Neuron, Vol. 19, 1271–1283, December, 1997, Copyright ©1997 by Cell Press

Vesicular Transport Regulates Monoamine Storage and Release but Is Not Essential for Amphetamine Action

Edward A. Fon,* Emmanuel N. Pothos,‡
Bao-Cun Sun,‡ Nigel Killeen,†
David Sulzer,‡ and Robert H. Edwards*§
*Graduate Programs in Neuroscience, Cell Biology, and Biomedical Science
Departments of Neurology and Physiology
†Department of Microbiology and Immunology
UCSF School of Medicine
University of California
San Francisco, California 94143
‡Departments of Neurology and Psychiatry
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

To assess the role of exocytotic release in signaling by monoamines, we have disrupted the neuronal vesicular monoamine transporter 2 (*VMAT2*) gene. *VMAT2*$^{-/-}$ mice move little, feed poorly, and die within a few days after birth. Monoamine cell groups and their projections are indistinguishable from those of wild-type littermates, but the brains of mutant mice show a drastic reduction in monoamines. Using midbrain cultures from the mutant animals, amphetamine but not depolarization induces dopamine release. In vivo, amphetamine increases movement, promotes feeding, and prolongs the survival of *VMAT2*$^{-/-}$ animals, indicating that precise, temporally regulated exocytotic release of monoamine is not required for certain complex behaviors. In addition, the brains of *VMAT2* heterozygotes contain substantially lower monoamine levels than those of wild-type littermates, and depolarization induces less dopamine release from heterozygous than from wild-type cultures, suggesting that VMAT2 expression regulates monoamine storage and release.

Introduction

Synaptic transmission mediates the processing of information in the central nervous system and involves the rapid, precise exocytotic release of neurotransmitter (Scheller, 1995; Sudhof, 1995). Although all of the classical transmitters undergo release by a similar exocytotic mechanism, the major inhibitory and excitatory transmitters γ-aminobutyric acid (GABA) and glutamate directly participate in information processing, whereas monoamine transmitters such as dopamine, norepinephrine, and serotonin appear to modulate neural circuits. Similar to other classical transmitters, signaling by monoamines involves two distinct classes of transport activity. One class resides at the plasma membrane and removes transmitter from the extracellular space, thereby terminating its action. These plasma membrane transport activities use the Na^+ gradient across the plasma membrane to drive reuptake (Amara and Kuhar, 1993). In the case of monoamines, cocaine and antidepressants act by inhibiting plasma membrane transport, thereby increasing the synaptic concentration of transmitter.

The other class of transport activity occurs in the membrane of the secretory vesicle and functions to package the transmitter for regulated exocytosis. Indeed, classical transmitters are synthesized in the cytoplasm, requiring transport into the vesicle, and several distinct vesicular transport activities have been identified for monoamines, acetylcholine, GABA, and glutamate (Schuldiner et al., 1995; Liu and Edwards, 1997). In contrast to plasma membrane reuptake, vesicular transport relies on a proton electrochemical gradient and in the case of monoamines involves the exchange of two lumenal protons for one cytoplasmic monoamine (Johnson, 1988). Classic pharmacologic studies have also indicated the potential for changes in vesicular monoamine transport to influence behavior. In contrast to cocaine and antidepressants, which inhibit plasma membrane monoamine transport, reserpine and tetrabenazine inhibit vesicular amine transport. Indeed, reserpine very effectively lowers blood pressure by depleting the pool of stored monoamine. Reserpine also produces a syndrome resembling depression, giving rise to the original hypothesis that monoamine transmitters have a role in affective disorders (Frize, 1954).

The action of amphetamines further indicates the potential for changes in both plasma membrane and vesicular transport to influence transmitter release and behavior. Amphetamines promote flux reversal by plasma membrane monoamine transporters, releasing cytoplasmic transmitter through a nonexocytotic mechanism (Di Chiara and Imperato, 1988; Rudnick and Wall, 1992; Sulzer et al., 1993; Giros et al., 1996). Neural activity can also induce transmitter release by promoting flux reversal through plasma membrane amino acid transporters (Schwartz, 1987; Attwell et al., 1993), suggesting a physiological as well as pharmacological role for this form of nonvesicular release. In addition to their action at the plasma membrane, amphetamines induce monoamine efflux from vesicles (Sulzer and Rayport, 1990; Rudnick and Wall, 1992; Sulzer et al., 1995), increasing the amount of cytoplasmic transmitter available for efflux from the cell. However, the role of a specific transport protein in vesicular efflux and in the action of amphetamines has remained unclear, since reserpine and tetrabenazine do not inhibit the efflux of monoamines from chromaffin granules (Maron et al., 1983). Nonetheless, the actions of both reserpine and amphetamines indicate that interference with vesicular monoamine storage has the potential to influence behavior.

Molecular cloning has recently led to the identification of several proteins involved in vesicular neurotransmitter transport. Selection of transfected cells for resistance to the Parkinsonian neurotoxin 1-methyl-4-phenylpyridinium (MPP^+) yielded a cDNA encoding vesicular monoamine transport, which protects against the toxin by sequestering it inside secretory vesicles and away

§To whom correspondence should be addressed.

from its primary site of action in mitochondria (Liu et al., 1992a, 1992b). The sequence predicts a polytopic membrane protein with twelve transmembrane domains and no similarity to plasma membrane neurotransmitter transporters (Erickson et al., 1992; Liu et al., 1992b). However, the sequence does show both structural and functional similarity to a class of bacterial antibiotic resistance proteins, supporting a role in detoxification as well as signaling. Indeed, the normal neurotransmitter dopamine readily oxidizes to produce free radicals and may contribute to the selective degeneration of dopamine cells that occurs in Parkinson's disease. Vesicular monoamine transport may thus protect against this endogenous toxin as well as exogenous toxins such as MPP^+. The sequence also defined a family of proteins that now includes two vesicular monoamine transporters (Erickson et al., 1992; Liu et al., 1992b) as well as an acetylcholine (ACh) transporter (VAChT; Alfonso et al., 1993). Vesicular monoamine transporter 1 (VMAT1) occurs in nonneural cells of the periphery, whereas VMAT2 occurs in neurons (Weihe et al., 1994; Peter et al., 1995). Biochemical analysis of transport function in transfected cells has also shown that the two VMATs differ in their affinity for substrates, in their pharmacology (Peter et al., 1994), and in their phosphorylation by endogenous kinases (Krantz et al., 1997). Overexpression of VMAT2 in rat pheochromocytoma PC12 cells increases quantal size ~3-fold (Sun et al., unpublished data). Similarly, overexpression of VAChT in cultured Xenopus motor neurons increases quantal size 2- to 3-fold (Song et al., 1997), suggesting that the expression of vesicular transporters determines the filling of synaptic vesicles and the amount of neurotransmitter released.

To characterize the role of vesicular monoamine transport in transmitter release, signaling, and behavior in vivo, we have disrupted the gene encoding the neuronal isoform VMAT2. The mutant mice move little, feed poorly, and do not survive more than a few days after birth. However, amphetamines promote locomotion, feeding, and survival, presumably by circumventing the block to exocytotic monoamine release. The precise mode of monoamine release thus does not appear crucial for certain complex behaviors, consistent with the role of monoamines as neuromodulators. The analysis of *VMAT2* heterozygotes also suggests that the expression of VMAT2 determines the rate of synaptic vesicle filling and the amount of monoamine released.

Results

To understand how vesicular transport influences monoamine release and behavior, we disrupted the *VMAT2* gene in mice. Transmembrane domain (TMD) 1 contains an aspartate required for transport activity (Merickel et al., 1995). Since the first protein-coding exon of the mouse *VMAT2* gene encodes the entire TMD1, we replaced this exon with a selectable marker for resistance to neomycin and introduced the construct, with 1.7 kb genomic DNA flanking the 5′ end of the marker and 4.9 kb flanking the 3′ end (Figure 1A), into 129/SvJ embryonic stem (ES) cells. From 400 stably transfected cell clones resistant to the neomycin analog G418, two showed homologous recombination at the anticipated site in the *VMAT2* gene by Southern analysis using both 5′ (Figure 1B) and 3′ probes (data not shown). Injection of these ES clones into blastocysts yielded multiple chimeras, and these were further bred with C57BL/6 animals to produce *VMAT2* heterozygotes (+/−). The heterozygotes were then mated with each other to produce homozygous (−/−) animals.

The knockout (−/−) mice lack VMAT2 by a variety of criteria. Western analysis using a polyclonal antibody that we have raised to rat VMAT2 (Peter et al., 1995) shows no detectable immunoreactive protein in membranes prepared from the homozygotes (Figure 1C). Heterozygotes contain less immunoreactivity, suggesting a lack of compensation by the remaining normal allele, at least in terms of the level of protein. Other synaptic vesicle proteins such as the vesicular acetylcholine transporter and synaptophysin show no generalized reduction, confirming the specificity of the defect in VMAT2. To determine whether the gene disruption has eliminated vesicular amine transport activity, we measured transport in the knockout animals. Membranes from *VMAT2*$^{-/-}$ mice show the same transport function as membranes from wild-type animals that have been treated with the potent inhibitor reserpine, confirming the loss of VMAT2 activity (Figure 1D). Importantly, the results also indicate a lack of compensation by other transport activities such as VMAT1, which occurs transiently in the brain during development (Hansson et al., 1996, Soc. Neurosci., abstract). *VMAT2*$^{+/-}$ heterozygous mice also show less transport activity than wild-type animals, but the low concentrations of ^{3}H-serotonin used for the assay do not provide a measure of the maximum rate of transport (V_{max}). To quantitate the reduction in VMAT2, we measured binding to tetrabenazine, another potent inhibitor of VMAT2 (Figure 1E). Brain membranes from *VMAT2* homozygotes show no specific binding to ^{3}H-tetrabenazine, whereas heterozygotes show 52% of the binding observed to membranes from wild-type littermates.

Mice homozygous for disruption of the *VMAT2* gene are born from heterozygote crosses at the expected Mendelian ratio of ~1/4, indicating survival through embryonic development. However, *VMAT2*$^{-/-}$ animals show differences from wild-type and heterozygous littermates soon after birth. The homozygous animals are small and move relatively little (Figure 2A). However, they do move vigorously in response to mild pain (e.g., tail pinching), excluding simple paralysis. Homozygous mice feed poorly, with little if any milk visible in their stomachs, do not gain weight (Figure 2B), and die within the first week of life (Figure 2C).

