A Stem Cell-Derived Platform for Studying Single Synaptic Vesicles in Dopaminergic Synapses

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ABSTRACT

The exocytotic release of dopamine is one of the most characteristic but also one of the least appreciated processes in dopaminergic neurotransmission. Fluorescence imaging has yielded rich information about the properties of synaptic vesicles and the release of neurotransmitters in excitatory and inhibitory neurons. In contrast, imaging-based studies for in-depth understanding of synaptic vesicle behavior in dopamine neurons are lagging largely because of a lack of suitable preparations. Midbrain culture has been one of the most valuable preparations for the subcellular investigation of dopaminergic transmission; however, the paucity and fragility of cultured dopaminergic neurons limits their use for live cell imaging. Recent developments in stem cell technology have led to the successful production of dopamine neurons from embryonic or induced pluripotent stem cells. Although the dopaminergic identity of these stem cell-derived neurons has been characterized in different ways, vesicle-mediated dopamine release from their axonal terminals has been barely assessed. We report a more efficient procedure to reliably generate dopamine neurons from embryonic stem cells, and it yields more dopamine neurons with more dopaminergic axon projections than midbrain culture does. Using a collection of functional measurements, we show that stem cell-derived dopamine neurons are indistinguishable from those in midbrain culture. Taking advantage of this new preparation, we simultaneously tracked the turnover of hundreds of synaptic vesicles individually using pH-sensitive quantum dots. By doing so, we revealed distinct fusion kinetics of the dopamine-secreting vesicles, which is consistent within both preparations.

SIGNIFICANCE

For the use of stem cell-derived neurons in clinical applications, improved differentiation efficiency and more careful characterization of resultant cells are needed. A procedure has been refined for differentiation of mouse embryonic stem cells into functional dopamine neurons. This preparation provides a high yield of dopaminergic cells that are morphologically and functionally similar to cultured midbrain dopamine neurons and can be used as a platform for thorough investigation of the mechanisms of dopaminergic neurotransmission.

INTRODUCTION

As one of the major neurotransmitters, dopamine (DA) plays an important role in various brain functions including learning, memory, emotions, and movement control. Dysfunctions of dopaminergic transmission in the brain are evidently associated with Parkinson’s disease and a number of psychiatric disorders, such as schizophrenia, drug addiction, and psychosis [1–3]. Although transplantation of embryonic DA neurons or dopaminergic progenitors has been explored for the treatment of DA-related neurological disorders [4, 5], this approach is clinically impractical because of ethical concerns. The recent advances in stem cell technology have made it possible to generate DA neurons from pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells. These stem cell-derived DA neurons have been used for drug screening, disease modeling, and even treatment [6–9]; however, the efficiency of DA neuronal differentiation is far from satisfactory. More importantly, the physiological features and molecular mechanisms of dopaminergic neurotransmission in these stem cell-derived DA neurons have not been thoroughly investigated and compared with native midbrain DA neurons.

In this study, we report a simple but more efficient protocol to differentiate mouse embryonic stem cells (mESCs) into DA neurons that express an array of dopaminergic neuron-specific markers. We also show that these neurons exhibit electrophysiological properties and pharmacological responses similar to those of cultured midbrain DA neurons. Using various techniques...
including single-vesicle imaging, we reveal that synaptic vesicles (SVs) in both types of DA neurons behave indistinguishably, and they both possess a pattern of spatial distribution and release kinetics distinct from those in other types of synapses. Because it provides significantly more mature DA neurons with many more dopaminergic projections, this mESC-derived preparation is a better in vitro platform for studying cellular and molecular mechanisms underlying vesicle-mediated DA release from DA neurons.

MATERIALS AND METHODS

This section is given in the supplemental online data.

