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Journal of Neuroscience Methods 146 (2005) 1–12

JOURNAL OF NEUROSCIENCE METHODS

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Use of TH-EGFP transgenic mice as a source of identified dopaminergic neurons for physiological studies in postnatal cell culture

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Received 25 June 2004; received in revised form 10 September 2004; accepted 14 October 2004

Abstract

The physiological and pharmacological properties of dopaminergic neurons in the brain are of major interest. Although much has been learned from cell culture studies, the physiological properties of these neurons remain difficult to study in such models because they are usually in minority and are difficult to distinguish from other non-dopaminergic neurons. Here we have taken advantage of a recently engineered transgenic mouse model expressing enhanced green fluorescence protein (EGFP) under the control of the tyrosine hydroxylase promoter to establish a more effective dopaminergic neuron cell culture model. We first evaluated the specificity of the EGFP expression. Although ectopic expression of EGFP was found in cultures derived from postnatal day 0 pups, this decreased over time in culture such that after 2 weeks, approximately 70% of EGFP-expressing neurons were dopaminergic. We next sought to validate this dopaminergic neuron culture model. We evaluated whether EGFP-expressing dopaminergic neurons displayed some of the well-established properties of dopaminergic neurons. Autoreceptor stimulation inhibited the activity of dopaminergic neurons while neurotensin receptor activation produced the opposite effect. Confocal imaging of the synaptic vesicle optical tracer FM4-64 in EGFP-expressing dopaminergic neurons demonstrated the feasibility of high resolution monitoring of the activity of single terminals established by these neurons. Together, this work provides evidence that primary cultures of postnatal TH-EGFP mice currently represent an excellent model to study the properties of these cells in culture.

Keywords: Dopamine; Culture; Fluorescence; Patch-clamp; Transgenic; Green fluorescent protein

1. Introduction

Dopaminergic neurons of the ventral mesencephalon are implicated in the regulation of motivated behavior. They are also a direct target of many drugs of abuse such as amphetamine and cocaine. Their physiological and pharmacological properties are thus of major interest. These neurons have been studied in vivo as well as in a number of more reduced models such as brain slices and primary cultures prepared from embryonic and postnatal rodent pups. Although primary culture models offer significant experimental advantages under many circumstances such as for fluorescence imaging experiments, for pharmacological approaches or for acute transfection strategies, the use of such models in single-cell physiological experiments is complicated by the fact that dopaminergic neurons typically account for only a small percentage of the total population of neurons. In embryonic cultures, dopaminergic neurons usually represent 1–5% of total neurons (di Porzio et al., 1987; Heyer, 1984; Silva et al., 1988) while in postnatal cultures, they usually account for 10–30% (Rayport et al., 1992; St-Gelais et al., 2004) (but see Masuko et al. (1992) and Shimoda et al. (1992) for microdissection strategies yielding higher percentages of dopaminergic neurons). A strategy to distinguish dopaminergic from non-dopaminergic neurons is thus required.

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doi:10.1016/j.jneumeth.2005.01.014

Differentiating between living dopaminergic and non-dopaminergic neurons on the basis of morphological features has been evaluated, but in practice this represents a highly unreliable method (Masuko et al., 1992; Ort et al., 1988). Electrophysiological criteria have also been proposed. For example, recordings made in vivo or in brain slice preparations have associated a unique action potential waveform with a duration of more than 2 s to dopaminergic neurons (Grace and Bunney, 1995). Other criteria such as a slow irregular (1–10 Hz) firing rate, a prominent afterhyperpolarization or the presence of an I_h-like hyperpolarization-activated inward current have also been described (Bunney et al., 1991; Johnson and North, 1992). Unfortunately, electrophysiological criteria of this type are highly unreliable in culture models. For instance, the firing pattern of identified dopaminergic neurons in culture was found to be regular (Cardozo, 1993) or silent (Rayport et al., 1992), this being probably due to the absence of the synaptic input that is normally present in the intact brain (Grace and Onn, 1989). Spike duration and the presence of an I_h current also do not allow to distinguish reliably between dopaminergic and non-dopaminergic neurons in culture (Cardozo, 1993; Masuko et al., 1992; Rayport et al., 1992).

To date the most reliable way to identify living dopaminergic neurons involves probing for their selective pharmacological properties (Johnson and North, 1992; Rayport et al., 1992). Unlike mesencephalic GABAergic neurons, dopaminergic neurons express D2-type autoreceptors. Therefore, only the latter respond to D2 agonists with a decrease in their firing rate. Conversely, mesencephalic GABAergic neurons, but not dopaminergic neurons, express somatodendritic μ-opioid receptors that lead to a decrease in firing when activated. Although such pharmacological criteria are as reliable in culture (Bergevin et al., 2002; Congar et al., 2002) as they are in vivo (Lacey et al., 1989), the application of receptor agonists and drug washout considerably lengthens experimental protocols and is not possible in dynamic fluorescence imaging experiments that are not accompanied by electrophysiological recordings.

The development of an alternative approach relying on optical signal would prove most useful. Such an attempt was made with the autofluorescent serotonin analogue, 5,7-dihydroxytryptamine (5,7-DHT) (Cardozo, 1993; Silva et al., 1988; Steensen et al., 1995). This experimental approach was based on the assumption that mesencephalic cultures are devoid of serotonin neurons and on the observation that serotonin derivatives, such as 5,7-DHT can be accumulated by catecholamine neurons through both serotonin and dopamine transporters (Dowling and Ehinger, 1975). However, one of the major disadvantages of this approach is the rapid photobleaching of the 5,7-DHT signal during UV illumination (Cardozo, 1993). More problematic is the fact that this approach has been found to be highly unreliable to identify dopaminergic neurons. Indeed in a recent study, this compound was found to accumulate preferentially in serotonin neurons and not in dopaminergic neurons (Franke et al., 2002). Another more effective strategy is to retrogradedly label dopaminergic neurons a few days before tissue dissection by injecting a fluorescent tracer in projection areas such as striatum (Rayport et al., 1992). Although this approach identifies dopaminergic neurons with high fidelity, it only labels a small proportion of dopaminergic neurons and is quite time-consuming.