To determine whether the elimination of VMAT2 impairs movement, feeding, and survival by interfering with neural development, we examined the brains of homozygous knockout mice. Although smaller than those of wild-type littermates, the brains of *VMAT2*$^{-/-}$ animals show no gross disturbance in morphology. Staining with cresyl violet also shows normal cell populations in the forebrain and brainstem (data not shown). Since a disturbance in monoamine transmission might have a relatively selective effect on the development of monoamine

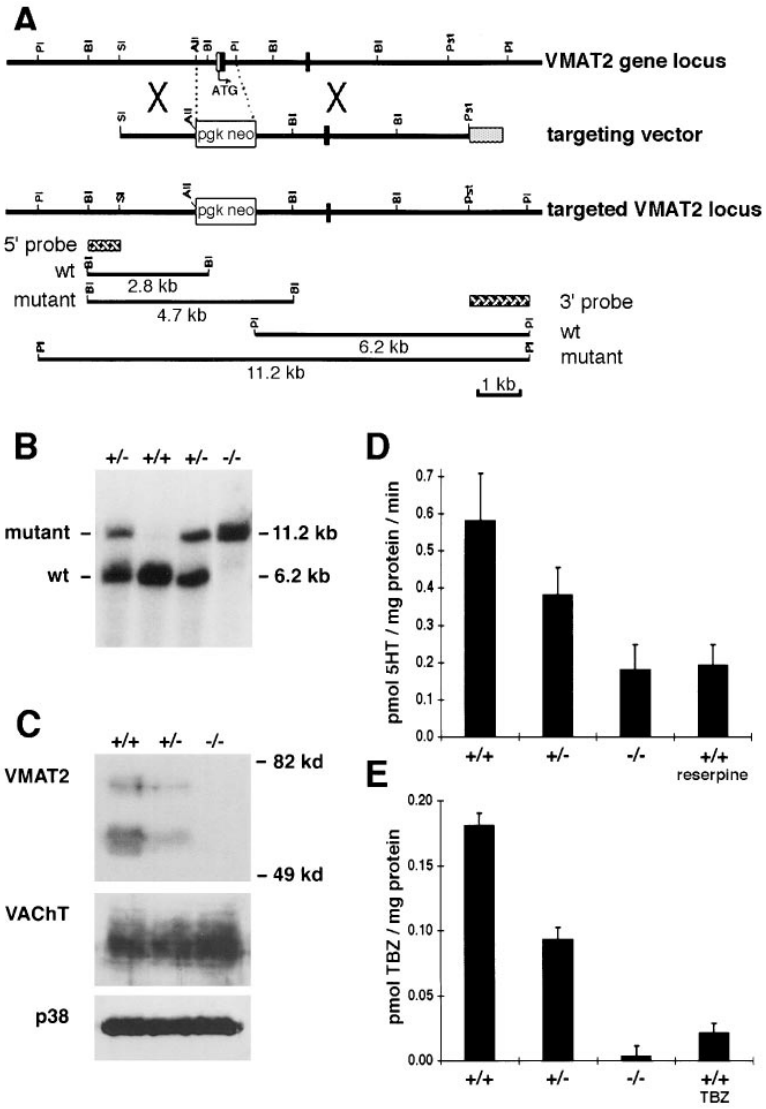

Figure 1. Production of *VMAT2* Mutant Mice

(A) Restriction maps of the native *VMAT2* locus, targeting vector, and recombinant allele. The boxes represent exons, with the open section indicating untranslated sequence and the filled section indicating protein-coding sequence. The arrow marks the site of translation initiation. To construct the targeting vector, the selectable marker for resistance to neomycin *PGK-neo* was used to replace an Aat II-Pflm I restriction fragment that contained the entire first protein-coding exon. The location of 5′ and 3′ probes used for genotype analysis (cross-hatched boxes) are shown along with the predicted size of Bam HI and Pflm I restriction fragments in the wild-type (wt) and mutant alleles. The shaded box indicates pBluescript vector sequences. Restriction sites for Pflm I are indicated by PI, sites for Bam HI by BI, Sac I by SI, Aat II by AII, and Pst I by Pst.

(B) Southern analysis of genomic DNA from the offspring of a cross between two *VMAT2* heterozygous (+/−) mice. Tail genomic DNA was digested with Pflm I, separated by electrophoresis through agarose, blotted to nylon, and hybridized to the 3′ probe described in (A). Both bands occur in the heterozygotes (+/−), whereas only the 6.2 kb wt band appears in the wild-type (+/+) mice and only the 11.2 kb mutant band in the homozygous knockout (−/−) mice. Southern analysis was also performed using digestion with Bam HI and hybridizion with the 5′ probe described in (A) (data not shown). A total of 172 mice born from crosses between *VMAT2* heterozygotes yielded the following genotypes: +/+, 23.8%; +/−, 49.4%; and −/−, 26.7%.

(C) Western analysis of membrane vesicles prepared by differential centrifugation from the brains of wild type (+/+), heterozygous (+/−), and knockout (−/−) mice. Membrane vesicles from the brains of newborn mice (100 μg protein) were separated by electrophoresis through polyacrylamide and transferred to nitrocellulose, and the blot was incubated with the indicated antibodies. Incubation with the VMAT2 antibody shows no detectable immunoreactivity in knockout animals and reduced staining in heterozygous relative to wild-type mice. Staining with the antibodies against two synaptic vesicle proteins, the vesicular acetylcholine transporter (VAChT) and synaptophysin (p38), shows no difference among $VMAT2^{+/+}$, $VMAT2^{+/-}$, and $VMAT2^{-/-}$ animals.

(D) Transport of serotonin into membrane vesicles prepared by differential centrifugation from the brains of $VMAT2^{+/+}$, $VMAT2^{+/-}$, and $VMAT2^{-/-}$ mice (n = 3 for each). Membrane vesicles (50 μg protein) were incubated in 20 nM ^{3}H-serotonin at 29°C for 2 min, a time at which the rate of uptake remains maximal. Membranes from $VMAT2^{-/-}$ animals show the same activity as membranes from wild-type mice treated with 5 μM reserpine. The results are presented as the mean ± standard error of the mean (SEM).

(E) Quantitation of VMAT2 protein by tetrabenazine (TBZ) binding to membrane vesicles prepared by differential centrifugation from the brains of $VMAT2^{+/+}$ (n = 4), $VMAT2^{+/-}$ (n = 4), and $VMAT2^{-/-}$ (n = 3) mice. Membrane vesicles (80 μg protein) were incubated in 5 nM ^{3}H-dihydrotetrabenazine at 29°C for 20 min. The level of binding seen with knockout membranes does not exceed binding to wild-type membranes in the presence of excess unlabeled TBZ (100 μM; n = 4), confirming the absence of VMAT2 protein in the brains of the knockout mice. Binding in the brains of $VMAT2^{+/-}$ animals was approximately half wild-type levels (51.8%; $p < 0.001$). The results are presented as the mean ± SEM.

cell populations, we immunostained for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. Dopamine neurons in the substantia nigra of $VMAT2^{-/-}$ mice (Figure 3B) show no clear difference from those in wild-type animals (Figure 3A). In addition, TH-immunoreactive fibers in the striatum of knockout mice (Figure 3D) show no substantial difference from those in wild-type littermates (Figure 3C). Dopamine islands that form transiently during development (Graybiel et al., 1981; Fishell and van der Kooy, 1987) occur in both wild-type and $VMAT2^{-/-}$ animals. Direct examination of the striatal patch-matrix system (Gerfen, 1992) by immunostaining for the μ opioid receptor also shows no difference between wild-type (Figure 3E) and knockout animals (Figure 3F). Thus, vesicular monoamine transport and hence exocytotic release of monoamines do not appear to be required for normal development of the mesostriatal projection.

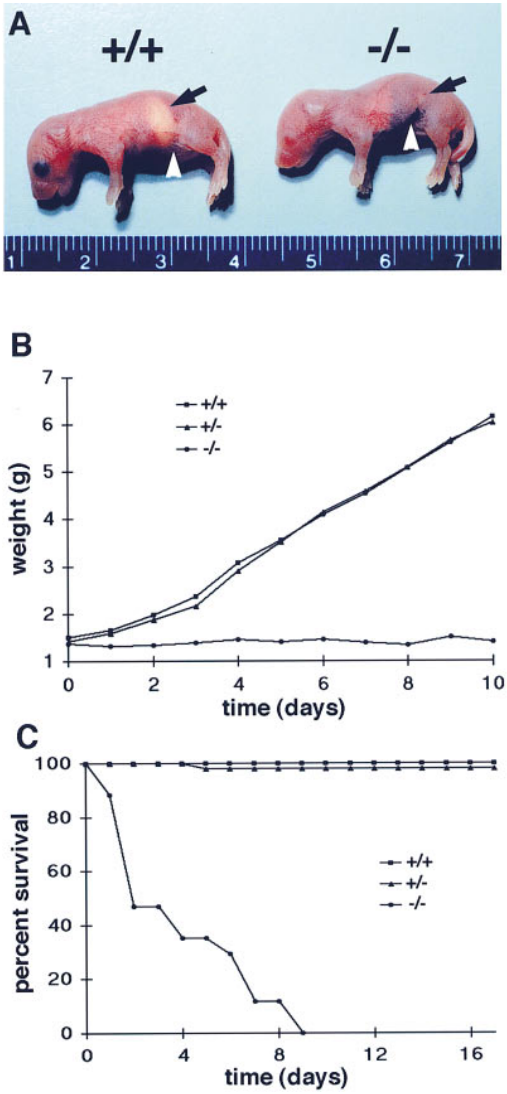

Figure 2. Behavioral Phenotype of *VMAT2* Mutant Mice

(A) Appearance of 1-day-old wild-type and *VMAT2* knockout mice. Compared to *VMAT2*$^{+/+}$ littermates, *VMAT2*$^{-/-}$ mice show little spontaneous movement. Twenty-four hours after the placement of ink marks (white arrowheads) on both animals, the wild-type but not the *VMAT2*$^{-/-}$ mice have rubbed them off. In addition, the mutant mice have little or no milk in their abdomens (black arrows), even after removal of wild-type and heterozygous pups from the litters to decrease competition for feeding.

(B) *VMAT2* knockout mice do not gain weight. Animals from the same litters (+/+, n = 27; +/−, n = 45; and −/−, n = 20) were weighed daily from P0 until the death of all *VMAT2*$^{-/-}$ mice.

(C) *VMAT2* knockout mice show reduced survival. Intact litters were monitored daily for survival (−/−, n = 12; +/−, n = 15; +/+, n = 10). Removal of *VMAT2*$^{+/+}$ and *VMAT2*$^{+/-}$ littermates increased maximum survival only slightly in remaining *VMAT2*$^{-/-}$ mice (to P10 versus P8 in intact litters).

