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Research report

Colocalization of neurotransmitter transporters on the plasma membrane of the same nerve terminal may reflect cotransmission

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ABSTRACT

There is increasing evidence for the neuronal coexistence of *classical* transmitters. Implications in favor of cotransmission have often been represented by the identification, in the same neuron, of the putative cotransmitters, their synthetic enzymes and/or their vesicular transporters. In contrast, coexpression of neurotransmitter transporters on the plasma membrane of the same nerve terminal, although a potentially important indication for cotransmission, has received poor attention. We here used preparations of isolated nerve endings to functionally identify transporters coexpressed on the plasma membrane of the same terminal, in order to verify if such transporter coexpression indeed exists in neuronal systems in which cotransmission has already been established or reasonably suspected through other technical approaches. We could observe that functional transporters for glycine and glutamate are coexpressed on nerve terminals in the cerebellum; transporters for dopamine and GABA coexist on striatal terminals; transporters for glycine and GABA, previously found to coexist as cotransmission markers on nerve terminals of spinal cord and cerebellum, are not coexpressed in neocortex and hippocampus, where cotransmission has not been proposed to occur; transporters for GABA, glycine and glutamate are colocalized on nerve terminals of the spinal cord. Confocal microscopy experiments were performed to substantiate functional data, highlighting the presence of the co-existing transporters under study on MAP-2 positive synaptosomes. It is concluded that investigating the colocalization of functional neurotransmitter transporters on the plasma membrane of nerve terminals can provide useful information on the possibility of cotransmission.

1. Introduction

The possibility that some nerve cells release more than one neurotransmitter was postulated by Burnstock (1976), based on studies in the autonomic nervous system showing corelease of ATP and noradrenaline or acetylcholine. Subsequently, cotransmission of ATP and classical transmitters has been thoroughly investigated in both peripheral and central nerves (see, for reviews, Burnstock, 2004, 2013, 2016; Burnstock et al., 2011; Kennedy, 2015). Studies on the colocalization, vesicular storage and release of peptides coexpressed with classical transmitters were also described (Hökfelt et al., 1977, 1987, 1998).

There is now increasing interest about neurons that can store and release two *classical* transmitters (for reviews see Hnasko and Edwards, 2012; Münster-Wandowski et al., 2013; Vaaga et al., 2014; Granger et al., 2016). The canonical version of cotransmission involving two transmitters includes synthesis of the two messengers in the same terminal, vesicular storage through appropriate transporters situated on different vesicles in the same terminal and release from the same terminal of the cotransmitters onto postsynaptic receptors.

Abbreviations: (*S*)AMPA, (*S*)α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; [(*RS*)-3,5-DHPG], (*RS*)-3,5-dihydroxyphenylglycine; 5,7-DCK, 5,7-dichlorokynurenate; AOAA, aminooxyacetic acid; DA, dopamine; DAT, dopaminergic transporter; DL-TBOA, DL-*threo*-β-benzyloxyaspartic acid; GABA, γ-aminobutyric acid; GAT1, GABA transporter 1; GLT1, glutamate transporter 1; NFPS, N-[3-([1,1-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine; NMDA, *N*-methyl-D-aspartate; SKF89976A, 1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride; SNc, substantia nigra pars compacta; SPNs, striatal projection neurons; VGAT, vesicular GABA transporter; VIAATs, vesicular inhibitory amino acid transporters; VMAT, vesicular monoamine transporter; VTA, ventral tegmental area.

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Exceptions to the canonical form of cotransmission are however frequent and many aspects of the process remain therefore only partly understood. There may exist nerve terminals unable to sufficiently synthesize one of the cotransmitters, which can be captured from the extracellular milieu by selective transporters situated on the plasma membrane of nerve terminals. This is what is likely to happen in the case of populations of GABAergic terminals endowed with glycine transporters which, being glycine synthesis inefficient, can perform uptake of glycine as potential GABA cotransmitter (Poyatos et al., 1997; Gomeza et al., 2003; Simat et al., 2007; Rousseau et al., 2008; Romei et al., 2012). Variants in the process of cotransmission can also be due to the storage of the two transmitters into the same synaptic vesicles. GABAergic and glycinergic nerve terminals contain vesicles endowed with transporters, termed vesicular inhibitory amino acid transporters (VIAATs), which are able to recognize both GABA and glycine as substrates (Chaudhry et al., 1998; Dumoulin et al., 1999). Thus, in the neurons where GABA and glycine are cotransmitters, the two amino acids are costored into and coreleased from the same synaptic vesicles (Jonas et al., 1998; Nabekura et al., 2004; Seal and Edwards, 2006). Surprisingly, in some cotransmission systems, synaptic vesicles have been found able to host 'foreign' transmitters together with the home-made transmitter. For example, striatal dopaminergic nerve terminals can corelease dopamine and GABA which are costored into the same vesicles through the vesicular monoamine transporter VMAT (Tritsch et al., 2012, 2014; Stensrud et al., 2014).

