable to the toxicity of either kainate or quisqualate. Thus, neurons containing NADPH-d may have an unusual distribution of receptors for excitatory amino acids, with a relative lack of *N*-methyl-D-aspartate receptors and a relative preponderance of kainate or quisqualate receptors. Since selective sparing of neurons containing NADPH-d is a hallmark of Huntington's disease, the results support the hypothesis that the disease may be caused by excess exposure to quinolinate or some other endogenous *N*-methyl-D-aspartate agonist.

A SMALL SUBPOPULATION OF NEURONS in the mammalian central nervous system (CNS) contains high activities of the enzyme nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d), the presence of which can be detected histochemically by the enzyme-mediated reduction of a tetrazolium dye to a visible reaction product (*1*). These NADPH-d–containing [NADPH-d(+)] neurons are scattered in "solitary" fashion throughout the neocortex, striatum, and other brain regions (*1, 2*), as well as in the retina (*3*). Neither the functional significance of the NADPH-d enzyme, nor the physiologic role of the NADPH-d(+) neurons is known, although in both the cortex and striatum the enzyme is colocalized with either somatostatin or avian pancreatic polypeptide–neuropeptide Y (or both) immunoreactivity (*4*).

Recently, this distinct class of neurons has been found to be selectively spared in the striatum of patients with Huntington's disease (HD) (*5*), in contrast to the general loss of intrinsic striatal neurons in HD. Biochemical markers for striatal γ-aminobutyric acid (GABA)–containing, cholinergic, and some peptidergic neurons (*6*) are all decreased in HD, but somatostatin levels are actually increased (*7*), probably reflecting

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the preservation of NADPH-d(+) cells amid local atrophy.

The critical defect in HD has been localized to the short arm of chromosome 4 (*8*), but the mechanism of neuronal degeneration orchestrated by this defect remains undefined. Present evidence suggests that an endogenous excitotoxic amino acid, in particular L-glutamate (GLU) (*9*) or quinolinate (QUIN) (*10*), may play a central role in the pathogenesis of HD. In our study, which utilized a simplified model system (mammalian cortical cell culture), we directly tested the possibility that NADPH-d(+) cells are intrinsically resistant to one or another of these endogenous excitotoxins. These NADPH-d(+) neurons survived controlled bath exposure to QUIN but not to GLU; this resistance to QUIN may result from an unusual distribution of excitatory amino acid receptor subtypes on these cells.

Dissociated cortical cell cultures were prepared from neocortices removed from mouse embryos at day 14 to 17 (*11*), and maintained at 37°C and 9% CO_2, in a medium of Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum, glucose (21 m*M*), and bicarbonate (38 m*M*). Culture dishes were selected for study after 15 to 23 days. At this time neurons (confirmed by intracellular recordings and Nissl stain) could be unambiguously identified by their extensive processes and phase-bright cell bodies, and the glia (selectively stained with antibody to glial fibrillary acidic protein) had formed a confluent background mat.

As previously reported, exposure of mature cultures to a 5-minute pulse of 0.5 m*M* GLU in the bathing solution invariably resulted in widespread neuronal disintegration by the following day (*11*) (Fig. 1B). Similar brief exposure to 1 m*M* QUIN had little effect (two experiments); however, with overnight exposure (18 to 24 hours), widespread neuronal disintegration invariably occurred, similar to that seen with GLU (Fig. 1A) (seven experiments) (*12*). Although the neuronal loss produced by exposure to either GLU or QUIN was massive, in both situations some intact neurons could be found a day after exposure. These surviving cells excluded trypan blue dye (5-minute incubation in a 0.4% solution) and remained morphologically stable for at least another day.