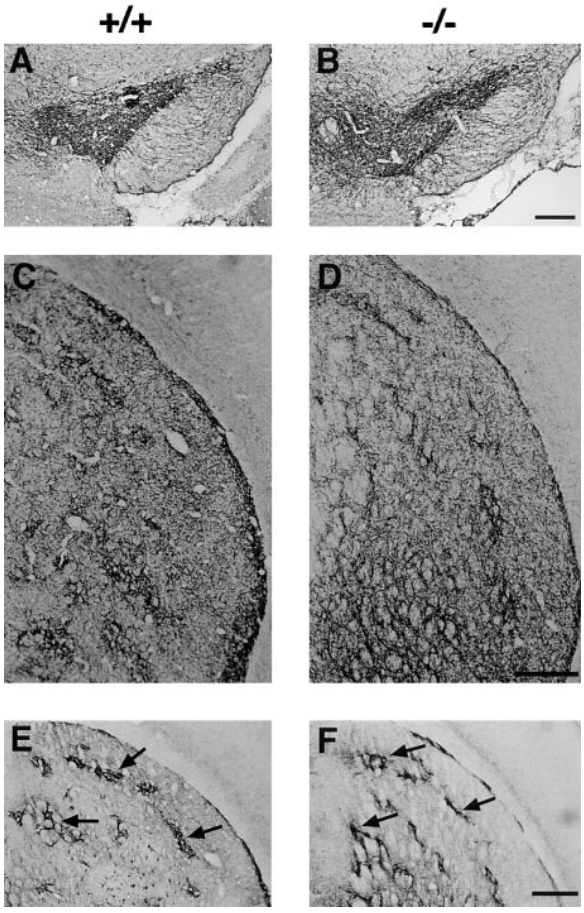

Figure 3. Midbrain Dopamine Neurons and their Striatal Projections in *VMAT2*$^{-/-}$ Mice

Coronal cryostat sections from the midbrain ([A] and [B]) and striatum ([C] through [F]) of wild-type ([A], [C], and [E]) and *VMAT2* knockout ([B], [D], and [F]) mice (n = 3 for each) were stained with antibodies to tyrosine hydroxylase (TH; [A] through [D]) and the μ opioid receptor ([E] and [F]). TH immunohistochemistry did not reveal any major differences between the brains of *VMAT2*$^{+/+}$ and *VMAT2*$^{-/-}$ mice, either in terms of cell bodies ([A] and [B]) or their projections ([C] and [D]). In particular, both wild-type and knockout mice show normal dendritic processes from the pars compacta of the substantia nigra into the pars reticulata ([A] and [B]). Clusters of increased TH staining in the striatum ("dopamine islands") also show no difference between wild-type and knockout mice ([C] and [D]). In addition, immunostaining using an antibody to the μ opioid receptor ([E] and [F]) did not reveal any differences in the striatal patch-matrix system between the wild-type and knockout mice. The arrows indicate patches (striosomes) that are known to stain intensely with this antibody.

Scale bars, 200 μm.

To characterize the defect in monoamine transmission in *VMAT2*$^{-/-}$ mice, we examined brain monoamines directly. Immunostaining for serotonin revealed no detectable reactivity in the cortex of knockout mice (Figure 4B), whereas wild-type animals contain profuse immunoreactive fibers (Figure 4A). However, the cell bodies of serotonergic neurons in the raphe of *VMAT2*$^{-/-}$ homozygotes still contain some immunoreactivity (Figure 4D), albeit considerably reduced from wild type (Figure 4C), suggesting residual accumulation of cytoplasmic serotonin in cell bodies but not processes. We also quantitated brain monoamines directly by high pressure liquid chromatography (HPLC). The brains of homozygous mice show extremely low monoamines (Figure 4E), with dopamine at ~1.5% of wild-type levels, norepinephrine at ~6%, and serotonin at ~1%. The results support the previous impression that an extremely large proportion of brain monoamines in normal animals occurs in the vesicular storage pool. The levels of dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and

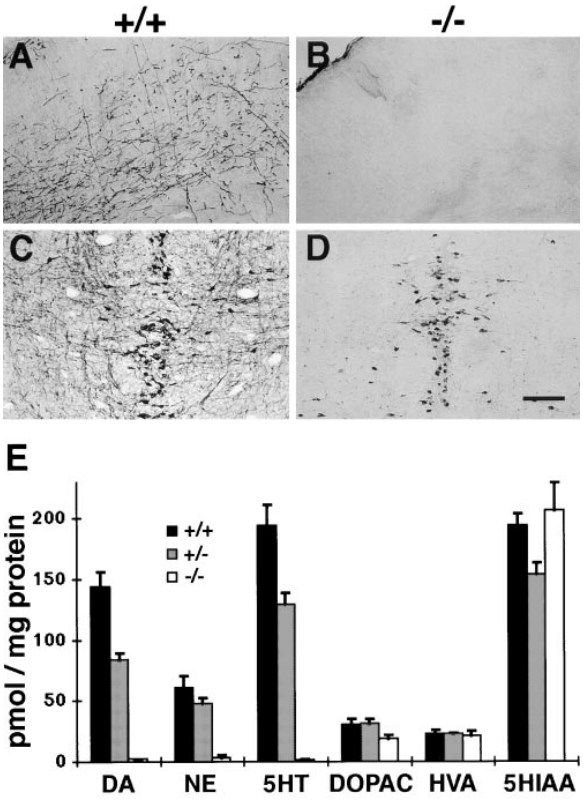

Figure 4. Reduced Brain Monoamine Levels in *VMAT2* Mutant Mice

(A–D) Coronal cryostat sections of the cerebral cortex ([A] and [B]) and the brainstem raphe nucleus ([C] and [D]) in wild-type ([A] and [C]) and *VMAT2* knockout ([B] and [D]) mice (n = 3 for each) were stained with an antibody to serotonin. The cortex of *VMAT2*$^{-/-}$ mice (B) shows no immunoreactivity, whereas the cortex of *VMAT2*$^{+/+}$ mice shows a rich network of reactive serotonergic fibers (A). The only immunoreactive structures in *VMAT2*$^{-/-}$ mice were the cell bodies of serotonergic neurons in the raphe nucleus (D). However, the staining in this region was limited to cell bodies (D) and excluded from processes observed in *VMAT2*$^{+/+}$ mice (C). In addition, the intensity of staining of cell bodies in the raphe of *VMAT2*$^{-/-}$ mice was considerably less than that of *VMAT2*$^{+/+}$ littermates. Scale bar, 100 μm.

(E) Quantitation of monoamine and metabolite levels in *VMAT2* mutant mice. Dopamine (DA), norepinephrine (NE), and serotonin (5-HT) as well as their metabolites, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5-HIAA) were measured in perchloric acid extracts prepared from the brains of newborn *VMAT2*$^{+/+}$ (n = 7), *VMAT2*$^{+/-}$ (n = 14), and *VMAT2*$^{-/-}$ (n = 9) mice using HPLC with electrochemical detection. Relative to the brains of wild-type animals, the brains of knockout mice show drastic reductions in the levels of dopamine (1.5%; $p < 0.0001$), norepinephrine (6%; $p < 0.001$), and serotonin (1%; $p < 0.0001$). In contrast, metabolite levels appear similar in all groups. The brains of the heterozygous mice also show reductions in dopamine (58% of wild-type levels; $p < 0.005$) and 5-HT (66% of wild type levels; $p < 0.01$). The results are presented as mean ± SEM.

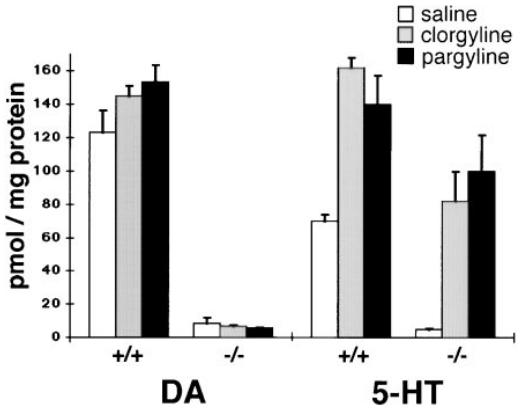

Figure 5. Inhibition of Monoamine Oxidase Increases Serotonin but Not Dopamine in *VMAT2*$^{-/-}$ Mice

Neonatal wild-type (+/+) and knockout (−/−) littermates were injected subcutaneously with either saline, the MAO-A inhibitor clorgyline (10 mg/kg), or the nonselective MAO inhibitor pargyline (75 mg/kg). Two hours after administration, dopamine (DA) and serotonin (5-HT) as well as their metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindolacetic acid (5-HIAA) were measured in perchloric acid extracts prepared from the brains of *VMAT2*$^{+/+}$ (n = 9 for both saline and clorgyline, n = 4 for pargyline) and *VMAT2*$^{-/-}$ (n = 4 for each treatment) mice using HPLC with electrochemical detection. Both clorgyline and pargyline increased brain serotonin in *VMAT2*$^{-/-}$ mice (P = 0.005 each) to levels observed in saline-treated *VMAT2*$^{+/+}$ mice. In contrast, brain dopamine levels do not increase in knockout animals after administration of MAO inhibitors. Metabolism by MAO therefore accounts for the low brain serotonin levels observed in the *VMAT2*$^{-/-}$ mice, whereas additional mechanisms maintain low brain dopamine levels. No major differences were observed for metabolite levels (data not shown). The results are presented as mean ± SEM.

5-hydroxyindolacetic acid (5-HIAA) appear normal in the mutant mice (Figure 4E) despite the extremely low levels of monoamine transmitters, suggesting rapid metabolism of the transmitter that cannot be stored in vesicles.

To assess the role of metabolism in the low monoamine levels of VMAT2-deficient mice, we have used inhibitors of monoamine oxidases (MAO) A and B, the principal enzymes involved in monoamine degradation (Chen and Shih, 1990). Clorgyline selectively inhibits MAO-A, the principal isoform found in newborn mice (Cases et al., 1996). Injection of clorgyline into newborn wild-type mice increases dopamine only slightly but increases serotonin by ~2-fold (Figure 5). Injection into *VMAT2*$^{-/-}$ mice also has little effect on dopamine levels but increases serotonin to wild-type levels. Thus, metabolism by MAO-A accounts for the low levels of serotonin in *VMAT2* knockout mice. To determine whether MAO-B accounts for the low levels of dopamine in *VMAT2* mutant animals, we injected the relatively MAO-B–selective inhibitor pargyline, which also inhibits MAO-A at the high doses that we used (Figure 5). However, similar to clorgyline, pargyline increases only serotonin levels and has little effect on dopamine levels in both wild-type and mutant mice. Interestingly, both clorgyline and pargyline increase the motor activity of knockout mice (data not shown), raising the possibility that dramatic elevations in cytoplasmic transmitter induce release through a nonexocytotic mechanism such as flux reversal by a plasma membrane transporter.

