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Dale's principle and glutamate corelease from ventral midbrain dopamine neurons

Review Article

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Summary. While direct application of dopamine modulates postsynaptic activity, electrical stimulation of dopamine neurons typically evokes excitation. Most of this excitation appears to be due to activation of collateral pathways; however, several lines of evidence have suggested that there is a monosynaptic component due to glutamate corelease by dopamine neurons. Recently, more direct evidence obtained in culture has shown that ventral midbrain dopamine neurons release both dopamine and glutamate. Moreover, they appear to do so from separate release sites, calling into question recent modifications of Dale's Principle. The neurochemical phenotype of a given synapse may be determined by subcellular neurotransmitter levels, uptake, or storage. However, the relationship between dopamine and glutamate release from dopamine neuron synapses in the intact brain – and the mechanisms involved – has yet to be resolved.

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Introduction

The idea that neurons release the same neurotransmitter from all of their synapses is associated with Sir Henry Dale, who suggested that this assumption would simplify the task of identifying neurotransmitters in the central nervous system (Dale, 1935). Sir John Eccles later elevated this notion to the status of a *Principle* (Feldman et al., 1997). Subsequent studies have required successive modifications of Dale's Principle. For instance, the discovery of stimulation-dependent corelease of neuropeptides (Hokfelt et al., 1984) forced modification of the Principle to the idea that neurons would release the

same mix of neurotransmitters from all their synapses. The discovery of stimulation-dependent corelease of additional neuroactive substances capable of diffusing across lipid bilayers, such as nitric oxide and arachidonic acid, reinforced that Dale's Principle needed to be modified further.

Less generally acknowledged was evidence that multiple *classical* neurotransmitters can be coreleased. Winkler and others demonstrated that ATP was present in the same adrenal chromaffin granules as catecholamines (Baumgartner et al., 1973). The adrenal chromaffin-derived PC12 cell line was shown by Greene and colleagues to release both acetylcholine and catecholamines (Greene and Rein, 1977). Furshpan and colleagues demonstrated that peripheral neurons convert from catecholaminergic to acetylcholinergic status depending on their postsynaptic target, and go through a phase when they show corelease (Furshpan et al., 1976). Subsequently, Stjarne and colleagues showed that peripheral neurons corelease norepinephrine and ATP (Stjarne et al., 1994; Stjarne and Stjarne, 1995).

These examples of corelease of classical transmitters involve catecholamines that act via G-protein coupled receptors rather than ionotropic receptors. Therefore, a short-lived reworking of Dale's Principle was that it only applied to *fast* neurotransmitters. However, spinal cord interneuron-motor neuron synapses coexpress GABA, acetylcholine, and glycine (Spike et al., 1993), and a recent report demonstrated corelease of GABA and glycine (Jonas et al., 1998) – all compounds that activate ionotropic receptors. Corelease of GABA and glycine could be seen as an aberrant exception to Dale's Principle, since the same synaptic vesicle GABA transporter is known to accumulate both transmitters (Burger et al., 1991; McIntire et al., 1997; Sagne et al., 1997; Chaudhry et al., 1998; Dumoulin et al., 1999). However, ATP – if this is to be considered a classical transmitter – is also coreleased with GABA from spinal neurons (Jo and Schlichter, 1999).

Given the evidence for corelease of neurotransmitters/classical neurotransmitters/fast classical neurotransmitters, could Dale's Principle be restated to hypothesize that the same classical neurotransmitters are released from each synapse of a given neuron? Certainly this is not true for non-classical transmitters, as peptide neurohormones derived from the same precursor are distributed to different synapses in the marine mollusk *Aplysia californica* (Sossin et al., 1990); however, it had been difficult to disprove this version of the Principle for classical transmitters.

Corelease of monoamine transmitters and glutamate

Recent studies demonstrating corelease of monoamines and glutamate now indicate that even this modification of Dale's Principle requires further modification. For instance, noradrenergic locus coeruleus neurons projecting to the spinal cord corelease glutamate (Liu et al., 1995). In this study, retrograde tracers injected into the spinal cord were used to label locus coeruleus neurons, which were then shown to immunostain for both tyrosine hydroxylase (TH) and glutamate. Electrical stimulation of the locus coeruleus produced lumbar motor neuron excitation measured in the ipsilateral ventral

root. Selective autoreceptor-mediated inhibition of noradrenergic neurons by infusion of the 2-andrenergic agonist clonidine into the locus coeruleus blocked the excitation, showing that it was mediated by the noradrenergic neurons. In the spinal cord, motor neuron excitation was reduced by both NMDA and 2-andrenergic antagonists, showing that the excitation was mediated both by glutamate and norepinephrine. Thus, spinally-projecting locus coeruleus neurons appear to release two classical neurotransmitters, namely norepinephrine and glutamate.