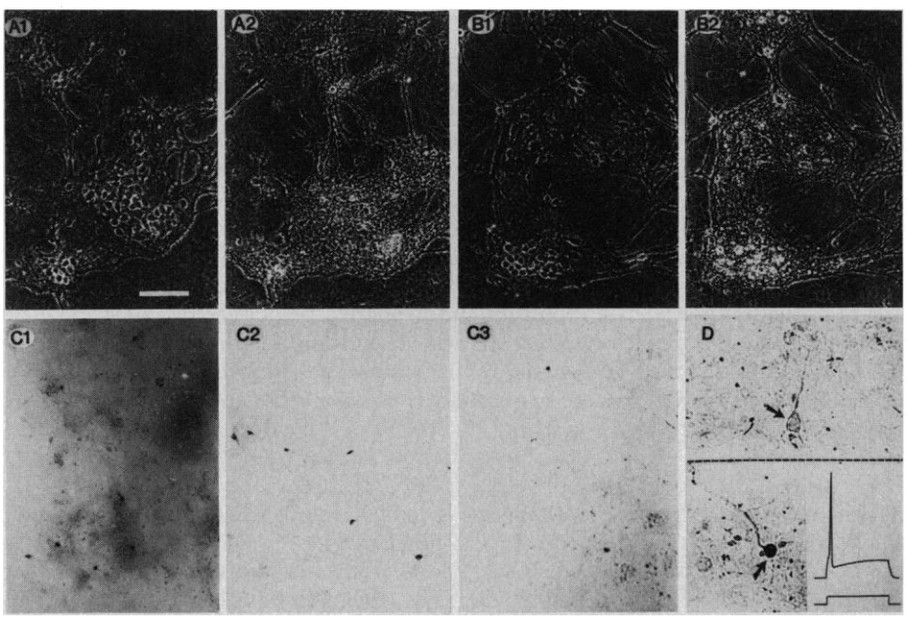

Fig. 1. (A) Phase contrast photomicrographs of an identified field of cortical neurons (200×) taken before (A1) and after (A2) a 24-hour incubation in 1 m*M* QUIN in the maintenance medium, but without serum. (B) Phase contrast photomicrographs of an identified field of cortical neurons [sister culture to the one shown in (A)], taken before (B1) and 24 hours after (B2) a 5-minute incubation in 0.5 m*M* GLU. (C) Representative low magnification bright-field photomicrographs (100×) of three separate cultures. (C1) Untreated; (C2) after 24-hour exposure to 1 m*M* QUIN; (C3) after 5-minute exposure to 0.5 m*M* GLU. (D) High magnification (400×) bright-field photomicrographs of unstained (upper) and NADPH-d(+) (lower) neurons (arrows) from the same culture dish after QUIN treatment. Before staining, we determined by intracellular recording that the NADPH-d(+) neuron was excitable to a depolarizing current pulse injected through the recording electrode by a bridge circuit (inset). The lower trace is injected current (210 pA, 80 msec), upper trace is membrane potential (resting membrane potential, −69 mV; action potential amplitude, 71 mV). Recordings were carried out at 34°C in Hanks balanced salt solution supplemented with calcium (total 4 m*M*) and 10 m*M* Hepes buffer. Calibration bar in A1 represents 100 μm in (A) and (B), 200 μm in (C), and 50 μm in (D).

Untreated (control) cultures, and cultures exposed the previous day to GLU or QUIN, were stained for NADPH-d by the method described by Scherer-Singler and colleagues (*2*). Cultures were fixed for 30 minutes in 4% paraformaldehyde at room temperature, and subsequently incubated in medium containing 1 m*M* NADPH (Sigma) and 0.2 m*M* nitro blue tetrazolium in 0.1 m*M* tris buffer (*p*H 8.2) at 37°C for 1 to 2 hours. The staining reaction was terminated by washing with water.

In control cultures, sparsely scattered NADPH-d(+) cells were readily identifiable by their darkly stained cell bodies and processes that were also often prominently stained (Fig. 1C1). The staining reaction sometimes resulted in some fine (1 to 3 μm) dark precipitates (visible in Fig. 1D), but these were too small to be confused with cells.