To examine monoamine storage and release directly, we used primary midbrain cultures. Although the knockout mice do not survive for more than a few days after birth, 4-week-old midbrain cultures from these animals show numbers of TH-positive cells at least as great as those from wild-type and heterozygous littermates (Figure 6A). The morphology of dopamine neurons from *VMAT2*$^{-/-}$ homozygotes also appears indistinguishable

from that of cells from normal littermates (Figure 6A). Similar to monoamine levels in the brain, total dopamine levels in the knockout cultures show a dramatic reduction (Figure 6B). To examine exocytotic release from the midbrain cultures, we have used K^+-evoked depolarization for 2 min and measured the dopamine released into the medium. Whereas cultures from wild-type animals show substantial release of dopamine, cultures from the *VMAT2*$^{-/-}$ mice show essentially no release (Figure 6C), confirming the requirement for vesicular transport to enable exocytotic release of transmitter. We have also used the midbrain cultures to determine the dependence of amphetamine action on VMAT2. Exposure to 10 μM D-amphetamine for 30 min induces dopamine release from the midbrain cultures of *VMAT2*$^{-/-}$ mice (Figure 7A), indicating that VMAT2 is not required for the action of this psychostimulant, at least in cells lacking VMAT2. On the other hand, cultures from the knockout animals release only ~35% as much dopamine as cultures from wild-type animals, demonstrating that VMAT2 and hence the vesicular pool nonetheless contribute the majority of dopamine released by amphetamine. Interestingly, the amount of dopamine not released by amphetamine (total minus released) appears to correlate with *VMAT2* gene dosage (Figures 7A and 7B), suggesting that the transport activity also reduces net efflux from vesicles.

Since *VMAT2* gene disruption reduces total brain monoamine and midbrain culture dopamine to <5% of wild-type levels, it is remarkable that amphetamine continues to induce release of greater than one-third as much dopamine from the knockout cultures as from wild-type cultures. Interestingly, amphetamine increases the total amount of dopamine stored in and released from midbrain cultures of wild-type mice by ~2.5-fold relative to cells depolarized with K^+ (Figure 7B), suggesting that flux reversal across the plasma membrane promotes synthesis by reducing the feedback inhibition of cytoplasmic dopamine on TH (Zigmond et al., 1989; Kumer and Vrana, 1996). Strikingly, midbrain cultures from *VMAT2*$^{-/-}$ homozygotes show a ~30-fold increase in total dopamine (stored and released) after exposure to amphetamine relative to K^+-evoked depolarization, presumably because the absence of a storage pool precludes vesicular efflux and, together with plasma membrane efflux, essentially eliminates feedback inhibition on TH. The ability of amphetamine to induce substantial dopamine release from the *VMAT2* knockout cultures also suggests a way to assess the effects of nonvesicular monoamine release in vivo.

Since amphetamine appears able to circumvent the defect in exocytotic monoamine release from *VMAT2* mutant cultures, we determined whether D-amphetamine (10 mg/kg, subcutaneously) would improve the feeding behavior of mutant animals. One hour after injection with D-amphetamine, a large proportion of *VMAT2*$^{-/-}$ animals were either actively suckling or had milk in their stomachs (Figure 8A). In contrast, uninjected or saline-injected mice showed very little feeding. To confirm that amphetamine action involved the nonvesicular release of monoamine through flux reversal mediated by plasma membrane transporters, we also injected the catecholamine transport inhibitor nomifensine. Nomifensine selectively inhibits the reuptake of catecholamines and

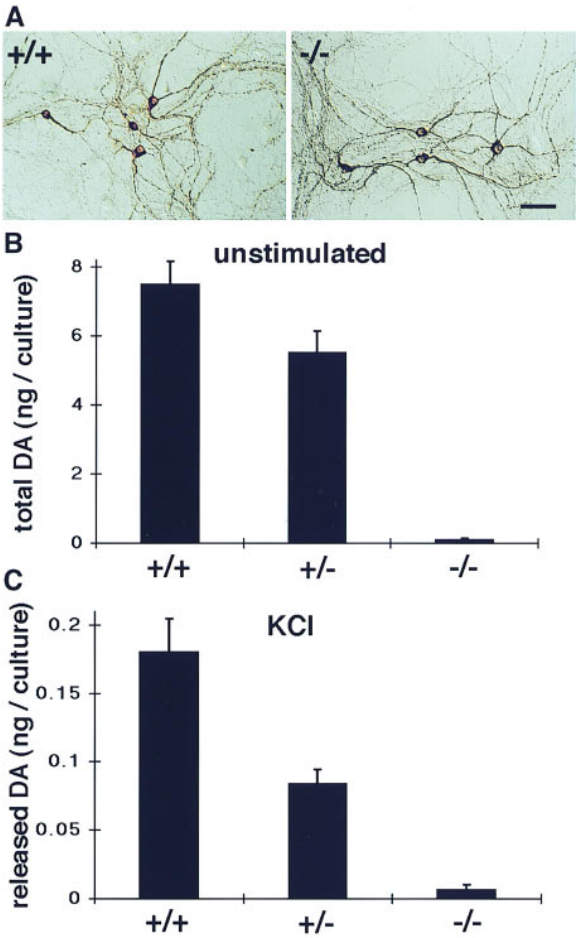

Figure 6. Primary Midbrain Cultures from *VMAT2* Mutant Mice

(A) Dopamine neurons from VMAT2-deficient mice survive in culture. Neurons derived from the midbrain of newborn *VMAT2*$^{+/+}$ and *VMAT2*$^{-/-}$ mice were grown in culture for 4 weeks and immunostained for tyrosine hydroxylase (TH) (Mena et al., 1997). The TH-immunoreactive cells from *VMAT2*$^{-/-}$ mice show no differences in size, morphology, process number, or length from cells derived from *VMAT2*$^{+/+}$ animals. Cultures from *VMAT2*$^{-/-}$ mice contained at least as many TH-positive cells (1277 ± 41; n = 2) as cultures from *VMAT2*$^{+/-}$ (993 ± 278; n = 4) and *VMAT2*$^{+/+}$ (731 ± 247; n = 2) animals. The results are presented as number of cells per plate ± SEM. Scale bar, 50 μm.

(B) Accumulation of dopamine (DA) depends on VMAT2. Primary cultures prepared from the midbrain of *VMAT2*$^{+/+}$ (n = 11), *VMAT2*$^{+/-}$ (n = 9), and *VMAT2*$^{-/-}$ (n = 6) mice were maintained in culture for 4 weeks. The sum of cellular and extracellular medium dopamine, as measured by HPLC coupled with electrochemical detection, was used to determine total dopamine in unstimulated cultures. Cultures from knockout mice show a marked reduction in total dopamine, containing 1.7% the levels of wild-type cultures ($p < 0.00001$). Cultures from heterozygous mice contain 73.6% the dopamine of wild-type cultures ($p < 0.05$). The results are presented as mean ± SEM.

(C) Depolarization-evoked release of dopamine (DA) depends on VMAT2. Primary cultures prepared from the midbrains of *VMAT2*$^{+/+}$, *VMAT2*$^{+/-}$, and *VMAT2*$^{-/-}$ mice (n = 4 for each) were maintained in culture for 4 weeks and stimulated with 40 mM KCl for 2 min, and the dopamine (DA) released into the medium was measured by HPLC. Cultures from knockout mice release only 3.9% of the dopamine released by wild-type cultures ($p < 0.005$). Cultures from heterozygous mice also release less dopamine than wild-type cultures in response to KCl-evoked depolarization (46.8%; $p < 0.01$). The difference between wild-type and heterozygous cultures in terms of depolarization-evoked dopamine release thus exceeds the difference in dopamine levels between these cultures when not stimulated (73.6%; Figure 6B). The results are presented as mean ± SEM.

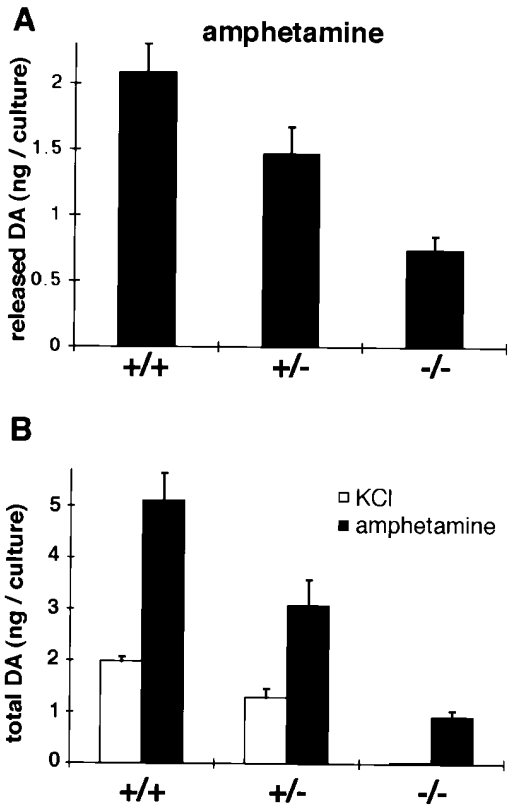

Figure 7. Amphetamine Induces Release in the Absence of Vesicular Transport

(A) Amphetamine induces release in cultures from *VMAT2*$^{-/-}$ mice. Primary cultures prepared from the midbrains of *VMAT2*$^{+/+}$, *VMAT2*$^{+/-}$, and *VMAT2*$^{-/-}$ mice (n = 4 for each) were maintained in culture for 4 weeks and stimulated with 10 μM D-amphetamine for 30 min, and the dopamine (DA) released into the medium was measured by HPLC. Amphetamine induces the release of 35.8% as much dopamine in the *VMAT2*$^{-/-}$ as in *VMAT2*$^{+/+}$ cultures ($p < 0.001$). The results are shown as the mean ± SEM.

(B) Amphetamine produces a greater increase in dopamine levels in cultures from *VMAT2*$^{-/-}$ mice than in those from *VMAT2*$^{+/+}$ or *VMAT2*$^{+/-}$ animals. Total dopamine was measured by adding the values for intracellular dopamine to the values for released dopamine in 4-week-old primary cultures generated from the midbrains of knockout, heterozygous, and homozygous mice (n = 4 for each) after stimulation with either 40 mM KCl or 10 μM D-amphetamine. Amphetamine increases dopamine levels 30-fold more than stimulation with KCl in the knockout cultures, but only ~2.5-fold more than KCl in the wild-type and heterozygous cultures. The results are presented as mean ± SEM.

strongly reduces the effect of amphetamine on feeding (Figure 8A). Thus, the nonvesicular release of catecholamine promoted by amphetamine can sustain certain complex behaviors in the absence of exocytotic release. Since poor feeding and reduced growth presumably account for the failure of *VMAT2*$^{-/-}$ mice to survive, we then injected the knockout mice with the same dose of D-amphetamine three times a day. As shown in Figure 8B, knockout animals injected with amphetamine survive substantially longer than knockout mice injected with saline. Indeed, they move more, show more directed movements such as crawling in the first week and walking in the third week (see supplemental video at http://www.neuron.org/supplemental/19/6/1271), feed better, and increase slightly in weight. Although they never attain the same weight as wild-type or heterozygous littermates and eventually die, amphetamine injection clearly increases the survival of *VMAT2*$^{-/-}$ animals into the third week after birth. Amphetamine can thus circumvent the defect in vesicular release of monoamine transmitters and promote complex behavioral phenomena such as locomotion and feeding, but it does not restore the knockout mice to normal.