Transgenic mouse models offer an alternate approach. To study dopaminergic neurons of the retina, Gustincich and co-workers (Gustincich et al., 1997) developed transgenic mice that express human placental alkaline phosphatase (PLAP) under the regulation of the tyrosine hydroxylase (TH) promoter. Catecholamine-containing neurons from these mice express PLAP on the outer surface of their cell membrane, allowing identification of living dopaminergic neurons using a PLAP antibody–fluorochrome conjugate. Although the use of these mice has been profitable to study retinal dopaminergic neurons in culture (Contini and Raviola, 2003; Feigenspan et al., 1998, 2000; Gustincich et al., 1999; Puopolo et al., 2001), this approach is relatively complex because it requires pre-exposure of living neurons to a fluorochrome–antibody conjugate. Although successful, this procedure can be quite expensive and under certain circumstances can be limited by poor penetration of antibodies in the experimental preparation. Antibodies could also possibly perturb living neurons. Finally, the usefulness of this model for the identification of midbrain dopaminergic neurons is unclear since only one previous study of mesencephalic dopaminergic neurons has been published (Mundorf et al., 2001).

More recently, Sawamoto and co-workers (Matsushita et al., 2002; Sawamoto et al., 2001) developed a new transgenic mouse model allowing direct visualization of midbrain dopaminergic neurons (see also Chuhma et al., 2004). Mice were engineered to express enhanced green fluorescent protein (EGFP) under the control of the TH promoter. The capacity of neurons derived from these mice to survive and differentiate in long-term culture has not yet been examined. The aim of this study was hence to develop a reliable primary culture model allowing a rapid and direct identification of postnatal dopaminergic neurons. Our first objective was to evaluate the selectivity of EGFP expression in dopaminergic neurons cultured from these mice. Our second objective was to evaluate whether the physiological properties of EGFP-expressing dopaminergic neurons were normal. We find that the majority of EGFP-expressing neurons cultured from the ventral mesencephalon of TH-EGFP mice are dopaminergic neurons and that EGFP-expressing dopaminergic neurons possess the typical physiological properties of dopaminergic neurons.

2. Materials and methods

2.1. Transgenic mice

All experiments were performed using the transgenic mice TH-EGFP/21-31 line carrying the EGFP gene under

the control of the TH promoter (Matsushita et al., 2002; Sawamoto et al., 2001). Offsprings that carried the transgene were identified by PCR on the genomic DNA extracted from tail biopsies. A 475 bp fragment of EGFP DNA was amplified by PCR, using the primers: AAGTTCATCTGCACCACCG and TGCTCAGGTATGGTTGTCG. Transgenic lines were maintained as heterozygous by breeding with C57BL/6J inbred mice.

2.2. Cell culture

Primary cultures of mesencephalic neurons from TH-GFP/21-31 transgenic mice were prepared according to recently described protocols (Bourque and Trudeau, 2000; Congar et al., 2002; Michel and Trudeau, 2000) derived from Cardozo (1993) and Sulzer et al. (1998). Dissociated neurons were plated on mesencephalic astrocytes grown in monolayers on pre-coated glass coverslips.

To prepare mesencephalic astrocyte cultures, TH-EGFP/21-31 mice pups (P0–P2) were cryoanasthetized. Cells from the mesencephalon were enzymatically dissociated using papain (Worthington Biochemical Corp., Lakewood, NJ, USA) and were grown in culture flasks for 5–10 days in Basal Medium Eagle with Earl's Salts (Sigma-Aldrich, Oakville, Ont., Canada) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco), Mito+ serum extender (VWR Canlab, Montreal, Canada) and 10% fetal calf serum (Gibco). A cold wash with vigorous shaking was used to dislodge neurons and microglial cells after 2 days in culture. After reaching confluence, astrocytes were trypsinized, washed, collected and plated at 100 000 living cells per milliliter on collagen/poly-L-lysine-coated coverslips. For single neuron microcultures, astrocytes were plated at a concentration of 60 000 living astrocytes per milliliter on poly-L-ornithine/agarose-covered glass coverslips, which had been sprayed with collagen (0.75 mg/ml) microdroplets (50–150 μM in diameter). This permitted the establishment of small groups of isolated cells (Segal et al., 1998).

To prepare neurons, a 1–2 mm thick coronal slice was cut at the level of the midbrain flexure. The ventral tegmental area and substantia nigra were isolated by microdissection. As for preparation of astrocytes, the tissue was digested with papain before being gently triturated. The dissociated cells were then collected by centrifugation and diluted at a density to optimize neuronal viability (240 000 living cells) and plated onto a pre-established mesencephalic astrocyte monolayer. For single neurons microcultures, dissociated cells were plated onto astrocyte microislands at a density of 80 000 living cells per milliliter. Cultures were incubated at 37 °C in 5% CO_2 atmosphere and maintained in Neurobasal-A/B27 medium (Gibco) supplemented with penicillin/streptomycin, GlutaMAX-1 (Gibco) and 10% fetal calf serum (Gibco, Logan, UT, USA). Astrocyte-conditioned Basal Medium Eagle was added to the standard Neurobasal-A medium to a proportion of 1:2.

2.3. Immunocytochemistry

Cultured neurons were fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS) (pH 7.4) and incubated with a monoclonal anti-TH antibody (TH-2 clone) (Sigma-Aldrich, Oakville, Ont., Canada) to confirm dopaminergic phenotype. The primary antibody was visualized using an Alexa-546-labeled secondary antibody (Molecular Probes Inc., Eugene, OR, USA). Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed by epifluorescence microscopy on a Nikon Eclipse TE-200 inverted microscope. Images of EGFP and TH immunofluorescence were acquired using a Hamamatsu Orca-II digital cooled CCD camera and an Inovision workstation using Isee software (Inovision Corporation, Raleigh, NC, USA).

Ectopic EGFP expression was evaluated by two complementary approaches. First, living, unfixed neurons were examined by epifluorescence microscopy. A field containing one or more EGFP-expressing neuron was randomly selected. All neurons showing unambiguous EGFP fluorescence, whether strong or weak, were counted. A phase-contrast image of the field was then captured and then blue fluorescent microspheres were deposited locally using a glass pipette. After fixation and immunocytochemistry against TH, the exact same field was localized using the fluorescent spheres and the phase-contrast image as an index. This permitted reliable determination of the dopaminergic or non-dopaminergic phenotype of all EGFP-expressing neurons. A second approach was to evaluate the phenotype of only the brightest EGFP-expressing neurons that were selected for electrophysiological experiments. Here again, a phase-contrast image was captured and fluorescent microspheres were used to identify the recorded neuron after fixation and immunocytochemistry.