In cultures exposed overnight to 1 m*M* QUIN, the NADPH-d(+) cells were largely preserved, in contrast to the reduction in overall neuronal number (Fig. 1C2) (six of six experiments). However, in sister cultures exposed the previous day to 0.5 m*M* GLU for 5 minutes, both the number of NADPH-d(+) cells and the overall neuronal number were reduced compared with controls (Fig. 1C3) (five of five experiments).

The morphology of the NADPH-d(+) cells suggested that they were neuronal, but because they were only a small fraction of the total neurons, their identity was confirmed electrophysiologically. Cells with neuronal morphology that survived the overnight incubation in 1 m*M* QUIN were impaled under direct visual guidance with 4*M* potassium acetate microelectrodes. Successful intracellular recordings (stable recording with membrane potential more negative than −50 mV) were obtained from 35 cells; the observed resting membrane potential [−63 ± 6 mV (SD), $n = 35$] and input resistance (100 ± 52 megohms, $n = 31$) were similar to those previously described for cortical neurons (*11, 13*). All 35 cells showed spontaneous or stimulus-evoked action potentials, consistent with a neuronal identity. After the electrophysiologic study, the cells were stained for NADPH-d and relocated with the aid of an objective field marker; the majority of the cells analyzed electrophysiologically did not stain for NADPH-d (Fig. 1D, upper), but two of these cells were found to be NADPH-d(+) (Fig. 1D, lower).

In a set of matched sister cultures (control, after exposure to QUIN and after exposure to GLU), the total number of morphologically intact neurons was determined in sample areas consisting of 40

arbitrarily selected 400× microscope fields (Fig. 2). The number of intact neurons was reduced to a similar extent by GLU or QUIN, from the control mean of 747 ± 99 (SEM) neurons to 256 ± 54 neurons (34% of control mean) after GLU, and to 281 ± 21 neurons (36% of control mean) after QUIN (Fig. 2A). However, the number of intact NADPH-d(+) neurons, determined in larger sample areas (0.125 section of a 35-mm dish) with bright-field optics, was only significantly reduced compared with control values in the cultures exposed to GLU—from 595 ± 28 neurons to 236 ± 22 neurons (Fig. 2A). In the dishes exposed to QUIN, selective preservation of NADPH-d(+) neurons resulted in an increase in the proportion of NADPH-d(+) neurons from the control value of 1.9 ± 0.3% (SEM) to 5.1 ± 0.6% (Fig. 2B). Although the percentage of NADPH-d(+) neurons remaining after GLU treatment tended to be slightly greater than control (2.7 ± 0.2%), the difference was not statistically significant.

Our experiments suggest that the small subpopulation of cortical neurons that contain high concentrations of NADPH-d are selectively resistant to injury after exposure to QUIN, but not to GLU. This finding is consistent with the recent report by Beal and colleagues (*14*) that rat striatal NADPH-d(+) neurons in vivo selectively survive after local intraparenchymal injection of QUIN, but not after injection of several other excitotoxins. Our results are also compatible with other observations in vivo indicating that not all central neurons in a zone of excitotoxic injury are damaged to the same degree (*15*). However, since the neurotoxicity of excitotoxins in intact systems can depend strongly on extrinsic environmental factors regulating the effective exposure of the neurons to the toxins [for example, uptake (*16, 17*) or secondary release of endogenous excitotoxins from excitatory pathway projections (*18*)] differences in neuronal vulnerability in intact systems may be due to differences in the neurons themselves or to local extrinsic factors. The demonstration that NADPH-d(+) neurons retain resistance to QUIN toxicity in dispersed cell culture, where known concentrations of drug can be delivered directly to exposed neurons and where extrinsic factors are likely attenuated, suggests that this resistance is an intrinsic property of NADPH-d(+) neurons.