Single neuron microcultures of serotonergic raphe neurons provided the first direct demonstration that central neurons corelease monoamines and glutamate (Johnson, 1994; Johnson and Yee, 1995). The neurons in these cultures form recurrent synapses, known as *autapses* (Van der Loos and Glaser, 1972). If autoreceptors are present, specific antagonists can be used to demonstrate that a given transmitter is released (Segal, 1991; Segal, 1994). Stimulation of single raphe neurons evoked either excitation, inhibition, or in about 10% of cells both excitation and inhibition; the excitation was blocked with glutamate antagonists while the inhibition was blocked with serotonin antagonists. At the ultrastructural level, individual serotonergic neurons formed both symmetric and asymmetric synapses. These synapses contained a mix of synaptic vesicles – small synaptic vesicles, clear round vesicles, and dense core granules. Although the mix varied from cell to cell, the mix of vesicles was the same at all of the synapses in a given cell. In the case of raphe neurons in microculture, both serotonin and glutamate components were blocked by the appropriate antagonists, clearly demonstrating corelease (Johnson, 1994). There was, however, no evidence that glutamate and serotonin were differentially localized to different synapses of a given neuron.

Corelease of dopamine and glutamate from ventral midbrain dopamine neurons

Several studies conducted in the 1970's indicated that direct application of dopamine (DA) tended to inhibit firing of medium spiny neurons in the striatum, while stimulation of the DA neurons in the SN or their axons in the medial forebrain bundle generally produced excitation, although depression of firing was noted in some studies (Siggins, 1978). These divergent responses to DA could be explained by recent findings that DA can either potentiate or depress excitatory synaptic transmission in the striatum or nucleus accumbens depending on the state of the postsynaptic neurons (Hernandez-Lopez et al., 1997; Surmeier and Kitai, 1997; Yan et al., 1997; Yan and Surmeier, 1997). In addition, DA can modulate activity of afferent pathways to the striatum (Nicola et al., 1996; Nicola and Malenka, 1997). Similar state-dependent responses to DA are also seen in the ventral midbrain depending on the state of the local GABAergic neurons (Bonci and Williams, 1996). Probably, most of the divergence between results with DA application versus stimulation of DA neurons can be attributed to activation of collateral pathways, as the magnitude of striatal EPSPs evoked by SN stimulation was greatly reduced by removal of cortex and transection of thalamic tracts (Wilson et al., 1982).

However, even after interruption of the collateral pathways, a small long latency EPSP was still evoked by SN stimulation, which Wilson and colleagues attributed to a direct monosynaptic excitation mediated by DA neurons.

More recently, a combined electrochemical/extracellular recording study showed that median forebrain bundle stimulation in anesthetized rats evoked two excitatory responses in a given striatal neuron, either rapid or delayed (Gonon, 1997). The delayed excitatory response was mediated by DA as it was blocked by the D1 receptor antagonists. In contrast, the fast component was not blocked by DA antagonists, and although that response was not sensitive to acute (one hour) exposure to the DA neurotoxin 6-hydroxydopamine, it could be due to glutamate release from ventral midbrain DA neurons if the toxin exposure was too brief. Consistent with Gonon's observation, stimulation of SN neurons in SN-striatum-cortex triple explant cultures has also been shown to evoke fast excitatory responses in striatal neurons (Plenz and Kitai, 1996).

While excitatory responses in the striatum due to activation of DA neurons could be explained by corelease of glutamate from midbrain DA neurons, this has not been explicitly considered despite evidence from morphological studies suggesting that glutamate might be a cotransmitter in midbrain dopamine neurons. Indeed, most DA neurons immunostain for phosphate-activated glutaminase, the major biosynthetic enzyme responsible for neurotransmitter glutamate in rat (Kaneko et al., 1990), DA neurons in rat and monkey immunostain for glutamate (Sulzer et al., 1998). Lesioning the dopaminergic projection to striatum reduced the number of asymmetric synaptic profiles by about 17% (Ingham et al., 1998) which could be explained by the loss of the glutamatergic terminals of DA neurons. Most directly, in an orthograde labeling study in combination with TH immunostaining, midbrain DA neurons were shown to have two types of synapses, ones with symmetric synaptic specializations that stained for TH, and asymmetric synapses labeled only by orthograde {^{3}H} leucine transport (Hattori et al., 1991). While these studies were consistent with glutamate corelease from DA, they did not provide a direct demonstration.

