Supporting Figures legends

Figure S1. *Purification of DA neurons by cell sorting*. Mesencephalic cell suspensions obtained from P14 mice were subjected to fluorescent-activated cell sorting. The fluorescent profile of WT (**A**) and TH-GFP (**B**) mice is shown. Note that no phycoerythrin labeling was used; the PEA filter was used to evaluate autofluorescence. Bidimensional plotting of GFP (fluorescent marked cells) vs PEA (autofluorescent cells) allowed us to discard cells that might have otherwise been collected using unidimensional sorting (only GFP) because of their high autofluorescence (more than 1000 fluorescent units, blue cell population). GFP-expressing DA neurons (P7 fraction) were sorted, their RNA extracted and subjected to RT-PCR (**C**). The expression of TH together with the absence of GAD-67 or GFAP (markers for GABA neurons and glía respectively) confirms the high purity level of these DA neuron preparations. β-actin expression was evaluated as a positive control for the presence of mRNA. FACS, FACS purified DA neurons; M, RNA extracted from whole mesencephalon was used as a positive control; -, water, used as internal control. **D**) Potassium-evoked [^{3}H]-labeled DA release was quantified following radioactive labeling in the presence of clorgyline and pargyline. Labeling was performed either in the presence (KS) or absence (K) of the D2 autoreceptor antagonist sulpiride. When labeling occurred in the presence of sulpiride, extensive potassium-evoked [^{3}H]-labeled DA release was detected (left column). When labeling was performed in the absence of sulpiride (right column), little potassium-evoked release was detectable, most likely due to strong inhibition of TH activity by the D2 autoreceptor. The residual labeling and release seen in the absence of sulpiride is likely to represent [^{3}H]-labeled tyrosine metabolites, whereas release detected following labeling in the presence of sulpiride likely represents DA produced *de novo* by DA neurons using [2, 3, 5, 6-^{3}H]-Tyrosine. n=6. In each experiment, release measured in the group treated with sulpiride was normalized relative to release detected in the group not treated with sulpiride (K), which was set to 100%. * $p<0.05$.

Figure S2. *Syt7 expression and basic characteristics of DA release in culture.* **A**) Cultured DA neurons were examined for Syt7 expression by immunocytochemistry. In addition to being found in the STD compartment (see Fig. 2), Syt7 (red signal, left panel) was also found to be localized in TH-positive (green signal, middle panel) axonal-like varicosities established by DA neurons. The right panel shows at higher magnification the colocalization of TH with Syt7 in the area identified by the white box in the central panel. **B**-**F**) The characteristics of DA release in rat cultures have been established previously; here we validate our radioassay method in mouse cultures. **B**) A decrease of extracellular Ca^{2+} from 2 to 0.5 mM reduced but did not block the release of [^{3}H]-DA. In the presence of 0.5 mM extracellular Ca^{2+}, conditions in which DA is released only from the somatodendritic compartment, [^{3}H]-DA release was decreased by blocking the spontaneous firing activity of DA neurons with 0.5 μM TTX, (**C**) and was increased when cells were depolarized with 40 mM KCl (**D**). (**E**) Blockade of the DA transporter with 1μM GBR1209 increased the extracellular levels of somatodendritically-released DA. Numbers inside the bars indicate the number of experiments performed in each group. * $p<0.05$ versus control group. Error bars represent SD. (**F**) Glutamate-mediated synaptic currents were recorded from GFP-expressing DA neurons using the patch-clamp technique. In normal saline (2 mM extracellular Ca^{2+}, top trace) numerous large amplitude synaptic currents were detected. In the presence of 0.5 mM extracellular Ca^{2+} (lower trace), such events disappeared completely, leaving only occasional action potential-independent miniature synaptic currents. The bottom graph summarizes the results of 9 recorded neurons. In the presence of 0.5 mM extracellular Ca^{2+}, all remaining events were miniature synaptic events of small amplitude.