Previous studies in several systems have suggested that QUIN acts primarily on the *N*-methyl-D-aspartate (NMDA) subclass of excitatory amino acid receptors (*19, 20*). The same situation exists in mouse cortical cell culture, as both QUIN neurotoxicity

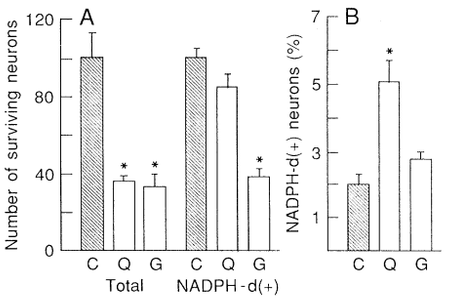

Fig. 2. (A) Effect of exposure to QUIN (Q) (1 m*M* for 20 hours) or GLU (G) (0.5 m*M* for 5 minutes) on numbers of intact neurons (left) and NADPH-d(+) neurons (right) per arbitrarily selected sample area (40 400× fields for the former, 0.125 dish for the latter), relative to controls (C, shaded bars). Cells were counted the day after initiation of appropriate toxic exposure or sham wash procedure (C, controls). Bars depict mean + SEM ($n = 3$) with the control mean in each group normalized to 100. The actual mean number of neurons counted in the control condition was 747 total neurons (left) and 595 NADPH-d(+) neurons (right). (B) Observed percentage of total neurons (per 40 400× field sample area) that stained for NADPH-d under control (C, shaded bar), QUIN exposed (Q), and GLU exposed (G) conditions. Mean (average of observed percentages) + SEM [$n = 3$]. *Significant difference ($P < 0.05$) compared with control (two-tail *t* test with Bonferroni correction to probabilities to account for multiple comparisons).

(Fig. 3A) and QUIN neuroexcitation (Fig. 3B) could be blocked by the selective NMDA antagonist, 2-amino-5-phosphonovalerate (APV) (*21*). In addition, QUIN neuroexcitation at hyperpolarized membrane potentials could be attenuated by the application of 1 m*M* Mg^{2+} (*22*), another characteristic of an NMDA receptor–mediated phenomenon (*23, 24*).

The intrinsic resistance of NADPH-d(+) neurons to QUIN toxicity might therefore be due to their relative lack of membrane NMDA receptors. To test this, we determined the vulnerability of NADPH-d(+) neurons to NMDA itself. Cultures exposed to 0.2 m*M* to 0.5 m*M* NMDA for 5 minutes resembled those exposed to QUIN, showing widespread neuronal loss but sparing of NADPH-d(+) neurons (three of three experiments). In contrast, exposure to 0.1 to 1 m*M* kainate (KAIN) (four of four experiments) or 50 μ*M* quisqualate (QUIS) (two of two experiments), agonists that define other subclasses of excitatory amino acid receptors (*25*), produced little generalized neuronal loss, but nearly total loss of the NADPH-d(+) subpopulation. Neuronal cell counts of a matched set of sister cultures confirmed both the selective resistance of the NADPH-d(+) neurons to NMDA, and the selective vulnerability of the neurons to KAIN and QUIS (Fig. 3C). In this set of cultures, the percentage of NADPH-d(+) cells in the neurons surviving NMDA exposure was significantly increased from the control value of 1.4 ± 0.2% (SEM) to 5.9 ± 0.9%. In comparison, the percentage of NADPH-d(+) neurons was significantly decreased to 0.2 ± 0.1% after QUIS exposure and significantly decreased to 0.1 ± 0.1% after KAIN exposure (Fig. 3D).