We have also analyzed *VMAT2*$^{+/-}$ heterozygotes to determine how the level of transporter expression influences transmitter release and behavior. The heterozygotes appear indistinguishable from wild-type littermates throughout the period of observation. Indeed, they move vigorously, feed well, and grow normally (Figures 2B and 2C). However, *VMAT2*$^{+/-}$ heterozygotes contain 58% as much dopamine, 77% as much norepinephrine, and 66% as much serotonin as wild-type animals (Figure 4E), indicating that a 50% reduction in VMAT2 protein expression produces a large reduction in monoamine stores. VMAT2 thus regulates the amount of neurotransmitter stored in vivo. In addition, midbrain cultures from heterozygous mice contain only ~75% as much dopamine as cultures from wild-type littermates and release 47% as much dopamine in response to depolarization for 2 min with elevated K^+ (Figures 6B and 6C). The regulated release of dopamine during continuous stimulation thus depends on VMAT2 expression, suggesting that VMAT2 determines the filling of recycling synaptic vesicles. If VMAT2 regulates the rate of refilling, this would predict that changes in neural activity and hence vesicle cycling would alter the amount of transmitter stored per vesicle.

Discussion

Disruption of the *VMAT2* gene in mice has dramatic consequences for the survival of the organism. Despite transient expression of the nonneural isoform VMAT1 in neurons during embryonic development (Hansson et al., 1996, Soc. Neurosci., abstract), *VMAT2*$^{-/-}$ mice show no vesicular monoamine transport activity in the brain as early as 1 day after birth. In addition, *VMAT2*$^{+/-}$ animals show little evidence of compensation by the intact allele. The brains of heterozygous mice contain substantially less VMAT2 protein than wild-type mice by Western analysis and ~50% as much binding to ^{3}H-tetrabenazine. The failure to observe either compensatory upregulation of VMAT1 in the absence of VMAT2 or of the remaining wild-type allele in the heterozygotes suggests that cell identity rather than monoamine neurotransmission determine both the isoform and level of VMAT expressed.

The absence of exocytotic monoamine release in *VMAT2*$^{-/-}$ mice has no discernible effect on brain development. The animals are born in the expected Mendelian proportions from heterozygote crosses and standard histological stains reveal no major abnormality in brain structure. Further, immunostaining of the brainstem for TH shows abundant catecholamine neurons with normal morphology. Mesostriatal dopamine projections also

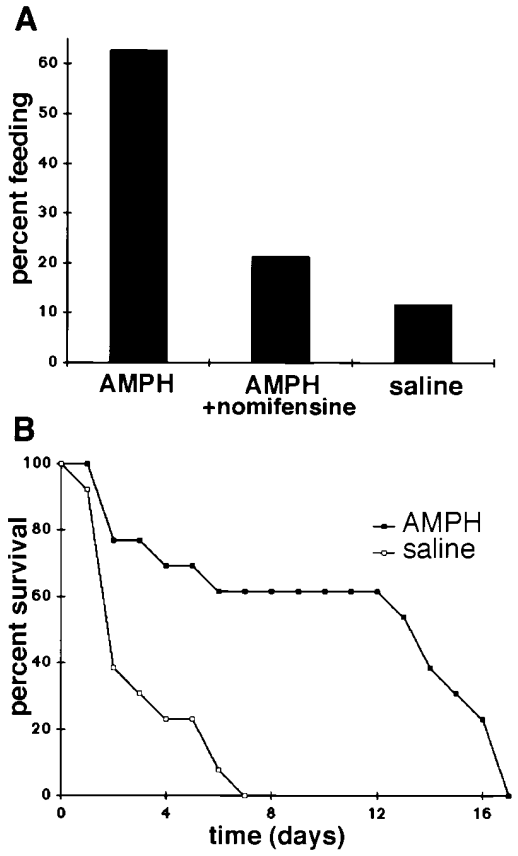

Figure 8. Behavioral Effects of Amphetamine in VMAT2-Deficient Mice

(A) Acute effect of amphetamine (AMPH) on feeding behavior in *VMAT2*$^{-/-}$ mice. D-amphetamine (10 mg/kg, subcutaneously), D-amphetamine coadministered with the plasma membrane uptake inhibitor nomifensine (10 mg/kg), and normal saline were alternately administered to neonatal *VMAT2*$^{-/-}$ mice (n = 28). The injections were initiated within 24 hr after birth and repeated twice daily for up to 7 days after birth. One hour after administration, the mice were scored for either active suckling or the presence of milk in the stomach. Feeding behavior was greatly enhanced by amphetamine compared to saline. This increased feeding was blocked by the specific plasma membrane catecholamine transport inhibitor nomifensine.

(B) Amphetamine increases survival in VMAT2-deficient mice. At birth, *VMAT2*$^{-/-}$ mice were alternately assigned to receive either D-amphetamine (AMPH; 10 mg/kg, subcutaneously; n = 13) or normal saline (n = 13), both administered three times per day. Half the mice that had received amphetamine survived to the third week after birth, whereas none of the mice who had received saline survived past the first week. Mean survival also increased from 3.2 ± 0.5 days in the saline-treated to 10.7 ± 1.8 days in the amphetamine-treated animals ($p < 0.001$). Amphetamine increased spontaneous activity and weight as well, with saline-treated mice reaching 1.36 ± 0.05 g, whereas amphetamine-treated littermates reached 2.26 ± 0.23 g ($p < 0.001$).

appear normal, including the presence of dopamine islands (Graybiel et al., 1981; Fishell and van der Kooy, 1987) and μ opioid receptor-positive patches (Gerfen, 1992). Although these structures presumably reflect finely tuned mechanisms that govern the development of striatal circuitry, the absence of dopamine release has no effect on their appearance, strongly suggesting their independence of monoamine neurotransmission, unless VMAT1 compensates for the absence of VMAT2 during a critical period early in embryonic development (Hansson et al., 1996, Soc. Neurosci., abstract). Consistent with the observed independence from vesicular monoamine transport, dopamine-deficient mice also show normal development of dopamine neurons and their striatal projections (Zhou and Palmiter, 1996). The formation of monoamine connections may thus depend either on signaling by transmitters other than monoamines or on intrinsic developmental mechanisms.

Although the brain appears to develop normally in *VMAT2* mutant mice, the animals survive for only a few days after birth. Nonetheless, they still live longer than mice deficient in TH that cannot produce catecholamines and mice deficient in dopamine β-hydroxylase that cannot produce norepinephrine, which largely die in utero. (Thomas et al., 1995; Zhou et al., 1995). Since elimination of vesicular transport also interferes with the release of catecholamines, the ability of *VMAT2* mutants to survive past birth suggests that these transmitters are released through a different mechanism, possibly through VMAT1 expression, which persists into adulthood in nonneural cells such as chromaffin cells of the adrenal medulla (Weihe et al., 1994; Peter et al., 1995). In contrast, the biosynthetic enzymes occur as only a single isoform, resulting in catecholamine dysfunction in both neural and nonneural tissues of knockout mice. Indeed, the embryonic lethality of knockouts in the biosynthetic enzymes may result from a disturbance in the peripheral cardiovascular system (Thomas et al., 1995; Zhou et al., 1995) rather than the brain. On the other hand, *VMAT2*$^{-/-}$ animals do not survive as long as dopamine-deficient mice (Zhou and Palmiter, 1996). The behavioral deficit in *VMAT2* mutant mice resembles that observed at a slightly greater age in dopamine-deficient animals, with reduced movement and feeding behavior, supporting the results of classic pharmacological studies showing that dopamine stimulates locomotion and mediates the rewarding action of food (Wise, 1990; Koob, 1992). However, the requirement of VMAT2 function for norepinephrine and serotonin as well as dopamine release from neural cells may account for onset of the syndrome immediately after birth in the *VMAT2* mutants rather than at 2 weeks as in the dopamine-deficient mice.

The dramatic reduction in brain monoamines observed in VMAT2-deficient animals reflects the absence of a storage pool and supports previous work suggesting that the bulk of brain transmitter resides in synaptic vesicles. Nonetheless, the level of monoamine metabolites remains similar to that of wild-type animals. The relatively high level of metabolites suggests that the failure to package transmitter into synaptic vesicles increases the time spent in the cytoplasm and hence the accessibility to enzymes such as monoamine oxidase (Napolitano et al., 1995). Indeed, inhibition of MAO-A increases serotonin levels dramatically in *VMAT2* knockout mice, indicating a central role for MAO-A in the metabolism of serotonin. The increased immunoreactive

serotonin in cell bodies but not processes of raphe neurons in untreated *VMAT2*$^{-/-}$ mice may result from reduced metabolism at this site (Westlund et al., 1988; Saura et al., 1992) or by less prominent feedback inhibition of tryptophan hydroxylase (Boadle-Biber, 1993). However, dopamine levels do not increase even after inhibition of both MAO-A and -B, suggesting that additional mechanisms prevent accumulation. The absence of an increase in metabolites in *VMAT2*$^{-/-}$ mice over wild type suggests a down-regulation of biosynthesis due to feedback inhibition of TH (Spector et al., 1967), although an overall increase in metabolic flux through the pathway is also possible. Indeed, the increased cytoplasmic concentrations of monoamine may promote nonvesicular release by the reversal of plasma membrane transporters even in the absence of drugs such as amphetamine. The increased motor activity observed after administration of the MAO inhibitors supports this possibility.

A variety of conditions such as disruption of the plasma membrane dopamine transporter dramatically reduce brain monoamines but do not impair survival (Giros et al., 1996), suggesting that VMAT2 disruption blocks monoamine release as well as reduces stores. Using primary midbrain cultures, K^+-evoked depolarization induces release of dopamine from wild-type but not from homozygous mutant cultures, confirming the requirement of vesicular transport for exocytotic release. Amphetamine continues to induce dopamine release from mutant cultures, indicating that the expression of VMAT2 and the presence of vesicular stores is not required for this in vitro measure of drug action, at least in VMAT2-deficient cells. (In wild-type cells, VMAT2 may be essential to promote vesicular efflux, increase cytoplasmic monoamine, and so promote efflux across the plasma membrane.) In cells lacking VMAT2, flux reversal by plasma membrane monoamine transporters apparently suffices to mediate the release of monoamines by amphetamine, consistent with both pharmacologic and genetic studies (Di Chiara and Imperato, 1988; Rudnick and Wall, 1992; Sulzer et al., 1993; Giros et al., 1996). Nonetheless, the amount of dopamine released by amphetamine from the mutant cultures was ~35% that released from wild-type cultures, indicating that the majority of dopamine is released from the vesicular compartment, at least under normal conditions (Florin et al., 1995; Sulzer et al., 1996). The observation that the amount of dopamine not released by amphetamine also correlates with *VMAT2* gene dosage may indicate that VMAT2 actually opposes the action of amphetamine, although residual lumenal dopamine bound to a matrix inside the vesicle could limit vesicular efflux in wild-type and to a lesser extent heterozygous mice.