2.4. Electrophysiology

Electrophysiological recordings were performed at room temperature on EGFP-expressing living neurons maintained for 10–18 days in culture. Cultures were transferred to a recording chamber that was fixed to the stage of an inverted Nikon Eclipse TE-200 microscope. The coverslip was constantly superfused with physiological saline solution using a gravity flow system (2.5–3 ml/min) with a standard extracellular bathing solution containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.35, ≅305 mOsm. Sucrose (6 mM) was added to the extracellular medium to adjust osmolarity. Drugs were bath applied, with a delay between valve opening and onset of drug action of approximately 15 s. Action potentials were recorded using the whole-cell current-clamp technique with a Warner PC-505 patch-clamp amplifier (Warner Instruments Corp., Hamden, CT, USA). Signals were filtered at 1 kHz, digitized at 10 kHz and recorded and analyzed using Pclamp7 software (Axon Instruments, Foster City, CA, USA) and

Mini Analysis software (Version 5.6) (Synaptosoft Inc., Leonia, NJ, USA), respectively. Borosilicate glass patch pipettes (5–7 MΩ) were filled with a potassium methylsulfate intrapipette solution containing (in mM): 145 $KMeSO_4$, 20 KCl, 10 NaCl, 0.1 EGTA, 2 ATP (Mg salt), 0.6 GTP (Tris salt), 10 HEPES, 10 phosphocreatine (Tris salt), pH 7.35, ≅295–300 mOsm.

Synaptic (or "autaptic") responses in single neurons were recorded with the perforated patch-clamp technique (amphotericin B, 150–200 μg/ml). Under conditions where dopaminergic neurons grow in isolation in microculture, they establish synaptic contacts on their own dendritic arbor and co-release glutamate together with dopamine. This allows the activity of synaptic terminals to be readily monitored (Bourque and Trudeau, 2000; Congar et al., 2002; Sulzer et al., 1998). During recordings, autaptic responses were evoked every 15 s by a brief (1 ms) depolarizing voltage step from a holding potential (V_H) of −50 mV. In dopaminergic neurons, this usually elicited a sodium "action current" followed by a 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)-sensitive glutamate-mediated inward autaptic EPSC. Input resistance was usually between 300 and 600 MΩ and was monitored periodically throughout experiments.

2.5. Calcium imaging

Changes in cytosolic intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured with Fura2-AM ratio fluorescence. Briefly, cells were loaded with Fura2-AM by incubating cells grown on 15-mm coverslips in saline containing 5 μM Fura2-AM and 0.02% pluronic acid (Molecular Probes, Eugene, OR, USA) for 50–60 min at room temperature. For $[Ca^{2+}]_i$ measurements, the coverslip was mounted in a recording chamber that was fixed to the stage of an inverted Nikon Eclipse TE-200 microscope. The coverslip was constantly superfused with physiological saline solution containing (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, 6 sucrose at a pH of 7.35. Imaging of Fura2-AM was performed using standard epifluorescence imaging through a GenIII+-intensified progressive line scan CCD camera (Stanford Photonics, Palo Alto, CA, USA) and a computer-controlled two-channel fast excitation wavelength switcher (DX-1000, Stanford Photonics). Standard image ratio pairs (340/380 nm) were acquired every 5 s and ratio values were analyzed using Axon Imaging Workbench software 4.0 (Axon Instruments).

2.6. Confocal imaging

Cells plated on 25 mm diameter coverslips were placed in an imaging chamber with integrated platinum stimulating electrodes (Warner Instruments, Hamden, USA), and the chamber was connected to a gravity perfusion system on the stage of the microscope. For loading with the vesicle recycling indicator FM4-64 (Molecular Probes), cells were exposed for 2 min to a saline solution containing 5 μM FM4-64 and 90 mM potassium (which replaced an equimolar concentration of NaCl). The cells were then rinsed for 10 min with saline solution. Images were taken with a point-scanning confocal microscope from Prairie Technologies LLC (Middleton, WI, USA). Excitation was performed with the 488 nm line of an argon ion laser and fluorescence emitted above 550 nm (yellow to orange) was measured. Images were taken every 15 s. After 2 min of recording, electrical field stimulation was applied at 2 Hz for 150 s or at 10 Hz for 60 s. Cells were then exposed to high potassium saline to completely release any remaining and releasable FM4-64 from the terminals. Images were analyzed using Metamorph software v4.5 from Universal Imaging Corp (USA).

3. Results

3.1. Selectivity of EGFP expression in dopaminergic neurons increases over time in culture

As previously reported (Matsushita et al., 2002), we first confirmed using immunocytochemistry that expression of EGFP co-localizes with TH in the ventral mesencephalon of TH-EGFP mice, thus validating expression in dopaminergic neurons of the substantia nigra and ventral tegmental area (not shown). Because postnatal cultures are usually prepared from P0 pups, we also evaluated co-localization at this developmental stage. We found that co-localization was also present but less extensive at this stage (not shown) perhaps due to lower or altered activity of the TH promoter around birth (Matsushita et al., 2002). All further experiments were performed on cultured mesencephalic neurons prepared from TH-EGFP mice. In total, 312 EGFP-expressing neurons were examined in detail.

We first evaluated the selectivity of EGFP expression in cultured dopaminergic neurons prepared from P0–P2 TH-EGFP mice. Ventral mesencephalic neurons were isolated by microdissection and enzymatically dissociated (Fig. 1A and B). They were then cultured together with mesencephalic astrocytes for periods of 24 h to 15 days. EGFP expression of living cultured neurons was detected by epifluorescence and confirmation of dopaminergic phenotype was obtained by immunocytochemistry using an antibody directed against TH. We observed numerous EGFP-expressing neurons in cultures prepared from these mice. Some neurons showed very bright EGFP fluorescence while others showed more modest signal. To directly quantify the selectivity of the expression of EGFP in dopaminergic neurons, we selected fields of living neurons where one or more EGFP-expressing neurons could be detected together with EGFP-negative neurons and locally deposited blue fluorescent microspheres so as to be able to localize the same field after immunocytochemistry against TH (Fig. 1C–E). In total, 220 EGFP-expressing neurons were evaluated in this set of experiments. We found