To test this directly, we placed ventral tegmental area dopamine neurons in single cell microcultures and examined their autaptic responses. Stimulation of DA neurons produced excitatory autaptic responses that could be blocked by both NMDA and AMPA-type glutamate receptor antagonists, indicating that glutamate itself was the likely transmitter (Sulzer et al., 1998). Similarly, when DA neurons were co-cultured with postsynaptic neurons from the nucleus accumbens they also made glutamatergic connections (Joyce et al., 1999). To date, neither a direct DA autaptic response nor a DA synaptic response has been seen; however, it is clear from amperometric recordings that DA neurons show quantal DA release at axonal sites that also show vesicular accumulation of the DA analog 5-hydroxy-DA (Pothos et al., 1998). Moreover, activity-dependent DA release produced a presynaptic inhibition of the glutamatergic autapses via D2 receptor activation.

Ultrastructural observation indicated that DA neurons in microculture, like raphe neurons, also formed both symmetric and asymmetric autapses.

However, unlike the results with raphe neurons, two classes of synapses were observed, rare ones that had symmetric synaptic profiles and stained for TH and more frequent synapses with asymmetric profiles that were comparatively unstained for TH. In cultures observed at the light level after immunostaining for glutamate and TH, there was a partially overlapping distribution of TH and glutamate at individual synapses.

Together these results indicate, in contrast to the most recent modification of Dale's Principle, that neurotransmitters may be segregated to the different synapses of individual central neurons. In the case of DA neurons, glutamate release from asymmetric synapses mediates the rapid excitation, similar to classic glutamate actions in the brain, while DA released from symmetric synapses, or axonal varicosities without obvious synaptic specializations, may be mostly active at extrasynaptic sites.

Control of neurochemical phenotype

Given the findings of plasticity and multiplicity of transmitter phenotypes in single neurons, one may ask, how is the neurochemical phenotype of neurons and their synapses controlled? For classical transmitters, the control could be exerted by pathways that provide cytosolic transmitter and control vesicular uptake. The regulation of these pathways, however, may prove to be quite complex, as seen with the myriad changes that occur in PC12 cells exposed to nerve growth factor, including the accumulation and release of acetylcholine from small synaptic vesicles (Greene and Rein, 1977; Tsao et al., 1990).

Cytoplasmic transmitter levels can control neurochemical phenotype. In acutely isolated *Xenopus* spinal motor neurons, glutamate loaded into the cytoplasm via patch pipettes enabled stimulation-dependent glutamate release from this normally cholinergic neuron; similarly, acetylcholine loaded into glutamatergic hippocampal neurons enabled stimulation-dependent acetylcholine release from this normally glutamatergic neuron (Dan et al., 1994). Therefore, it appears that synaptic vesicles can be somewhat promiscuous and package and release false transmitters present at sufficiently high cytosolic levels.

The role of vesicular transporters is shown by transfection experiments with recombinant VMAT2, the CNS vesicular monoamine transporter. In AtT-20 cells, a cell line that normally secretes neuropeptides transfected with TH alone, there was very little quantal DA release, as measured by amperometry. However, with coexpression of both TH and VMAT2, the cells showed robust stimulation-dependent DA release (Pothos et al., 1999b). Hippocampal neurons in culture will also release DA in a stimulation-dependent manner, if they are transfected with VMAT2 and bathed in high external DA concentrations (Pothos et al., 1999a).

How then could a given neuron regulate local neurochemical phenotype at a given synapse? In addition to Hattori's ultrastructural studies mentioned above, it appears that TH is targeted selectively to a subset of axonal varicosities in cultured ventral midbrain neurons (Sulzer et al., 1998), pre-

sumably controlling the local cytosolic transmitter pool of DA. TH activity and expression are regulated by numerous pathways (Kumer and Vrana, 1996) and phosphate-activated glutaminase could be regulated as well. For instance, the inorganic phosphate transporter that provides a necessary co-factor for glutaminase activity and has been localized to excitatory synaptic terminals, could be differentially distributed (Bellocchio et al., 1998). Interestingly, culturing midbrain DA neurons in the presence of the glutamate receptor antagonist kynurenate increased the percentage of DA neurons that expressed glutamate autapses; however, this may be due to an upregulation of glutamate receptors rather than a potentiation of presynaptic function (Sulzer et al., 1998). Alternatively, vesicular transporters could be differentially trafficked to different synapses. When the vesicular glutamate transporter is cloned, this issue should become tractable.