In light of the drastically reduced dopamine stores observed in both the brains and cultures of *VMAT2*$^{-/-}$ mice, the ability of amphetamine to release relatively large amounts of dopamine is remarkable. Midbrain cultures from both wild-type and mutant mice show an increase in dopamine after exposure to amphetamine relative to depolarization. However, the increase in mutant cultures is 10-fold greater than the increase in wild-type cultures, apparently accounting for the ability of amphetamine to induce substantial dopamine release even in the absence of vesicular stores. Amphetamine thus appears to have a greater effect on dopamine synthesis when the vesicular store of transmitter is depleted, possibly because the reduced efflux from vesicles together with increased efflux across the plasma membrane reduces cytoplasmic concentrations to extremely low levels and essentially eliminates feedback inhibition on the rate-limiting biosynthetic enzyme TH (Zigmond et al., 1989; Kumer and Vrana, 1996). In addition, the absence of vesicular monoamine transport may cause the accumulation of cytoplasmic transmitter to levels that promote efflux across the plasma membrane. Amphetamine action may thus require vesicular stores and hence VMAT2 function in wild-type animals to elevate cytoplasmic monoamines and promote efflux. Nonetheless, the extraordinary ability of amphetamines to induce release even in the absence of vesicular stores may account for persistent effects even after repeated administration and hence underlie their potential for abuse (Robinson, 1991; Seiden et al., 1993; Pierce and Kalivas, 1995; Melega et al., 1995).

The ability of amphetamine to induce dopamine release from VMAT2-deficient neurons also enabled us to assess the biological effects of nonvesicular release. Administration of L-DOPA to patients with Parkinson's disease has suggested that tonic rather than phasic release of dopamine may suffice to preserve motor control (Birkmayer and Hornykiewicz, 1961; Cotzias et al., 1969). However, the dopamine derived from exogenous L-DOPA presumably enters the remaining dopamine terminals from which it undergoes exocytotic release (Pothos et al., 1996). The progressive loss of dopamine neurons in the disease and the reduced ability to store and release dopamine in a regulated manner may indeed underlie the eventual failure of L-DOPA replacement therapy (Brannan et al., 1996). To determine whether tonic monoamine release can support complex behavioral phenomena, we took advantage of the ability of amphetamine to induce nonvesicular monoamine release in the absence of VMAT2. Systemic administration to newborn *VMAT2*$^{-/-}$ mice increases directed locomotor behavior, including crawling in the first few days after birth and walking in the third week. In addition, amphetamine increases suckling and the presence of milk in the abdomen. The catecholamine reuptake inhibitor nomifensine blocks these phenomena, indicating that nonvesicular release through a plasma membrane transporter can induce certain complex behaviors and phasic release is not required for these phenomena. The ability of nomifensine to block amphetamine stimulation of these behaviors also strongly suggests that they are mediated by catecholamines. Clorgyline stimulates similar behaviors to amphetamine but elevates only serotonin. However, catecholamine efflux induced by clorgyline may not result in a net increase in steady-state levels due to metabolism unrelated to MAO.

Although amphetamine prolongs the survival of *VMAT2* mutant mice, they still do not grow normally and die in the third week of life. The inability of amphetamine to rescue the VMAT2-deficient phenotype completely may result from insufficiently frequent administration,

reduced efficacy with repeated use, or the inadequacy of nonvesicular monoamine release to sustain more complex behaviors required for survival beyond 2–3 weeks of age. It also remains unclear whether amphetamine improves viability by releasing monoamines from central or peripheral sympathetic stores. Nonetheless, the ability to prolong survival of *VMAT2*$^{-/-}$ animals into the third week after birth should enable analysis of such postnatal developmental events as barrel field formation, in which the plasma membrane serotonin transporter and VMAT2 have both been implicated (Lebrand et al., 1996).

In addition to its essential role in exocytotic release, the level of VMAT2 expression influences the amount of monoamine stored and released. Although the *VMAT2* heterozygotes appear normal in behavior, growth and survival, their brains contain substantially less dopamine and serotonin than wild-type animals. VMAT2 thus determines the storage of these transmitters. If synaptic vesicles have adequate time to fill with neurotransmitter, the level of VMAT expression should make little difference to the amount of transmitter stored at equilibrium. However, if synaptic vesicles do not have adequate time to fill, VMAT2 expression would influence the amount of transmitter stored.

Recent studies have demonstrated rapid recycling of synaptic vesicles within 1–2 min after exocytosis (Ryan et al., 1993; Lagnado et al., 1996). If each synaptic vesicle contains ~5000 molecules of serotonin (Bruns and Jahn, 1995), it would take a single VMAT2 protein with a turnover number of 5/s at 29°C (Peter et al., 1994) ~15 min to fill the vesicle at saturating concentrations of transmitter. Although the rate would be greater at 37°C, and the turnover of dopamine by VMAT2 is faster than that of serotonin (Finn and Edwards, 1997), the filling would still take several minutes at saturating substrate concentrations. In addition, the cytoplasmic concentration of monoamines appears considerably lower than the K_m for transport (Perlman and Sheard, 1982), making the time required to fill the vesicle considerably longer. Indeed, the release of 47% as much dopamine by heterozygote *VMAT2*$^{+/-}$ cultures as by wild type suggests that synaptic vesicles do not have adequate time to fill.

Unstimulated midbrain cultures show little spontaneous activity and hence should allow sufficient time for vesicle filling. It is therefore surprising that heterozygote cultures contain only ~75% as much dopamine as wild type. This raises the possibility that VMAT2 expression does not influence solely the rate of filling but also the number of vesicles that can be filled. The VMATs are expressed at low levels relative to other synaptic vesicle proteins and may not occur in all synaptic vesicles. Changing the level of expression may thus alter the number of vesicles available for release as well as the amount of transmitter per vesicle. The level of norepinephrine in the brains of heterozygous mice approaches that of wild-type animals, suggesting that VMAT2 may normally occur at higher levels in noradrenergic neurons, so that heterozygous levels still suffice to ensure adequate rates of refilling or at least one transporter per vesicle. Alternatively, lower levels of activity in these cells may not require high rates of vesicle recycling and hence refilling.

These results support recent studies of vesicular acetylcholine transport in a culture model of the neuromuscular junction. In this system, elevated expression of VAChT increased quantal size (Song et al., 1997), presumably because this protein also influences the rate of synaptic vesicle filling. The expression of vesicular neurotransmitter transport proteins thus limits vesicle filling in the presence of wild-type as well as reduced transporter expression. This predicts that changes in activity and hence vesicle cycling will influence the amount of transmitter stored per vesicle. Elevated VAChT expression also increased the frequency of release (Song et al., 1997), possibly because the larger quantal size facilitated detection. However, increased VAChT may also recruit synaptic vesicles not previously capable of storing neurotransmitter, a possibility supported by the observation that unstimulated wild-type midbrain cultures store substantially more dopamine than *VMAT2* heterozygote cultures even though the unstimulated state should permit greatly increased time for vesicle refilling.

In summary, the results show that VMAT2 is essential for exocytotic monoamine release but not release induced by amphetamine, at least in the absence of vesicular monoamine stores. Indeed, amphetamine partially rescues the phenotype of VMAT2-deficient mice, indicating that nonvesicular release suffices for behavior dependent on monoamines, consistent with their role as neuromodulators. The analysis of heterozygous mice also demonstrates that the level of VMAT2 expression influences the storage and release of monoamines, presumably because the rate of synaptic vesicle recycling exceeds the rate of refilling with transmitter but possibly because transporter expression also determines the number of vesicles capable of storing transmitter.

Experimental Procedures

Production of *VMAT2* Mutant Mice

Using the rat *VMAT2* cDNA as a probe for hybridization, several phage clones were isolated from a mouse 129/SvJ strain genomic library. Restriction and Southern analysis identified a 7.5 kb fragment containing the first and second protein-coding exons. After subcloning into pBluescript (Stratagene), a 0.9 kb Aat II-Pflm I fragment containing the entire first protein-coding exon was excised and replaced with a 1.3 kb selectable marker for resistance to neomycin (*PGK-neo*). The resulting 11 kb targeting vector thus contained the inserted *PGK-neo* cassette flanked on the 5′ side by 1.7 kb and on the 3′ side by 4.9 kb of genomic *VMAT2* sequence.

Strain 129/SvJ ES cells were grown in the presence of leukemia inhibitory factor (LIF) on a monolayer of neomycin-resistant, γ-irradiated, mouse primary embryonic fibroblasts. The targeting vector was linearized by digestion with Sal I, and 20 μg DNA was introduced into 2×10^7 ES cells by electroporation at 250 V and 500 μF. After selection for 10 days in the neomycin analog G418 (effective dose, 250 μg/ml), resistant colonies were picked and transferred to microtiter plates. The colonies were grown for an additional 4 days and then split into duplicate plates, with one used to prepare genomic DNA and the other to propagate the clones. Southern analysis of genomic DNA digested with Pflm I and hybridized with a 1.3 kb Pst I-Pflm I fragment from genomic DNA flanking the 3′ end of the targeting vector identified 2 out of 400 G418-resistant colonies with an enlarged 11.2 kb Pflm I fragment as well as the wild-type 6.2 kb allele. Southern analysis with a probe flanking the 5′ end of the targeting vector confirmed the presence of homologous recombination in these two cell clones (data not shown).

Both ES cell clones demonstrating homologous recombination at

the *VMAT2* locus were microinjected into C57BL/6 blastocysts. Male chimeras derived from these blastocysts were mated with C57BL/6 females and germline transmission was assessed by coat color. Tail genomic DNA was used to genotype the agouti offspring, either by Southern analysis using the 3′ probe described above or by PCR. One pair of primers (5′-CATCGTGTTCCTCGCGCTGC-3′ and 5′-GGGATGCTGTCACCTGGG-3′) was designed to amplify a 181 bp fragment in the wild-type but not the mutant allele, whereas a second primer pair (5′-CCGCTCCCGATTCGCAGCG-3′ and 5′-GCAGCAGC TTAGCACACTGG-3′) was designed to amplify a 292 bp fragment in the mutant but not the wild-type allele. Heterozygous mice were then mated to generate homozygous mutant mice.

Western Analysis

To prepare membrane vesicles, the brains of newborn mice were dissected on ice, frozen immediately, and stored in liquid nitrogen. Using a chilled porcelain mortar and pestle, the brains were ground to a fine powder and dissolved in ~5 ml cold 0.32 M sucrose, 10 mM HEPES-KOH (pH 7.4; SH buffer) containing 5 mM Mg EGTA, 0.4 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2 μg/ml pepstatin A, 2 μg/ml E64, and 2 μg/ml aprotinin (homogenization buffer). The samples were then disrupted with a Teflon homogenizer and the debris sedimented at 1600 × g for 10 min at 4°C. Membrane vesicles in the supernatant were then collected by sedimentation at 175,000 × g for 1 hr at 4°C. The pellet was resuspended in ~200 μl homogenization buffer and either used immediately or stored at −70°C. The protein concentration of the sample was determined using the Bradford assay (Bio-Rad).