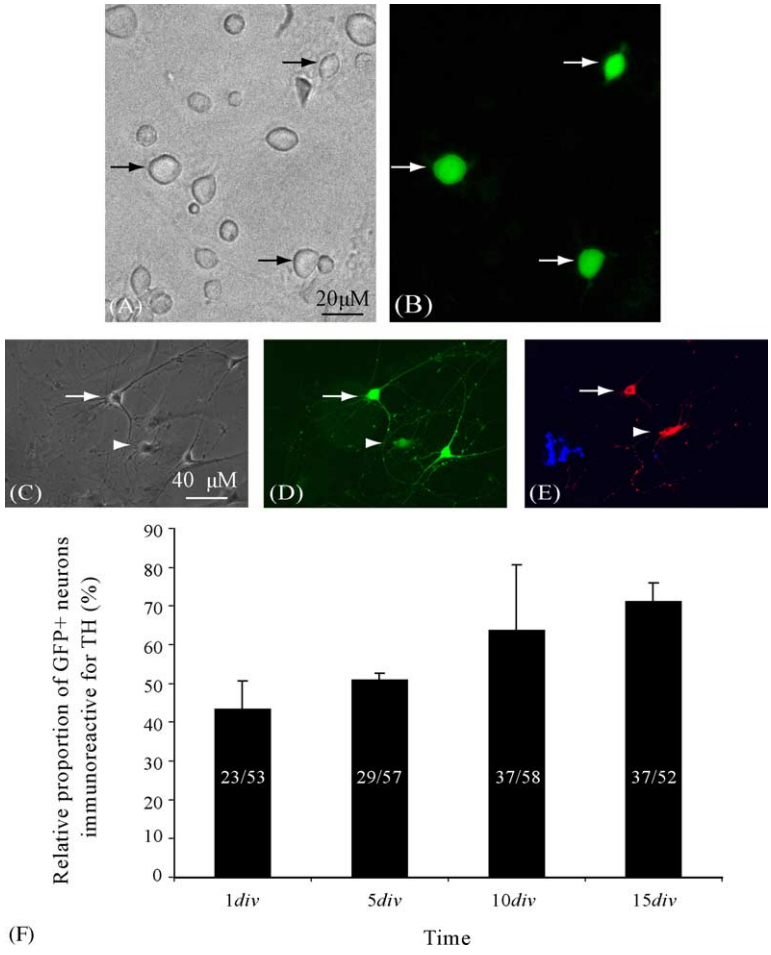

Fig. 1. Selectivity of EGFP expression in dopaminergic neurons increases over time in culture. (A and B) Living acutely dissociated neurons from the mesencephalon of TH-EGFP mice in phase contrast (A) and during epifluorescence (B). The white arrows identify three neurons that were EGFP-positive. (C–E) Live cultured neurons from the mesencephalon of EGFP-TH mice in phase contrast (C), during epifluorescence (D) and after fixation and post-hoc immunocytochemistry against TH (E). The white arrows identify two EGFP-positive neurons that were also TH-immunopositive. Note the presence of blue fluorescent microspheres that were used to localize neurons after immunocytochemistry. Ectopic expression of EGFP was found in a variable, but minor proportion of neurons. (F) Summary graph showing the selectivity of EGFP expression in dopaminergic neurons over time in culture (1–15 days in vitro; div). The proportion of EGFP expressing neurons that was immunoreactive for TH increases over time in culture. $\chi^2(3) = 10.225$, $p = 0.017$.

that 1 day after cell plating, $43 \pm 7\%$ of EGFP-expressing neurons were immunoreactive for TH (Fig. 1F). This proportion gradually increased over time spent in culture, reaching $71 \pm 4\%$ (after 15 days (Fig. 1F)) ($\chi^2(3) = 10.225$, $p = 0.017$). These experiments also revealed that a population of TH-immunoreactive neurons did not express EGFP (not shown).

To evaluate the usefulness of EGFP expression as a criterion for selecting dopaminergic neurons in physiological experiments, we observed living cultured dopaminergic neurons by epifluorescence and selected for patch-clamp recordings neurons that appeared to be the brightest EGFP-expressing cells. After recording, we deposited fluorescent microspheres next to the recorded neurons and processed the cells for TH immunocytochemistry to identify all dopaminergic neurons. We found that 90% of recorded neurons (36 of 40) were indeed dopaminergic neurons.

3.2. *Electrophysiological characteristics cannot help identify cultured dopaminergic neurons*

As described previously, dopaminergic neurons in vivo or in brain slices can be identified using a number of electrophysiological characteristics (Bunney et al., 1991). For example, in brain slice preparations, the major electrophysiological characteristic that allows discrimination of dopaminergic from non-dopaminergic neurons is the presence of an I_h current induced by hyperpolarizing steps (Grace and Bunney, 1995; Jiang et al., 1993; Lacey et al., 1989). We thus evaluated the presence of an I_h-like current in EGFP-expressing and EGFP-negative neurons in culture. For these experiments, the dopaminergic phenotype of neurons was confirmed by post-recording immunocytochemistry for TH. Although an I_h current could be detected in a minor proportion of neurons, it could not be reliably detected in EGFP-expressing dopaminergic neurons (Fig. 2A).