Supporting Materials and Methods:

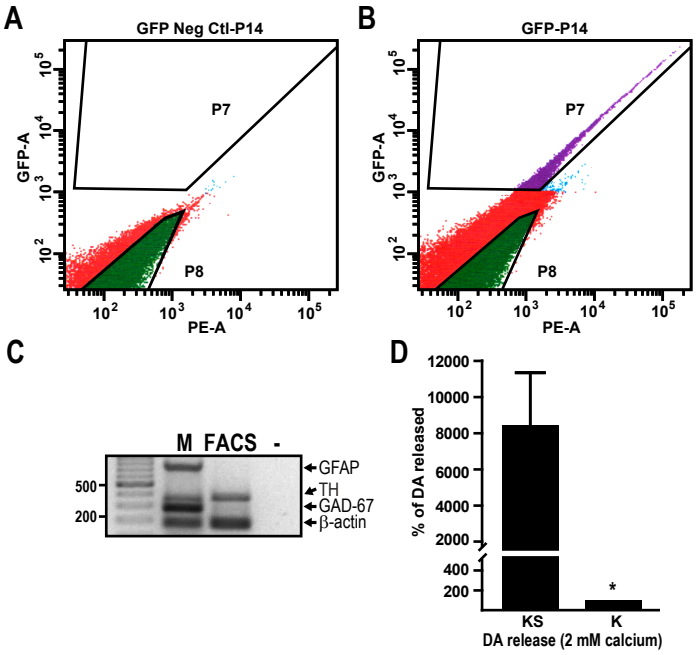
The labeling of *de novo* synthesized DA with radioactivity was performed based upon the rationale that the limited enzymatic repertoire for tyrosine metabolism of mesencephalic DA neurons allows the production of a minimal amount of tyrosine metabolites if monoamine oxidases (MAOs) are inhibited

with clorgyline (5μM) and pargyline (100 μM) to block DA degradation. To ensure high production of ^{3}H-labeled DA, intracellular DA stores were reduced by potassium-induced depolarization and TH activity was promoted by using sulpiride to block feedback inactivation of TH secondary to D2 autoreceptor stimulation (1). According to the enzymatic repertoire of mouse DA neurons, the radioactivity-labeled metabolites likely to be produced upon [2, 3, 5, 6-^{3}H]-Tyrosine treatment in our cultures are: (*i*) 4-hydroxyphenyl pyruvate, which can be only converted into (*ii*) hydroxyphenyl lactate (in a reversible reaction) that is not further metabolized; the trace amine (*iii*) tyramine, which cannot be further metabolized into 4-hydroxyphenyl acetaldehyde because MAOs are blocked by clorgyline and pargyline; (*iv*) L-DOPA and (*v*) DA which cannot be further catabolized into DOPAC nor homovanillic acid because of the actions of clorgyline and pargyline. DA can however be catabolized into (*vi*) 3-methoxy tyramine. In addition, [2, 3, 5, 6-^{3}H]-Tyrosine can be incorporate into proteins (*vii*). The negligible contribution of these metabolites is revealed by the fact that D2 receptor-mediated feedback inhibition of TH activity and DA release, secondary to the release of DA in response to potassium-induced depolarization, greatly reduces the release of ^{3}H-labelled products in our model. As can be observed in Fig. S1D, the D2 receptor antagonist sulpiride lifts this feedback inhibition and restores potassium-evoked release of ^{3}H-DA, clearly showing that the vast majority of radioactivity we recovered depends on the activity of TH and of DA neurons and is therefore ^{3}H-DA. Such an effect would not be expected for products # *i-iv* mentioned previously. The last product to consider is methoxy tyramine. However, this product is produced by Catechol-O-methyltransferase (COMT), an enzyme that is expressed in DA postsynaptic areas, but not in the SN nor in the VTA (2), therefore making it highly improbable to be an important contributor to the radioactivity signal we sampled in our experiments. Moreover, 3-methoxy tyramine is not released by exocytosis, therefore making it unlikely that changes in external Ca^{2+} concentration or changes in Syt's expression would alter its non-regulated release.