For Western analysis, 100 μg membrane protein extracted from the brains of wild-type, heterozygous, and homozygous mice was separated by electrophoresis through 10% polyacrylamide and transferred to nitrocellulose. The blots were then incubated with a polyclonal, affinity-purified, anti-peptide antibody (at a dilution of 1:1000) that we have previously raised to rat VMAT2 (Peter et al., 1995). Since the antibody recognizes the C terminus of VMAT2, it has the potential to detect forms of the protein truncated at the N terminus that might persist despite the gene disruption. As a control, the blots were also incubated with a polyclonal antibody to the rat vesicular acetylcholine transporter (Gilmor et al., 1996) and with a monoclonal antibody to rat synaptophysin (p38; Sigma), both diluted 1:5000. After incubation with the primary antibody, the blots were incubated with the appropriate secondary antibodies conjugated to peroxidase and the deposits visualized by enhanced chemiluminescence (Amersham).

Transport Measurement

Transport assays were initiated by adding membrane extracts (50 μg protein) to 200 μl SH buffer containing 4 mM KCl, 2.5 mM $MgSO_4$, 4 mM ATP, and 20 nM ^{3}H-serotonin (DuPont NEN), either with or without 5 μM reserpine. The reactions were incubated at 29°C for varying time intervals and terminated by dilution into 3 ml cold SH buffer followed by filtration through Supor 200 membranes (Gelman). Filters were dried and the bound radioactivity measured by scintillation counting in 2.5 ml of cytoscint (ICN). Background uptake was determined at 0°C for 0 min. Transport activity was calculated using a 2 min time point during which the rate of uptake remained linear (data not shown). The activity was measured using at least three different membrane preparations for each genotype.

Tetrabenazine Binding

Tetrabenazine binding was initiated by adding membrane extracts (80 μg protein) to 200 μl SH buffer containing 5 nM ^{3}H-dihydrotetrabenazine (Amersham), either with or without excess unlabeled tetrabenazine (100 μM). The reactions were incubated at 29°C for 20 min and terminated by dilution into 4 ml cold SH buffer containing 125 μM unlabeled tetrabenazine, followed by filtration through Supor 200 membranes (Gelman). Filters were dried and the bound radioactivity measured by scintillation counting in 2.5 ml of cytoscint (ICN). Background binding was determined using the reaction solution alone without added membrane extracts. Tetrabenazine binding was measured using at least three different membrane preparations for each genotype.

Histology

Mice aged 0–7 days after birth were anesthetized and perfused for 15 min with cold 4% paraformaldehyde–4% sucrose in 0.1 M phosphate buffer (pH 7.4). The brains were then dissected and immersed in the same fixative overnight at 4°C, cryoprotected in increasing concentrations of sucrose (10%, 20% and 30%), frozen in O. C. T. (Tissue-Tek), and sectioned at 15–30 μm using a cryostat. All histologic analysis involved comparison of *VMAT2*$^{-/-}$ mice with wild-type (+/+) mice from the same litter.

For immunocytochemistry, brain sections were rinsed in phosphate buffered saline (PBS), pretreated with 0.3% H_2O_2 in PBS for 30 min, blocked in PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 hr, and then incubated at 4°C overnight in the same buffer with either antibodies to TH (Eugene Tech), μ opioid receptor (a generous gift of Chris Evans, UCLA), or serotonin (Incstar) at dilutions of 1:1000, 1:100, and 1:25,000, respectively. After washing in PBS containing 1% normal goat serum and 0.3% Triton X-100 (PBT) three times for 10 min each, the sections were incubated for 30 min at room temperature with biotinylated goat anti-rabbit secondary antibody (Vector) diluted 1:200 in PBT, washed three times again in PBT, and incubated for 30 min with an avidin-biotin complex conjugated to horseradish peroxidase (Vector), again in PBT. The sections were then washed twice in PBT and once in PBS, and the deposits were visualized with 0.04% 3′,3′-diaminobenzidine and 0.0001% H_2O_2, using 0.3% $NiSO_4$ to enhance the reaction.

Quantitation of Brain Monoamines

Whole brains from neonatal mice were dissected on ice and disrupted with a Teflon homogenizer in 1 ml cold 0.1 N perchloric acid and 0.1 mM EDTA. The extracts were then sedimented at 16,000 × g for 20 min at 4°C, and the supernatant was collected and stored at −70°C. Protein concentrations were determined using the Bradford assay (BioRad).

Monoamines and metabolites were measured using high performance liquid chromatography coupled with electrochemical detection (HPLC-EC) on an ESA Coulochem II detector equipped with a model 5011 analytical cell (ESA) set at an applied potential of 400 mV and a Velosep RP-18 column (Applied Biosystems). The mobile phase contained 6.9 g/l $NaH_2PO_4 \cdot H_2O$, 80 mg/l EDTA, 750 mg/l heptasulfonic acid, and 4% methanol, adjusted to pH 3.6 with phosphoric acid. Norepinephrine, DOPAC, 5-HIAA, dopamine, HVA, and serotonin were measured in 100 μl samples from the whole brain homogenates injected in a 200 μl injection loop. The neurochemicals eluted in the above order and the total processing time was 25 min. The chromatography peaks of interest were compared to 10 pg/μl external standards injected in the HPLC on a daily basis.

Primary Midbrain Neuronal Cultures and Stimulation

Postnatal mouse midbrain cultures were established as previously described (Mena et al., 1997), except that the serum-free medium was supplemented with 10 ng/ml glial-derived neurotrophic factor (Intergen) and cortical astrocyte monolayers were derived from Sprague-Dawley rats (Rayport et al., 1992). Briefly, the ventral midbrain from 0–24 hr neonatal mice was dissected, the tissue from each animal dissociated in papain for 2 hr in separate vials, and 80,000 cells per culture plated on the astrocyte monolayer. Each animal yielded two to three cultures. One day after plating, cultures were treated with 6.6 ng/ml 5-flurodioxyuridine in 16.4 ng/ml uridine to suppress cell division. All experiments compared cultures derived from the same litter. TH staining was performed as previously described (Mena et al., 1997). For cell counts, all TH-stained cell bodies in the dish were counted using differential interference contrast (DIC) optics in an inverted microscope.

In unstimulated primary midbrain cultures, dopamine levels were measured in both the medium and perchloric acid cell extracts using HPLC with electrochemical detection, and the values were combined to yield total dopamine. For depolarization-evoked release of dopamine from primary midbrain neurons, the cultures were incubated in elevated KCl (40 mM KCl, 112 mM NaCl, 1 mM NaH_2PO_4, 10 mM HEPES, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM glucose [pH 7.4]) for 2 min, the medium was collected immediately, and

the released dopamine was measured by HPLC. For amphetamine-evoked release, cultures were incubated with D-amphetamine (10 μM) for 30 min, the medium was collected immediately, and the released dopamine was measured using HPLC. To determine total dopamine in the stimulated cultures, the amount in perchloric acid extracts from the cells immediately after stimulation was added to the amount of dopamine released.

Drug Studies

To determine whether the low brain dopamine and serotonin levels in VMAT2-deficient mice resulted from metabolism by monoamine oxidase (MAO), the MAO-A inhibitor clorgyline (10 mg/kg) and the nonselective MAO inhibitor pargyline (75 mg/kg) were administered subcutaneously to *VMAT2*$^{+/+}$ and *VMAT2*$^{-/-}$ mice within 24 hr after birth. Two hours after injection, monoamine and metabolite levels were measured in perchloric acid extracts from the brains of these animals.

To assess the acute effects of amphetamine administration on the behavior of *VMAT2*$^{-/-}$ mice, we alternately injected them with (1) D-amphetamine (10 mg/kg, subcutaneously), (2) nomifensine (10 mg/kg, subcutaneously) followed 30 min later by D-amphetamine, and (3) normal saline. The injections were started within 24 hr after birth and repeated twice daily. One hour after each injection, the mice were scored for the presence of either active suckling or milk in the stomach. The injections were administered and the animals scored by an investigator blind to the treatment.

To test whether chronic administration of amphetamine prolongs survival in *VMAT2*$^{-/-}$ mice, mutant pups were given either D-amphetamine (10 mg/kg, subcutaneously) or normal saline three times a day (8 a.m., 4 p.m., and 10 p.m.). Because newborn animals were alternately assigned to the two treatment groups at the time of birth, both amphetamine and placebo-treated animals were present together in most litters. Behavior, weight, and survival were monitored daily.

Statistical Analysis

Statistical analysis was performed using the two-tailed Student's t test.

Acknowledgments

We thank J. Kwan and E. Friesen for technical assistance, the members of the Edwards lab for thoughtful discussion, Y. Liu for help with the graphics, D. Krantz for helpful comments on the text, and Bigzig video (www.blasto.com//bigzig) for the Web movie. This work was supported by a clinician-scientist award from the Medical Research Council of Canada (E. F.), an Aaron Diamond Foundation Fellowship (E. P.), a Young Investigator Award from the National Alliance for Research on Schizophrenia and Affective Disorders (NARSAD; E. P.), the Aaron Diamond Foundation (D. S.), NARSAD (D. S.), and the National Institutes of Mental Health (R. H. E.) and Drug Abuse (D. S. and R. H. E.).

Received August 20, 1997; revised November 12, 1997.

References

Alfonso, A., Grundahl, K., Duerr, J.S., Han, H.-P., and Rand, J.B. (1993). The *Caenorhabditis elegans unc-17* gene: a putative vesicular acetylcholine transporter. Science *261*, 617–619.

Amara, S.G., and Kuhar, M.J. (1993). Neurotransmitter transporters: recent progress. Annu. Rev. Neurosci. *16*, 73–93.

Attwell, D., Barbour, B., and Szatkowski, M. (1993). Nonvesicular release of neurotransmitter. Neuron *11*, 401–407.

Birkmayer, W., and Hornykiewicz, O. (1961). Der 1-dioxyphenylalinin-(=L-Dopa)-Effekt bei der Parkinson-Akinesie. Wien Med. Wochenschr. *73*, 787–788.

Boadle-Biber, M.C. (1993). Regulation of serotonin synthesis. Prog. Biophys. Mol. Biol. *60*, 1–15.

Brannan, T., Prikhojan, A., and Yahr, M.D. (1996). An in vivo comparison of the capacity of striatal versus extrastriatal brain regions to form dopamine from exogenously administered L-DOPA. J. Neural Transm. *103*, 1287–1294.

Bruns, D., and Jahn, R. (1995). Real-time measurement of transmitter release from single synaptic vesicles. Nature *377*, 62–65.

Cases, O., Vitalis, T., Seif, I., De Maeyer, E., Sotelo, C., and Gaspar, P. (1996). Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: role of a serotonin excess during the critical period. Neuron *16*, 297–307.

Chen, K., and Shih, J.C. (1990). Monoamine oxidase A and B: Structure, function and behavior. In Catecholamines: Bridging Basic Science with Clinical Medicine, S. Goldstein, G. Eisenhofer, and R. McCarty, eds. (San Diego: Academic Press), pp. 292–296.