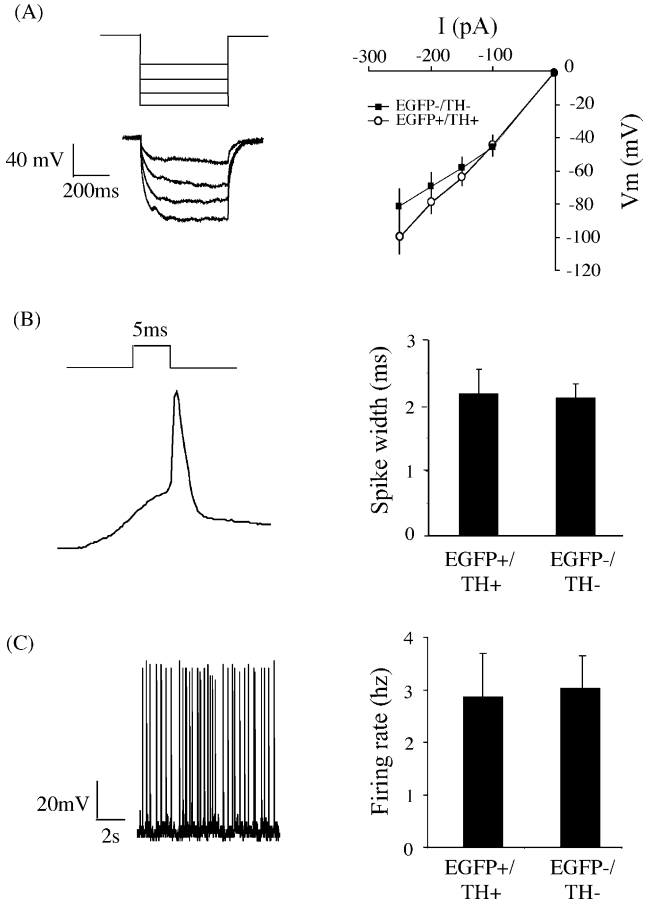

Fig. 2. Electrophysiological characteristics cannot help discriminate dopaminergic neurons in culture. (A) I–V relation of neurons expressing EGFP ($n = 5$, circles) and of other neurons not expressing EGFP ($n = 5$, black squares). The hyperpolarizing stimulation protocol and representative responses are shown to the left. (B) Action potential width of dopaminergic (EGFP+/TH+) ($n = 7$) and non-dopaminergic (EGFP−/TH−) ($n = 6$) neurons induced by a short depolarizing step. A typical action potential recorded from an EGFP+ neuron is shown to the left. (C) Spontaneous firing rate of dopaminergic and non-dopaminergic neurons. A typical firing rate pattern recorded from an EGFP+ neuron is shown to the left. Neurons expressing EGFP were confirmed to be dopaminergic by post-recording immunolabelling with an anti-TH antibody.

A comparison of current–voltage relationships in EGFP-expressing and EGFP-negative neurons confirmed that there was no significant difference in the inward current induced by hyperpolarizing steps between these two populations of neurons (two-way ANOVA, $F(1,24) = 1.985$, $p = 0.702$). Moreover, there was no difference between EGFP-expressing and EGFP-negative neurons in spike width, evaluated from spikes evoked by a short depolarizing step at hyperpolarized membrane potential (*t*-test, $p = 0.88$) (Fig. 2B), nor in spontaneous firing rate (*t*-test, $p = 0.89$) (Fig. 2C). Accommodation, evaluated during a prolonged depolarizing step, was also not different between the two types of neurons. Accommodation, defined by a cessation of firing during the 500 ms depolarizing step, occurred in 2/5 EGFP-expressing dopaminergic neurons and in 2/5 EGFP-negative neurons.

3.3. Activation of somatodendritic dopamine D2 receptors inhibits firing rate

To validate the use of cultures prepared from TH-EGFP mice, we evaluated whether dopaminergic neurons in these cultures possess some of the well-known physiological and pharmacological properties of dopaminergic neurons. We first evaluated the ability of a D2 receptor agonist to activate D2-like receptors and inhibit the firing rate of cultured dopaminergic neurons. Whole-cell patch-clamp recordings were obtained from EGFP-expressing dopaminergic neurons, identified by epifluorescence prior to recording and by post-recording immunocytochemistry against TH. As expected, quinpirole (1 μM), a D2 receptor selective agonist, significantly reduced spontaneous firing rate ($51 \pm 11\%$ inhibition; $n = 7$, *t*-test, $p = 0.002$) in comparison to the mean frequency observed in the baseline period (Fig. 3A–C). These results show that cultured dopaminergic neurons prepared from TH-EGFP mice possess functional somatodendritic D2 autoreceptors.

3.4. Terminal D2 receptors inhibit neurotransmitter release in dopaminergic neurons

D2 autoreceptors are also located on the axon terminals of dopaminergic neurons. To evaluate the function of such receptors in cultured EGFP-expressing dopaminergic neurons, we took advantage of the ability of isolated dopaminergic neurons in culture to co-release glutamate together with dopamine (Bourque and Trudeau, 2000; Sulzer et al., 1998). The glutamate-mediated synaptic currents recorded from neurons under such conditions are known to be robustly inhibited by D2 receptor activation (Congar et al., 2002). Experiments were thus performed on single EGFP-expressing dopaminergic neurons in a microisland culture system. During whole-cell recording a brief (1 ms) depolarizing voltage step evoked a fast inward sodium current (generating an unclamped action potential) followed by a postsynaptic, CNQX-sensitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated EPSC (autaptic EPSC) (Fig. 4A and B). Bath application of quinpirole (1 μM) reversibly decreased the peak amplitude of autaptic EPSCs in EGFP-expressing dopaminergic neurons (Fig. 4A and B). Quinpirole caused a $34.5 \pm 7.4\%$ inhibition of autaptic EPSCs amplitude when neurons were recorded in whole-cell mode (Fig. 4C) ($n = 6$, *t*-test, $p = 0.07$) whereas this inhibition reached $66.8 \pm 10.6\%$ when the recordings were made in perforated-patch configuration (Fig. 4D) ($n = 5$, Student's paired *t*-test, $p \leq 0.05$). These observations show that cultured dopaminergic neurons prepared from TH-EGFP mice possess functional terminal D2 autoreceptors.

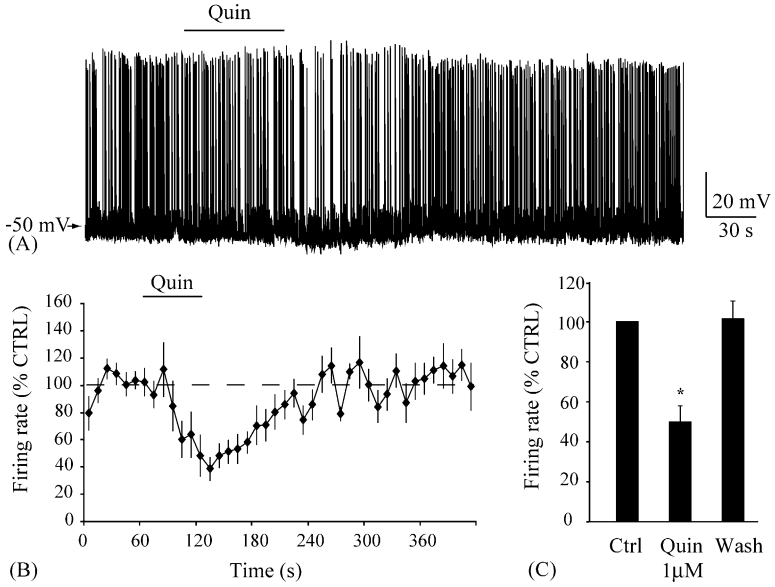

Fig. 3. Activation of somatodendritic dopamine D2 receptors inhibits firing rate. (A) Patch-clamp recording of spontaneous action potentials in an EGFP-expressing dopaminergic neuron. Quinpirole (1 μM) caused a strong reduction in firing rate. (B) Time-course of quipirole's effect on firing rate ($n = 7$). Firing rate was measured as the number of action potentials per 10 s bins and expressed as percent of control. (C) Summary graph of the effect of quinpirole on firing frequency in EGFP-expressing dopaminergic neurons ($n = 7$). The magnitude of the response to quinpirole was measured for 1 min at the maximum and normalized to that observed during the baseline period. Data are expressed as mean ± S.E.M. $^{*}p \leq 0.05$.