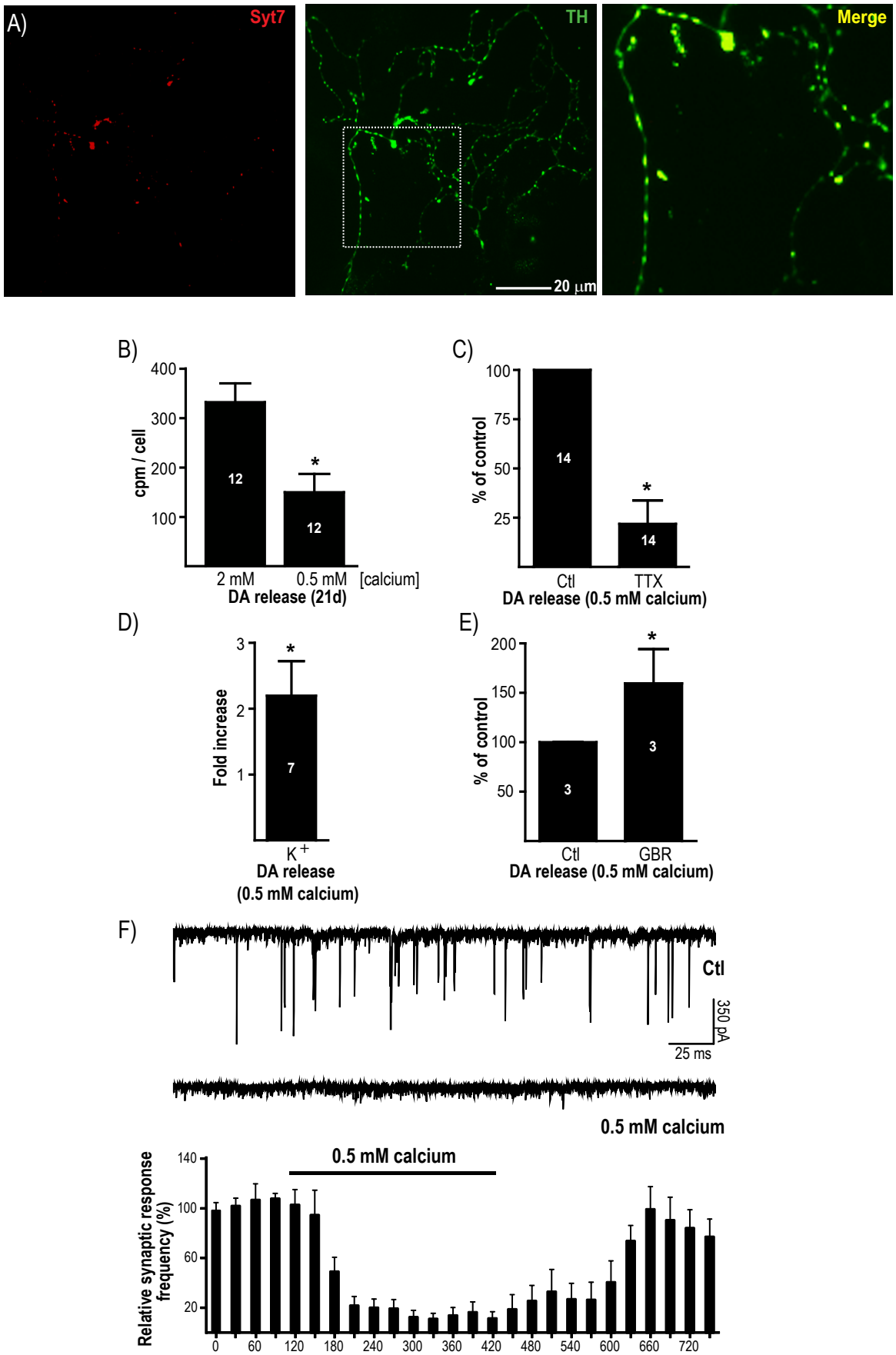
References

1. Lindgren, N., Xu, Z. Q., Herrera-Marschitz, M., Haycock, J., Hokfelt, T., and Fisone, G. (2001) *The European journal of neuroscience* **13**, 773-780
2. Mannisto, P. T., and Kaakkola, S. (1999) *Pharmacol Rev* **51**, 593-628

Suppl. Fig. 1, Mendez et al., 2010



Suppl. Fig. 2


A)
Syt7
TH
Merge
20 µm
B)
cpm / cell
400
300
200
100
12
12
*
2 mM
0.5 mM
[calcium]
DA release (21d)
C)
% of control
100
75
50
25
14
14
*
Ctl
TTX
DA release (0.5 mM calcium)
D)
Fold increase
3
2
1
7
*
K+
DA release
(0.5 mM calcium)
E)
% of control
200
150
100
50
3
3
*
Ctl
GBR
DA release (0.5 mM calcium)
F)
Ctl
350 pA
25 ms
0.5 mM calcium
0.5 mM calcium
Relative synaptic response frequency (%)
140
100
60
20
0
60
120
180
240
300
360
420
480
540
600
660
720