RESULTS AND DISCUSSION

We started with a two-step monolayer protocol (i.e., 6-day induction followed by 3-week terminal differentiation) to generate DA neurons from mESCs. Similar to previous reports [10, 11], we found only ∼2% cells positive for tyrosine hydroxylase (TH) and expressing neuron-specific class III β-tubulin (Tuj1) using immunocytochemistry (supplemental online Fig. 1). As reported previously [11, 12], some cells remained pluripotent (i.e., Oct-4 positive) after the induction (supplemental online Fig. 2A), suggesting that the induction was not comprehensive enough. We replated the culture for another round of induction (Fig. 1A), after which Oct-4 was no longer detectable (supplemental online Fig. 2B, 2C), whereas the number of Tuj1 or microtubule-associated protein 2 (MAP2)-positive cells expressing TH or vesicular monoamine transporter 2 (VMAT2) increased to 25%–30% (supplemental online Fig. 3). The increased yield was further confirmed by the expression of other dopaminergic markers [10, 13], including nuclear receptor related 1 protein (Nurr1), Lim-1/2, Engrailed 1 (En1), and forkhead box protein A2 (FOXA2) (supplemental online Fig. 4). It is noteworthy that more rounds of induction did not increase the yield further (data not shown), indicating that our extended induction was sufficient for subsequent differentiation. To directly distinguish differentiated DA neurons, mESCs were transfected with *phTH-GFP* plasmid (*phTH* is human tyrosine hydroxylase gene promoter; *GFP* is green fluorescent protein) [14].

**Figure 1.** Differentiation of mouse embryonic stem cells (mESCs) to dopamine (DA) neurons. (A): The scheme of the two-phase differentiation procedure. (B): Sample images of DA neurons derived from mESCs carrying *phTH-GFP*, immunostained for specific markers for neurons (Tuj1), synapses (Syn), DA neurons (TH), and dopamine-containing synaptic vesicles (VMAT2). Scale bar = 50 μm. (C): Sample images of GFP-positive neurons in MDNs and EDNs. Scale bar = 50 μm. (D): Sholl analysis shows more branching of GFP-positive neurons in EDNs than those in MDNs. n = 8 for each group thereafter. *, p < .05; **, p < .01. (E): Comparison of synapse numbers between GFP-positive neurons in EDNs and MDNs. **, p < .01. (F): Comparison of lateral density of synapses between the neurites of GFP-positive neurons in EDNs and MDNs. **, p < .01. (G): Comparison of VMAT2 staining between GFP-positive neurons in EDNs and MDNs. *, p < .05. Abbreviations: AA, ascorbic acid; B27, B-27 supplements; BDNF, brain-derived neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole; dcAMP, dibutyryl cyclic-AMP; DMEM/F12, Dulbecco’s modified Eagle’s medium with nutrient mixture F-12; EDN, embryonic stem cell–derived neuronal culture; FN, fibronectin; F2, basic fibroblast growth factor; F8, fibroblast growth factor 8; GDNF, glia-derived neurotrophic factor; GFP, green fluorescent protein; MDN, midbrain–derived neuronal culture; PO, polyornithine; SHH, sonic hedgehog; Syn, synapsin 1/2; TH, tyrosine hydroxylase; Tuj1, β-III tubulin; VMAT2, vesicular monoamine transporter 2.
GFP-positive cells expressed VMAT2 and TH (Fig. 1B), suggesting that they were DA neurons.

For comparison, we produced midbrain culture from phTH-GFP transgenic mice in which DA neurons expressed GFP [15]. Using immunocytochemistry, we compared the morphology of GFP-positive cells in ESC-derived neuronal culture (EDN) and midbrain-derived neuronal culture (MDN). The number of GFP-positive cells in EDN was greater than in MDN (Fig. 1C), and those GFP-positive cells in EDN had more complex neurites (Fig. 1C, 1D; supplemental online Table 1). In EDN, both the number (Fig. 1E) and the density (Fig. 1F) of VMAT2 puncta that overlapped with GFP-positive neurites were significantly higher, as was the average fluorescence intensity of those VMAT2 puncta (Fig. 1G), all of which suggests that EDN had more mature dopaminergic axon projections than MDN. Given that only a fraction of Tuj1-positive cells bore DA neuron-specific markers (supplemental online Fig. 4) in EDN, we reasoned that the presence and activity of non-DA neurons might facilitate the differentiation and survival of DA neurons [16].