3.5. Dopaminergic neurons show an increased firing rate in response to neurotensin

To extend our physiological profiling of EGFP-expressing cultured dopaminergic neurons, we evaluated the effect of the peptide neurotensin on the firing rate of these neurons. The excitatory effect of this tridecapeptide on dopaminergic neurons is well established (Legault et al., 2002; Pinnock, 1985; Seutin et al., 1989; St-Gelais et al., 2004). The effect of the active fragment of neurotensin (NT(8–13)) on neuronal excitability was evaluated by whole-cell current-clamp recordings. Similarly to the response previously described in cultured rat dopaminergic neurons (St-Gelais et al., 2004), cultured dopaminergic neurons from TH-EGFP mice showed a significant increase in their firing rate in response to 100 nM NT(8–13) (115.6 ± 37.7%; $n = 8$, t-test, $p = 0.01$) (Fig. 5). Intracellular calcium imaging experiments with Fura-2 showed that NT(8–13) (100 nM) also caused a significant increase in intracellular calcium concentration in cultured dopaminergic neurons (average ratio increase was 1.74 ± 0.13, $n = 12$, Student's paired t-test, $p < 0.001$) (Fig. 6). This response was quantitatively similar to what was previously shown in cultured dopaminergic neurons from rat (St-Gelais et al., 2004).

3.6. Confocal imaging of EGFP-expressing dopaminergic neuron axon terminals with FM4-64

The ability to culture dopaminergic neurons and to identify them using EGFP fluorescence will facilitate investigations of the function of the axon terminals established by these neurons. For instance, the synaptic vesicle cycle can be studied optically using fluorescent styryl dyes that act as activity-dependent indicators of endocytosis and exocytosis (Aravanis et al., 2003; Betz et al., 1996). To establish the feasibility of such experiments, we evaluated the use of the styryl dye FM4-64 in EGFP-expressing single dopaminergic neurons in microisland cultures. Because this molecule emits at wavelengths above 550 μm (Wang and Zucker, 1998), measurements of its fluorescence emission should be possible without interference from EGFP, thus allowing us to study the exocytotic kinetics of dopaminergic terminals.

Axon terminals were first loaded by depolarizing cells with saline containing 90 mM K^+ together with 5 μM FM4-64. After thoroughly rinsing the non-sequestered FM4-64, action potentials were evoked by electrical field stimulation at either 2 Hz for 150 s (300 pulses) or at 10 Hz for 60 s (600 pulses). Neurons were then exposed to high K^+ saline to release any remaining and releasable FM4-64 from the terminals. Fig. 7 shows the characteristic dotted or punctate appearance of the fluorescent signal (Fig. 7A and B), which has been attributed to the varicose nature of axonal terminals, where the dye accumulates within small vesicles (Everett et al., 2002). The two stimulation protocols elicited release along different kinetics and, as expected, exocytosis occurred at a faster rate at the higher stimulation frequency (Fig. 7C). Together these data demonstrate the feasibility of using FM4-64 to image synaptic vesicle cycling in single EGFP-expressing dopaminergic neurons.

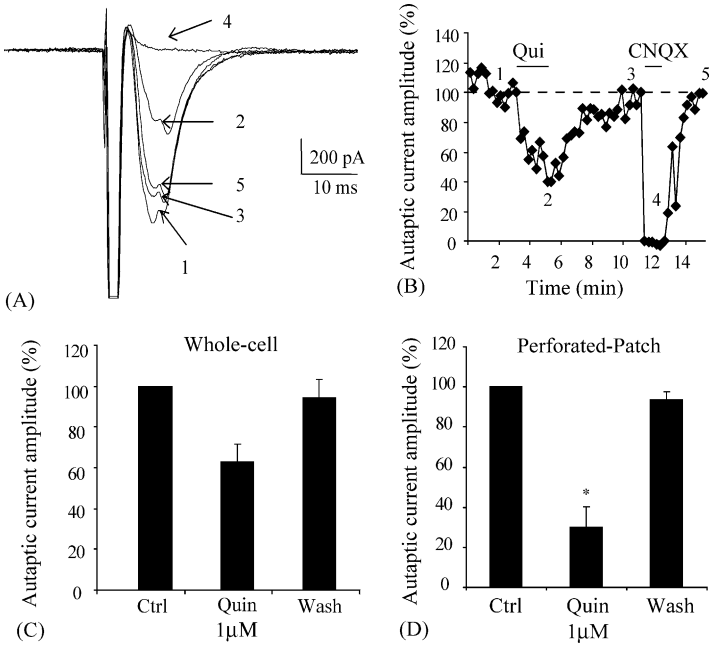

Fig. 4. Terminal D2 receptors inhibit neurotransmitter release in dopaminergic neurons. (A and B) Patch-clamp recording of EPSCs evoked by a single action potential in an isolated EGFP-expressing dopaminergic neuron. CNQX-sensitive glutamate-mediated autaptic EPSC were recorded in dopaminergic neurons (trace 4). Quinpirole (1 μM) caused a strong reduction of the amplitude of the autaptic current (trace 2). The baseline period and quinpirole and CNQX washout are shown, respectively, as traces 1, 3 and 5. (C and D) Summary graph of the effect of quinpirole on the amplitude of autaptic responses in EGFP-expressing dopaminergic neurons when recorded in whole-cell configuration (C) ($n = 6$) or in perforated patch configuration (D) ($n = 5$). The amplitude of autaptic responses was normalized to that observed during the baseline period. Data are expressed as mean ± S.E.M. $^{*}p \leq 0.05$.