Conclusion

Dale's Principle continues to inspire neuroscientists to define and test the basic rules governing synaptic transmission. This impetus now drives the investigation of neurochemical phenotype. In particular, there is presently little understanding of the subcellular control of neurochemical identity, particularly if and how different synapses of a given neuron maintain different transmitters. There is also a large gap between the known range of possible phenotypes for a given neuron observed in culture and what the neuron actually does *in vivo* – it will be necessary to devise new strategies to study these issues *in vivo*. If past work provides any indication, it seems safe to predict that neurons are both more plastic and more complex than our models.

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References

Baumgartner H, Winkler H, Hortnagl H (1973) Isolated chromaffin granules maintenance of ATP content during incubation at 31 degrees C. Eur J Pharmacol 22: 102–104

Bellocchio EE, Hu H, Pohorille A, Chan J, Pickel VM, Edwards RH (1998) The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. J Neurosci 18: 8648–8659

Bonci A, Williams JT (1996) A common mechanism mediates long-term changes in synaptic transmission after chronic cocaine and morphine. Neuron 16: 631–639

Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F, Jahn R (1991) GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron 7: 287–293

Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic

vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18: 9733–9750
Dan Y, Song HJ, Poo MM (1994) Evoked neuronal secretion of false transmitters. Neuron 13: 909–917
Dumoulin A, Rostaing P, Bedet C, Levi S, Isambert MF, Henry JP, Triller A, Gasnier B (1999) Presence of the vesicular inhibitory amino acid transporter in GABAergic and glycinergic synaptic terminal boutons. J Cell Sci 112: 811–823
Furshpan EJ, MacLeish PR, O'Lague PH, Potter DD (1976) Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. Proc Natl Acad Sci USA 73: 4225–4229
Gonon F (1997) Prolonged and extrasynaptic excitatory action of dopamine mediated by D1 receptors in the rat striatum in vivo. J Neurosci 17: 5972–5978
Greene LA, Rein G (1977) Synthesis, storage and release of acetylcholine by a noradrenergic pheochromocytoma cell line. Nature 268: 349–351
Hattori T, Takada M, Moriizumi T, Van der Kooy D (1991) Single dopaminergic nigrostriatal neurons form two chemically distinct synaptic types: possible transmitter segregation within neurons. J Comp Neurol 309: 391–401
Hernandez-Lopez S, Bargas J, Surmeier DJ, Reyes A, Galarraga E (1997) D1 receptor activation enhances evoked discharge in neostriatal medium spiny neurons by modulating an L-type Ca2+ conductance. J Neurosci 17: 3334–3342
Hokfelt T, Johansson O, Goldstein M (1984) Chemical anatomy of the brain. Science 225: 1326–1334
Ingham CA, Hood SH, Taggart P, Arbuthnott GW (1998) Plasticity of synapses in the rat neostriatum after unilateral lesion of the nigrostriatal dopaminergic pathway. J Neurosci 18: 4732–4743
Jo YH, Schlichter R (1999) Synaptic corelease of ATP and GABA in cultured spinal neurons. Nat Neurosci 2: 241–245
Johnson MD (1994) Synaptic glutamate release by postnatal rat serotonergic neurons in microculture. Neuron 12: 433–442
Johnson MD, Yee AG (1995) Ultrastructure of electrophysiologically-characterized synapses formed by serotonergic raphe neurons in culture. Neurosci 67: 609–623
Jonas P, Bischofberger J, Sandkuhler J (1998) Corelease of two fast neurotransmitters at a central synapse. Science 281: 419–424
Joyce MP, Lin L, Rayport S (1999) Mesoaccumbens dopamine neuron synapses *in vitro*. Soc Neurosci Abstr 25 (in press)
Kaneko T, Akiyama H, Nagatsu I, Mizuno N (1990) Immunohistochemical demonstration of glutaminase in catecholaminergic and serotoninergic neurons of rat brain. Brain Res 507: 151–154
Kumer SC, Vrana KE (1996) Intricate regulation of tyrosine hydroxylase activity and gene expression. J Neurochem 67: 443–462
Liu RH, Fung SJ, Reddy VK, Barnes CD (1995) Localization of glutamatergic neurons in the dorsolateral pontine tegmentum projecting to the spinal cord of the cat with a proposed role of glutamate on lumbar motoneuron activity. Neurosci 64: 193–208
McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. Nature 389: 870–876
Nicola SM, Kombian SB, Malenka RC (1996) Psychostimulants depress excitatory synaptic transmission in the nucleus accumbens via presynaptic D1-like dopamine receptors. J Neurosci 16: 1591–1604
Nicola SM, Malenka RC (1997) Dopamine depresses excitatory and inhibitory synaptic transmission by distinct mechanisms in the nucleus accumbens. J Neurosci 17: 5697–5710
Plenz D, Kitai ST (1996) Organotypic cortex-striatum-mesencephalon cultures: the nigrostriatal pathway. Neurosci Lett 209: 177–180