These additional observations support the possibility that the selective resistance of NADPH-d(+) neurons to QUIN may be caused by a paucity of membrane NMDA receptors. Resistance to QUIN could be caused by alterations other than a lack of membrane receptors, but such alterations could not as easily be reconciled with the selective vulnerability of NADPH-d(+) neurons to KAIN or QUIS toxicity. This neuronal subpopulation may also have unusually large numbers of KAIN or QUIN receptors—a suggestion supported by in vivo observations that striatal NADPH-d(+) neurons are more vulnerable to KAIN neurotoxicity than GABA-containing or cholinergic neurons (*14, 26*). NADPH-d(+) neurons may thus have an unusual distribution of excitatory amino acid receptors, with a low proportion of the NMDA subtype and a high proportion other excitatory amino acid receptor (non-NMDA) subtypes. The intermediate vulnerability of these neurons to GLU neurotoxicity probably reflects the fact that GLU is a mixed agonist that is active at all three receptor subtypes (*25*), although perhaps relatively weakly at non-NMDA receptors (*17*).

The fact that these neurons were resistant to NMDA, a compound not normally present in the mammalian CNS, as well as to QUIN argues against the possibility that resistance to QUIN might be due to compound-specific metabolic protection by the enzyme NADPH-d itself (*5, 14*). After in vivo injection of NMDA, Beal and colleagues did not see sparing of striatal somatostatin or neuropeptide Y (*14*). This may be because the neurotoxic effect of NMDA in the intact CNS could be contaminated by the neurotoxicity of endogenous neurotransmitters (for example, glutamate) released from depolarized presynaptic pathways to a greater extent than in cell culture (see above). Although some secondary release of transmitters would be expected to occur with QUIN injection as well, QUIN has been shown to be 40 times less potent than NMDA in evoking the release of acetylcholine from striatal slices (*24*), a finding consistent with electrophysiological studies showing that QUIN is less potent as a neuroexcitant than NMDA (*27*).

Our results add support to the proposal of other investigators that QUIN, or another endogenous NMDA agonist, may be the

actual pathogenic agent in HD. Although the NADPH-d(+) cells in our system were derived from cortex and not striatum, substantial neuronal loss also occurs in the cortex in HD (*28*), and cortical NADPH-d(+) neurons are immunoreactive for somatostatin or neuropeptide Y (NPY) as are their striatal counterparts (see above). Furthermore, an examination of brains from 24 HD patients by Beal, Martin, and colleagues indicated that somatostatin and NPY levels in the frontal cortex were increased by almost a third as compared with those in control patients, suggesting that the selective sparing of somatostatin-NPY-NADPH-d(+) neurons is not restricted to the striatum, but also extends to the cortex of patients with HD (*29*).