4. Discussion

On the basis of the data presented here, we conclude that primary cultures prepared from TH-EGFP mice reliably allow the identification of dopaminergic neurons and hence provide an excellent model to study their physiological regulation. Indeed, dopaminergic neurons cultured from TH-EGFP mice show physiological properties very similar to those previously reported for these neurons in vivo, such as an inhibition of firing rate and neurotransmitter release in

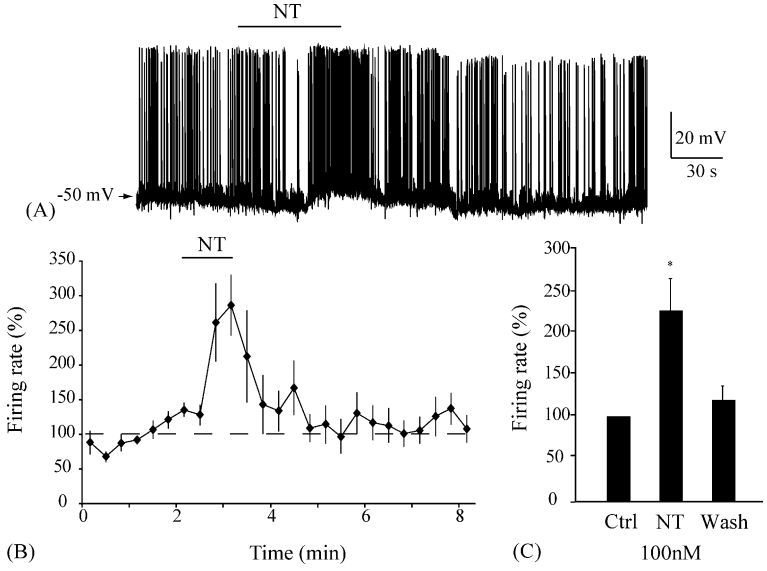

Fig. 5. Dopaminergic neurons show an increased firing rate in response to neurotensin. (A) Patch-clamp recording of spontaneous action potentials in an EGFP-expressing dopaminergic neuron. NT(8–13) (100 nM) produced an increase in firing rate. (B) Time-course of the response to NT(8–13) on dopaminergic neurons firing rate ($n = 8$). (C) Summary graph showing the average enhancement in firing rate caused by NT(8–13) (100 nM) ($n = 8$). The magnitude of response to NT(8–13) was measured for 1 min at the maximum and normalized to that observed during the baseline period. Data are expressed as means ± S.E.M. $^{*}p \leq 0.05$.

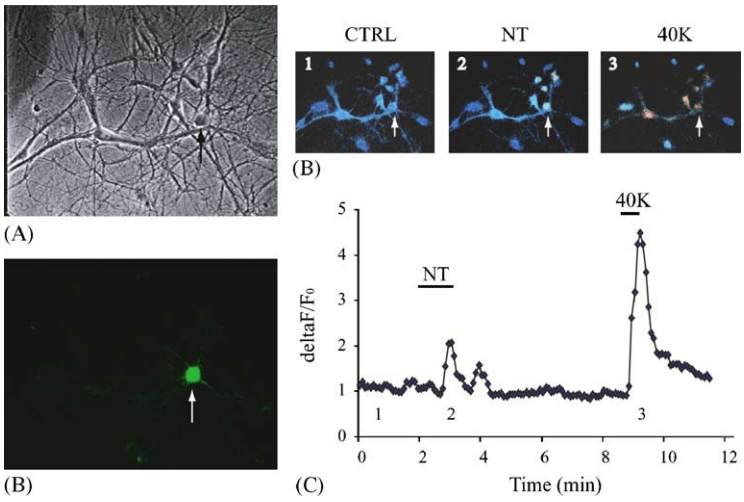

Fig. 6. Neurotensin induces an increase in intracellular calcium in dopaminergic neurons. (A) Phase contrast (upper image) and epifluorescence (lower image) were used to identify EGFP-expressing dopaminergic neurons prior to Fura-2 calcium imaging experiments. (B) False-colored image sequence illustrating that NT(8–13) (middle panel) caused a rise in intracellular calcium ($[Ca^{2+}]_i$) in an EGFP-positive dopaminergic neuron (white arrow). It should be noted that some EGFP-negative but dopaminergic neurons in the field also responded to NT(8–13). (C) Time-course of the rise in $[Ca^{2+}]_i$ in an EGFP-expressing dopaminergic neuron during a 60-s exposure to 100 nM NT(8–13) ($n = 12$). The cells were exposed to saline containing 40 mM K^+ (40 K) to depolarize neurons and evaluate their viability at the end of experiments. Data are represented as the mean fluorescence ratio as a function of time ($\Delta F/F_0$).

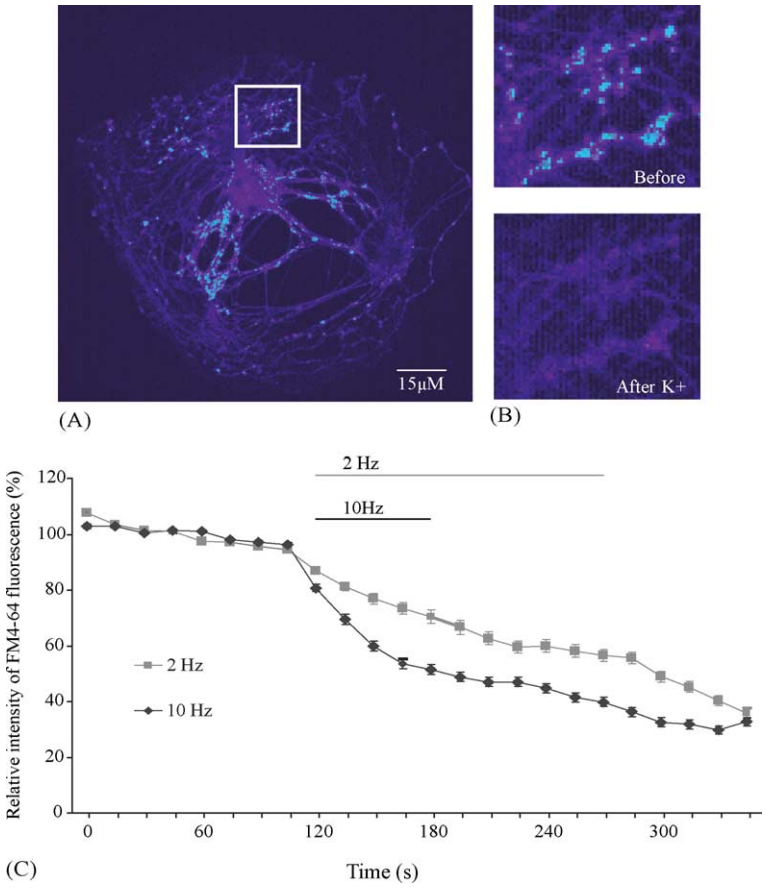

Fig. 7. FM4-64 allows the study of neurotransmitter release kinetics in dopaminergic neurons. (A) Cells exposed to FM4-64 and 90 mM potassium for 2 min and subsequently rinsed for 10 min in physiological saline display a dotted fluorescent signal. This signal represents internalized FM4-64 in presumed axon terminals. (B) Enlargement of (A) showing the FM4-64 signal before and after stimulation with 40 mM potassium. (C) Confocal imaging of FM4-64 showing the release kinetics upon electrical stimulation at 2 Hz for 150 s (300 pulses) and 10 Hz for 60 s (600 pulses). Images are taken every 15 s and data are expressed relative to the first 2 min of the control period. Bars represent the mean ± S.E.M. ($n = 5$ cells for 2 Hz; $n = 4$ cells for 10 Hz).