Cotzias, G.C., Papavasiliou, P.S., and Gellene, R. (1969). Modification of parkinsonism—chronic treatment with levodopa. N. Engl. J. Med. *180*, 337–345.

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Natl. Acad. Sci. USA *85*, 5274–5278.

Erickson, J.D., Eiden, L.E., and Hoffman, B.J. (1992). Expression cloning of a reserpine-sensitive vesicular monoamine transporter. Proc. Natl. Acad. Sci. USA *89*, 10993–10997.

Finn, J.P., and Edwards, R.H. (1997). Individual residues contribute to multiple differences in ligand recognition between vesicular monoamine transporters 1 and 2. J. Biol. Chem. *272*, 16301–16307.

Fishell, G., and van der Kooy, D. (1987). Pattern formation in the striatum: developmental changes in the distribution of striatonigral neurons. J. Neurosci. *7*, 1969–1978.

Florin, S.M., Kuczenski, R., and Segal, D.S. (1995). Effects of reserpine on extracellular caudate dopamine and hippocampus norepinephrine responses to amphetamine and cocaine: mechanistic and behavioral considerations. J. Pharmacol. Exp. Ther. *274*, 231–241.

Frize, E.D. (1954). Mental depression in hypertensive patients treated for long periods with high doses of reserpine. N. Engl. J. Med. *251*, 1006–1008.

Gerfen, C.R. (1992). The neostriatal mosaic: multiple levels of compartmental organization in the basal ganglia. Annu. Rev. Neurosci. *15*, 285–320.

Gilmor, M.L., Nash, N.R., Roghani, A., Edwards, R.H., Yi, H., Hersch, S.M., and Levey, A.I. (1996). Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. J. Neurosci. *16*, 2179–2190.

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., and Caron, M.G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature *379*, 606–612.

Graybiel, A.M., Pickel, V.M., Joh, T.H., Reis, D.J., and Ragsdale, C.W. (1981). Direct demonstration of a correspondence between the dopamine islands and acetylcholinesterase patches in the developing striatum. Proc. Natl. Acad. Sci. USA *78*, 5871–5875.

Johnson, R.G. (1988). Accumulation of biological amines into chromaffin granules: a model for hormone and neurotransmitter transport. Physiol. Rev. *68*, 232–307.

Koob, G.F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol. Sci. *13*, 177–184.

Krantz, D.E., Peter, D., Liu, Y., and Edwards, R.H. (1997). Phosphorylation of a vesicular monoamine transporter by casein kinase II. J. Biol. Chem. *272*, 6752–6759.

Kumer, S.C., and Vrana, K.E. (1996). Intricate regulation of tyrosine hydroxylase activity and gene expression. J. Neurochem. *67*, 443–462.

Lagnado, L., Gomis, A., and Job, C. (1996). Continuous vesicle cycling in the synaptic terminal of retinal bipolar cells. Neuron *17*, 957–967.

Lebrand, C., Cases, O., Adelbrecht, C., Doye, A., Alvarez, C., El Mestikawy, S., Sief, I., and Gaspar, P. (1996). Transient uptake and storage of serotonin in developing thalamic neurons. Neuron *17*, 823–835.

Liu, Y., and Edwards, R.H. (1997). The role of vesicular transport

proteins in synaptic transmission and neural degeneration. Annu. Rev. Neurosci. *20*, 125–156.

Liu, Y., Roghani, A., and Edwards, R.H. (1992a). Gene transfer of a reserpine-sensitive mechanism of resistance to MPP^+. Proc. Natl. Acad. Sci. USA *89*, 9074–9078.

Liu, Y., Peter, D., Roghani, A., Schuldiner, S., Prive, G.G., Eisenberg, D., Brecha, N., and Edwards, R.H. (1992b). A cDNA that suppresses MPP^+ toxicity encodes a vesicular amine transporter. Cell *70*, 539–551.

Maron, R., Stern, Y., Kanner, B.I., and Schuldiner, S. (1983). Functional asymmetry of the amine transporter from chromaffin granules. J. Biol. Chem. *258*, 11476–11481.

Melega, W.P., Williams, A.E., Schmitz, D.A., DiStefano, E.W., and Cho, A.K. (1995). Pharmacokinetic and pharmacodynamic analysis of the actions of D-amphetamine and d-methamphetamine on the dopamine terminal. J. Pharmacol. Exp. Ther. *274*, 90–96.

Mena, M., Khan, U., Togasaki, D.M., Sulzer, D., Epstein, C.J., and Przedborski, S. (1997). Effects of wild-type and mutated copper/zinc superoxide dismutase on neuronal survival and L-DOPA-induced toxicity in postnatal midbrain culture. J. Neurochem. *69*, 21–33.

Merickel, A., Rosandich, P., Peter, D., and Edwards, R.H. (1995). Identification of residues involved in substrate recognition by a vesicular monoamine transporter. J. Biol. Chem. *270*, 25798–25804.

Napolitano, A., Cesura, A.M., and Da Prada, M. (1995). The role of monoamine oxidase and catechol-O-methyltransferase in dopaminergic neurotransmission. J. Neural Transm. *45*, 35–45.

Perlman, R.L., and Sheard, B.E. (1982). Estimation of the cytoplasmic concentrations of dopamine in pheochromocytoma cells. Biochim. Biophys. Acta *719*, 334–340.

Peter, D., Jimenez, J., Liu, Y., Kim, J., and Edwards, R.H. (1994). The chromaffin granule and synaptic vesicle amine transporters differ in substrate recognition and sensitivity to inhibitors. J. Biol. Chem. *269*, 7231–7237.

Peter, D., Liu, Y., Sternini, C., de Giorgio, R., Brecha, N., and Edwards, R.H. (1995). Differential expression of two vesicular monoamine transporters. J. Neurosci. *15*, 6179–6188.

Pierce, R.C., and Kalivas, P.W. (1995). Amphetamine produces sensitized increases in locomotion and extracellular dopamine preferentially in the nucleus accumbens shell of rats administered repeated cocaine. J. Pharmacol. Exp. Ther. *275*, 1019–1029.

Pothos, E., Desmond, M., and Sulzer, D. (1996). L-3,4-dihydroxyphenylalanine increases the quantal size of exocytotic dopamine release in vitro. J. Neurochem. *66*, 629–636.

Rayport, S., Sulzer, D., Shi, W.-X., Sasasdikosol, S., Monaco, J., Batson, D., and Ragendran, G. (1992). Identified postnatal mesolimbic dopamine neurons in culture: morphology and electrophysiology. J. Neurosci. *12*, 4264–4280.

Robinson, T.E. (1991). The neurobiology of amphetamine psychosis: evidence from studies with an animal model. Neurosci. Res. Suppl. *14*, 185–201.

Rudnick, G., and Wall, S.C. (1992). The molecular mechanism of "ecstasy" [3,4-methylenedioxymethamphetamine (MDMA)]: serotonin transporters are targets for MDMA-induced serotonin release. Proc. Natl. Acad. Sci. USA *89*, 1817–1821.

Ryan, T.A., Reuter, H., Wendland, B., Schweizer, F.E., Tsien, R.W., and Smith, S.J. (1993). The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. Neuron *11*, 713–724.

Saura, J., Kettler, R., Da Prada, M., and Richards, J.G. (1992). Quantitative enzyme radioautography with ^{3}H-Ro 41–1049 and ^{3}H-Ro 19–6327 in vitro: Localization and abundance of MAO-A and MAO-B in rat CNS, peripheral organs and human brain. J. Neurosci. *12*, 1977–1999.

Scheller, R.H. (1995). Membrane trafficking in the presynaptic nerve terminal. Neuron *14*, 893–897.

Schuldiner, S., Shirvan, A., and Linial, M. (1995). Vesicular neurotransmitter transporters: from bacteria to humans. Physiol. Rev. *75*, 369–392.

Schwartz, E.A. (1987). Depolarization without calcium can release gamma-aminobutyric acid from a retinal neuron. Science *238*, 350–355.

Seiden, L.S., Sabol, K.E., and Ricaurte, G.A. (1993). Amphetamine: effects on catecholamine systems and behavior. Annu. Rev. Pharmacol. Toxicol. *32*, 639–677.

Song, H.-J., Ming, G.-L., Fon, E., Bellocchio, E., Edwards, R.H., and Poo, M.-M. (1997). Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. Neuron *18*, 815–826.

Spector, S., Gordon, R., Sjoerdsma, A., and Udenfriend, S. (1967). Endproduct inhibition of tyrosine hydroxylase as a possible mechanism for regulation of norepinephrine synthesis. Mol. Pharmacol. *3*, 549–555.

Sudhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature *375*, 645–653.

Sulzer, D., and Rayport, S. (1990). Amphetamine and other psychostimulants reduce pH gradients in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. Neuron *5*, 797–808.

Sulzer, D., Maidment, N.T., and Rayport, S. (1993). Amphetamine and other weak bases act to promote reverse transport of dopamine in ventral midbrain neurons. J. Neurochem. *60*, 527–535.

Sulzer, D., Chen, T.-K., Lau, Y.Y., Kristensen, H., Rayport, S., and Ewing, A. (1995). Amphetamine redistributes dopamine from synaptic vesicles to the cytosol and promotes reverse transport. J. Neurosci. *15*, 4102–4108.

Sulzer, D., St. Remy, C., and Rayport, S. (1996). Reserpine inhibits amphetamine action in ventral midbrain culture. Mol. Pharmacol. *49*, 338–342.

Thomas, S.A., Matsumoto, A.M., and Palmiter, R.D. (1995). Noradrenaline is essential for mouse fetal development. Nature *374*, 643–646.

Weihe, E., Schafer, M.K., Erickson, J.D., and Eiden, L.E. (1994). Localization of vesicular monoamine transporter isoforms (VMAT1 and VMAT2) to endocrine cells and neurons in rat. J. Mol. Neurosci. *5*, 149–164.

Westlund, K.N., Denney, R.M., Rose, R.M., and Abell, C.W. (1988). Localization of distinct monoamine oxidase A and monoamine oxidase B cell populations in human brainstem. Neuroscience *25*, 439–456.

Wise, R.A. (1990). The role of reward pathways in the development of drug dependence. In Psychotropic Drugs of Abuse, D.J.K. Balfour, ed. (Oxford: Pergamon), pp. 23–57.

Zhou, Q.Y., and Palmiter, R.D. (1996). Dopamine-deficient mice are severely hypoactive, adipsic, and aphagic. Cell *83*, 1197–1209.

Zhou, Q.Y., Quaife, C.J., and Palmiter, R.D. (1995). Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. Nature *374*, 640–643.

Zigmond, R.E., Schwarzschild, M.A., and Rittenhouse, A.R. (1989). Acute regulation of tyrosine hydroxylase by nerve activity and by neurotransmitters via phosphorylation. Annu. Rev. Neurosci. *12*, 415–461.