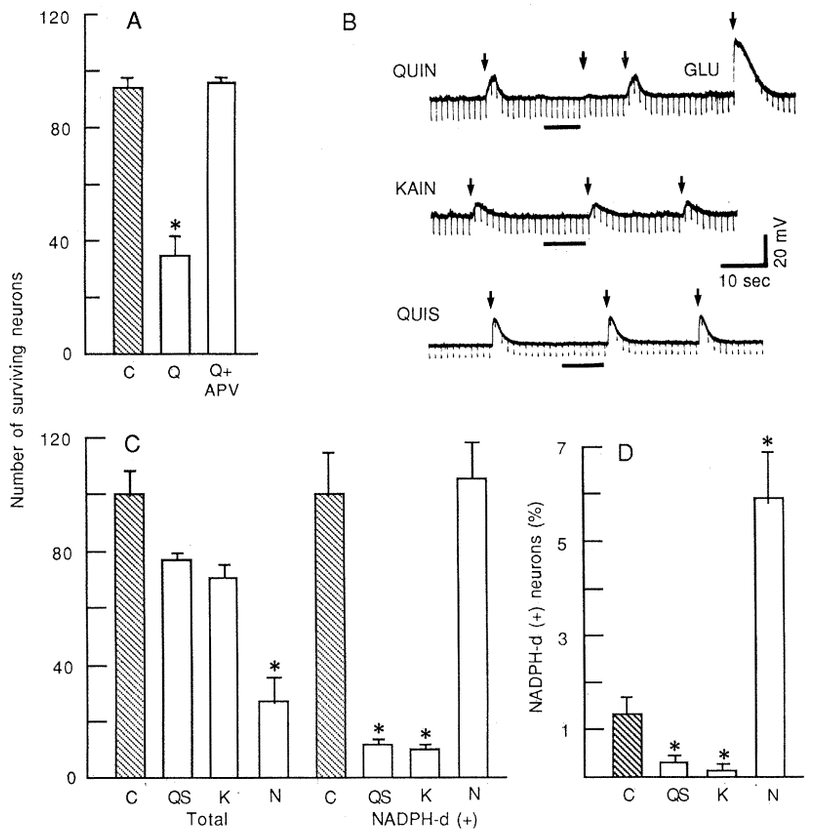

Fig. 3. (A) Blockade of QUIN neurotoxicity by the NMDA receptor antagonist APV. Bars depict the mean number (+ SEM) of intact neurons in several 200× microscope fields (C, $n = 6$; Q, $n = 3$; Q + APV, $n = 6$) after experimental manipulation, normalized to the number of neurons present in those same fields before manipulation (assigned a value of 100). This normalization method differs from that used in Figs. 2, 3C, and 3D—each field is normalized to itself. Even the control condition (shaded bar) shows a mean count slightly less than 100, signifying a small loss of previously identified neurons, probably due to the wash procedure. The chosen fields were relocated with the aid of an objective marker. Dishes exposed to 1 m*M* QUIN for 20 hours (Q) showed a large neuronal loss, whereas addition of 50 μ*M* APV to the QUIN (Q + APV) resulted in neuronal loss at control levels. (B) Selective blockade of QUIN neuroexcitation by APV. Penwriter records show intracellular voltage recordings from three neurons, bathed in the same solution as in Fig. 1 except for the removal of Mg^{2+} (to increase the QUIN responses); downward deflections are produced by the injection of constant current pulses through the recording electrode. Agonists (arrows) and antagonist (horizontal bars) were applied by pressure ejection from separate micropipettes positioned within 10 μm of the soma. The response to 2 m*M* QUIN was consistently and reversibly abolished by 1 m*M* APV and was much smaller than the response elicited by 0.2 m*M* GLU (top trace). On the other hand, responses to 0.1 m*M* KAIN (middle trace) and 0.1 m*M* QUIS (bottom trace) were unaffected by 1 m*M* APV. Irregularity in the records represents spontaneous synaptic activity. (C) Effect of exposure to QUIS (QS) (50 μ*M* for 5 minutes), KAIN (K) (0.1 m*M* for 5 minutes), and NMDA (N) (0.2 m*M* for 5 minutes) on numbers of intact neurons (left) and NADPH-d(+) neurons (right) per chosen sample area (40 400× fields for the former, 0.125 dish for the latter) relative to controls (C, shaded bars). As in Fig 2A, bars depict mean + SEM ($n = 3$) with the control mean in each group normalized to 100. The actual mean number of neurons counted in the control condition was 280 total neurons (left) and 144 NADPH-d(+) neurons (right). Low concentration of QUIS and KAIN (especially QUIS) were intentionally chosen to highlight the selective vulnerability of NADPH-d(+) neurons; higher concentrations produced increased generalized neuronal loss in addition to destroying the NADPH-d(+) population. (D) Percentage of total neurons per 40 400× field sample area that stained for NADPH-d, under the same conditions as (C). Data are expressed as the mean (average of observed percentages) + SEM ($n = 4$). *Significant difference ($P < 0.05$) compared with control (two-tail *t* test with Bonferroni correction to probabilities to account for multiple comparisons).

Detailed study of the cellular characteristics special to NADPH-d(+) neurons may yield additional insights into the pathogenesis of HD, as well as into the pathogenesis of other acute and chronic neurological diseases possibly mediated by endogenous NMDA agonists (*30*).

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