Pothos EN, Davila V, Sulzer D (1998) Presynaptic recording of quanta from midbrain dopamine neurons and modulation of the quantal size. J Neurosci 18: 4106–4118

Pothos EN, Krantz D, Edwards R, Sulzer D (1999a) Expression of VMAT2 in hippocampal neurons as a tool for exploring the plasticity of quantal transmission. Soc Neurosci Abstr

Pothos EN, Larsen KE, Krantz DE, Liu Y-J, Edwards RH, Sulzer D (1999b) Synaptic vesicle transporter expression regulates vesicle phenotype and quantal size. (in submission)

Sagne C, El Mestikawy S, Isambert MF, Hamon M, Henry JP, Giros B, Gasnier B (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. FEBS Lett 417: 177–183

Segal MM (1991) Epileptiform activity in microcultures containing one excitatory hippocampal neuron. J Neurophysiol 65: 761–770

Segal MM (1994) Endogenous bursts underlie seizurelike activity in solitary exocitatory hippocampal neurons in microcultures. J Neurophysiol 72: 1874–1884

Siggins GR (1978) Electrophysiological role of dopamine in the striatum: excitatory or inhibitory? In: Psychopharmacology: a generation of progress. Raven Press, New York, pp 143–157

Sossin WS, Sweet-Cordero A, Scheller RH (1990) Dale's hypothesis revisited: different neuropeptides derived from a common prohormone are targeted to different processes. Proc Natl Acad Sci USA 87: 4845–4848

Spike RC, Todd AJ, Johnston HM (1993) Coexistence of NADPH diaphorase with GABA, glycine, and acetylcholine in rat spinal cord. J Comp Neurol 335: 320–333

Stjarne L, Astrand P, Bao JX, Gonon F, Msghina M, Stjarne E (1994) Spatiotemporal pattern of quantal release of ATP and noradrenaline from sympathetic nerves: consequences for neuromuscular transmission. Adv Second Messenger Phosphoprotein Res 29: 461–496

Stjarne L, Stjarne E (1995) Geometry, kinetics and plasticity of release and clearance of ATP and noradrenaline as sympathetic cotransmitters: roles for the neurogenic contraction. Prog Neurobiol 47: 45–94

Sulzer D, Joyce MP, Lin L, Geldwert D, Haber SN, Hattori T, Rayport S (1998) Dopamine neurons make glutamatergic synapses *in vitro*. J Neurosci 18: 4588–4602

Surmeier DJ, Kitai ST (1997) State-dependent regulation of neuronal excitability by dopamine. Nihon Shinkei Seishin Yakurigaku Zasshi 17: 105–110

Tsao H, Aletta JM, Greene LA (1990) Nerve growth factor and fibroblast growth factor selectively activate a protein kinase that phosphorylates high molecular weight microtubule-associated proteins. Detection, partial purification, and characterization in PC12 cells. J Biol Chem 265: 15471–15480

Wilson CJ, Chang HT, Kitai ST (1982) Origins of postsynaptic potentials evoked in identified rat neostriatal neurons by stimulation in substantia nigra. Exp Brain Res 45: 157–167

Yan Z, Song WJ, Surmeier DJ (1997) D2 dopamine receptor reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein kinase C-insensitive pathway. J Neurophysiol 78: 1003–1015

Yan Z, Surmeier DJ (1997) D5 dopamine receptors enhance Zn2+-sensitive GABAA currents in striatal cholinergic interneurons through a PKA/PP1 cascade. Neuron 19: 1115–1126

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