response to a D2 receptor agonist as well as an increase in excitability and in intracellular calcium in response to the neuromodulatory peptide neurotensin. Moreover, as EGFP expression by dopaminergic neurons does not interfere with fluorescent dyes like Fura-2 or FM4-64, this model can be useful for a number of dynamic fluorescence imaging studies. Overall, the use of the present model for the study of identified living dopaminergic neurons in culture is reliable and shows numerous advantages over alternate approaches.

In several previous studies, discrimination between dopaminergic and non-dopaminergic neurons was performed on the basis of a number of electrophysiological characteristics. This electrophysiological signature included a broad action potential, a slow irregular (1–10 Hz) firing rate, a prominent afterhyperpolarization and the presence of an I_h-like hyperpolarization-activated inward current (Bunney et al., 1991). However, consistent with previous findings (Masuko et al., 1992; Rayport et al., 1992), we show here that such properties cannot adequately identify dopaminergic neurons in culture. Indeed, we found that contrarily to the situation in vivo, these properties are variably expressed both in dopaminergic and non-dopaminergic mesencephalic neurons in culture. The reason for this difference is unclear but the absence of appropriate synaptic inputs or some regulatory signals under culture conditions could readily influence the level of expression of ionic channels that mediate I_h or influence cellular excitability. An alternate explanation could be that a proportion of dopaminergic neurons express very low levels of TH and are thus mistakenly identified as non-dopaminergic, thus masking any differences between dopaminergic and non-dopaminergic neurons. This possibility cannot be rejected off hand, but TH immunoreactivity is very efficient and even low level expression is readily detected, making it unlikely that a significant proportion of dopaminergic neurons were misclassified.

Dopaminergic neurons also display a unique pharmacological signature (Lacey et al., 1989). This includes (1) inhibitory response to a D2 autoreceptor agonist, (2) excitatory response to neurotensin and (3) lack of response to a μ-opioid receptor agonist (not shown). We find that similar to the in vivo situation, this pattern is found in cultured TH-EGFP dopaminergic neurons. Expression of EGFP therefore visibly does not interfere with the major signalling pathways activated by D2 and neurotensin receptors. In the present set of experiments, we have not re-investigated the responsiveness of mesencephalic GABA neurons to μ-opioid receptor agonists. However, in a recent study we have confirmed that cultured rat mesencephalic GABA neurons indeed continue to express this receptor (Bergevin et al., 2002). Although the pharmacological signature is a reliable strategy to identify dopaminergic neurons in culture, it is a time-consuming approach and it is incompatible with dynamic fluorescence imaging experiments that are not accompanied by electrophysiological recordings.

The use of primary cultures derived from TH-EGFP mice is also advantageous relative to other strategies that have been previously established as effective to identify dopaminergic neurons in culture. First, the approach is less time-consuming than microdissection strategies (Masuko et al., 1992) or retrograde labelling approaches (Rayport et al., 1992). Relative to the first of these two approaches, it also has the advantage of preserving the usual complement of dopaminergic and non-dopaminergic neurons present in the ventral mesencephalon.

Although we believe that using TH-EGFP mice is currently the most advantageous strategy to identify living dopaminergic neurons in culture, this model is not without its shortcomings. First, although the vast majority of EGFP-expressing neurons were confirmed to be bona fide dopaminergic neurons, ectopic expression of EGFP was detected in a small proportion of non-dopaminergic neurons. Fortunately, we found that ectopic expression decreased with time in culture such that this does not represent a significant problem unless one wishes to study acutely dissociated dopaminergic neurons from neonatal mice. This limited ectopic expression of EGFP has also been reported in vivo in these mice (Matsushita et al., 2002) and is likely to result from the absence of some regulatory elements in the TH promoter that was used to prepare the mice. However, the level of ectopic expression reported by this group (8%) is significantly lower than the level that we have estimated here (29% after 2 weeks in culture). This apparent discrepancy is likely to be due to methodological considerations. Indeed, Matsushita and co-workers examined EGFP-expression by dopaminergic neurons in fixed brain section. This is likely to have somewhat underestimated the level of ectopic EGFP expression since tissue fixation usually attenuates the brightness of EGFP fluorescence. To avoid this limitation, we counted live EGFP positive neurons and re-localized them after TH immunocytochemistry, an approach that would not be easy to implement in tissue sections. In support of this interpretation, we found that ectopic expression was only 10% when only the bright EGFP neurons were considered in patch-clamp experiments. This selectivity of EGFP expression in dopaminergic neurons is practically the same as that determined by Matsushita and colleagues (2002) and is low enough to insure that most EGFP-expressing neurons selected in physiological experiments will be dopaminergic.

A second limitation of the present model is that the specific TH-EGFP line that we used requires to be maintained as heterozygotes. The reason is that we have noted that homozygote pups display a slowed growth curve and eventually die after approximately 2–3 weeks. The reason for this is unclear but most likely results from insertion of one of the transgene copies within the coding region or the promoter region of an important gene. It should be possible to circumvent this problem by preparing additional mice lines or by using alternate engineering strategies (see for example, Chuhma et al. (2004)). In conclusion, this work provides evidence that primary cultures of postnatal TH-EGFP mice currently represent an excellent model to study the properties of these cells in culture.

Acknowledgements

This work was supported by a grant from the Canadian Institutes of Health Research to L.-E.T. and by a grant from the Ministry of Education, Science, Sports, Culture, and Technology of Japan to K.K. and O.H.L.-E.T. receives salary support from the Fonds de la Recherche en Santé du Québec (FRSQ). C.J., F.St-G. and G.D.F. were supported by scholarships from the FRSQ.

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