Differently from colocalization of transmitter transporters situated on distinct sets of synaptic vesicles inside the same axon terminal, which has been relatively often studied, coexpression of transporters on the plasma membrane of nerve terminals have received poor consideration. Yet, coexpression of two transporters on the plasma membrane of nerve terminals containing two classical transmitters would provide relevant information on the involvement of cotransmission. In fact, the coexpression on nerve terminals of transport systems performing transmitter uptake to replenish the messenger stores is considered one of the major criteria to be satisfied in order to establish that the transporter substrates are actually cotransmitters (Burnstock, 2004).

In the present work, superfused synaptosomes (Raiteri and Raiteri, 2000; see also Discussion) were utilized to investigate the coexistence on the plasma membrane of the same nerve terminal of functional transporters for glutamate and glycine, dopamine and GABA, glycine and GABA, as well as glutamate and GABA, in discrete regions of the adult mouse central nervous system. These transporter pairs were chosen because evidences suggesting cotransmission for the corresponding neurotransmitters have already been provided, using technical approaches different from that here employed (see Discussion, for references). Our results show that the functional determination of transporter coexpression on the plasma membrane of the same nerve terminal may represent a good marker for cotransmission.

2. Materials and methods

2.1. Animals

Adult male Swiss mice (weighing 20–25 g; Charles River, Calco, Italy) were used. Animals were housed at constant temperature ($22 \pm 1\,^{\circ}C$) and relative humidity (50%) under a regular light/dark schedule (light 7.00 a.m. to 7.00 p.m.). Food and water were freely available. All experiments were carried out in accordance with the guidelines established by the European Communities Council (Directive 114 2010/63/EU of September 22nd, 2010) and were approved by the Italian Ministry of Health. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

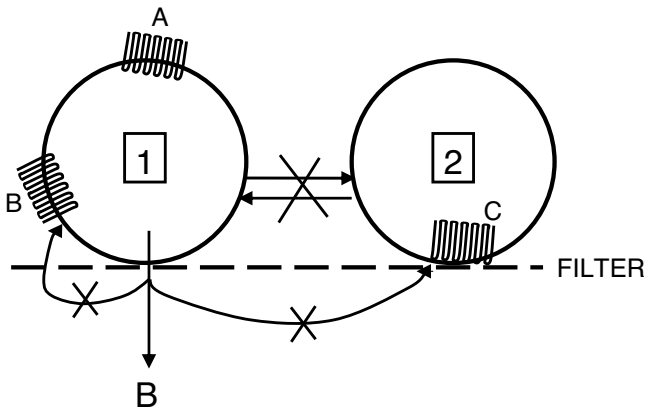

Fig. 1. Scheme representating some critical features of superfused synaptosomes. Suspensions of purified nerve terminals are filtered through large (2.5 cm diameter) microporous filters placed at the bottom of parallel superfusion chambers to form monolayers of particles. Two nerve terminals (1 and 2) are shown. Compelling evidence exists that compounds released from nerve terminal 1 are immediately removed (and possibly collected for analysis) by the solutions that up-down perfuse the synaptosomal monolayer before they can feedback on targets situated on terminal 1 or reach targets, like C, present on the neighboring terminal 2 (see text). A = transporter A; B = transporter B; C = presynaptic target C.

2.2. Isolation and purification of synaptosomes

Animals were sacrificed by cervical dislocation and the spinal cord, cerebellum, cortex, hippocampus and striatum were quickly removed. Tissues were homogenized in 10 vol. of 0.32 M sucrose buffered at pH 7.4 with Tris–HCl, using a glass-Teflon tissue grinder (clearance 0.25 mm, 12 up-down strokes at 900 rpm in about 1 min in the case of cortex, hippocampus and striatum; 24 up-down strokes at 90 rpm in about 2 min for the cerebellum and spinal cord). The homogenate was centrifuged (5 min, 1000*g* at $4\,^{\circ}C$) to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll® gradient (2%, 6%, 10% and 20% in Tris-buffered sucrose) and centrifuged at 33,500*g* for 5 min. The layer between 10% and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation and resuspended in a physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; glucose, 10; HEPES, 10; pH adjusted to 7.4 with NaOH. All the above procedures were performed at 0–4 °C.

2.3. Experiments of release

Synaptosomes were incubated at 37 °C for 15 min with the appropriate radioactive tracer: [^{3}H]L-Glutamate (0.1 μM); [^{3}H]GABA (0.02 μM); [^{3}H]dopamine (0.05 μM). At the end of incubation, identical aliquots of the synaptosomal suspension (each corresponding to about 40 μg protein for spinal cord, cerebellum and cortex and 25 μg for hippocampus and striatum) were distributed on microporous filters placed at the bottom of a set of parallel superfusion chambers maintained at 37 °C and superfused with standard medium at a rate of 0.5 ml/min (Raiteri and Raiteri, 2000; see also Fig. 1 and Discussion). In the experiments with [^{3}H]GABA, aminooxyacetic acid (AOAA) was present at 50 μM in all the solutions, to prevent neurotransmitter catabolism. In the experiment with [^{3}H]L-glutamate, aminooxyacetic acid was present at 1 mM to prevent catabolism of glutamate by blocking the glutamic acid decarboxylase (Cavallero et al., 2009). After 36 min of superfusion with standard medium, to equilibrate the system,