In addition, more DA neurons might result in more dopamine, which, via autoreceptors, could promote neurogenesis and synaptogenesis [17, 18].

Next, we measured the membrane excitability of those GFP-positive neurons (Fig. 2A; supplemental online Fig. 5). In both groups, the stepwise depolarization triggered tetrodotoxin (TTX)-sensitive inward currents (Fig. 2B). The averaged peak values of inward and outward currents were similar in both groups (Fig. 2C, 2D). The resting membrane potentials (Vrest) were also indistinguishable. In current-clamp mode, current injection depolarized both groups of neurons and triggered action potentials (Fig. 2E). The threshold, amplitude, and duration of action potentials in GFP-positive EDNs were essentially the same as those of GFP-positive MDNs (supplemental online Table 2).

Membrane depolarization in response to the DA receptor D2 antagonist raclopride is a characteristic feature of midbrain DA neurons [19]. We tested the reactions of those GFP-positive neurons to raclopride. In the presence of raclopride, the Vrest of both groups rose to the same extent (Fig. 2F, 2G).
Accordingly, the thresholds for action potential were lowered by raclopride in both types of cells (Fig. 3H). These data indicate that both groups of GFP-positive neurons possess functional D2 receptors. We also looked for the hyperpolarization-activated current (Ih), another known conductance of dopaminergic neurons [20]; however, we could not detect Ih in either group of GFP-positive neurons, possibly because its expression is extremely variable in different subtypes of DA neurons in vivo [21]. In 5 of 6 GFP-positive cells among EDNs and 3 of 5 among MDNs, we were able to detect spontaneous postsynaptic currents and miniature postsynaptic currents that were resistant to TTX (1 μM) but blocked by the addition of NBQX (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[f] quinoxaline-2,3-dione; 10 μM), and bicuculline (30 μM) (Fig. 2I). The amplitude and frequency of the miniature events were similar between both groups of GFP-positive neurons (Fig. 2J, 2K).

A defining characteristic of DA neurons is activity-dependent release of DA [22, 23]. Using high-performance liquid chromatography (HPLC), we examined the cellular DA content in cell lysates from MDN and EDN and bath solutions from EDN on stimulation. n = 3 for each group. *, p < .05; **, p < .01. (B): Sample images of FM4-64 staining in MDNs or EDNs expressing phTH-GFP. Scale bar = 5 μm. (C): Scatterplot of average fluorescence intensities (FM4-64 vs. GFP) in FM4-64-defined synaptic boutons. All images and measurements were taken with the same settings. The green dashed line indicates the threshold of GFP fluorescence to determine GFP-positive or GFP-negative synapses. Six fields of view of each group. The same set of symbols will be used hereafter. (D): Scatterplot of FM4-64 fluorescence intensities versus areas from the same sets of images. The linear regression fittings of the four types of synapses show significantly less FM4-64 in GFP-positive synapses for both MDNs and EDNs. *, p < .05. (E): Loss of synaptic FM4-64 fluorescence upon 90-mM K+ stimulation. The insert shows that the decay time constant for GFP-positive synapses in both MDNs and EDNs (solid line) is significantly slower than that for GFP-negative synapses (dashed line). n = 6 for each group. *, p < .05. Abbreviations: a.u., arbitrary unit; DA, dopamine; EDN, embryonic stem cell-derived neuronal culture; GFP, green fluorescent protein; MDN, midbrain-derived neuronal culture.
Figure 4. Behavior of single synaptic vesicles (SVs) in MDNs and EDNs. (A): Sample images of MDNs (Ai) or EDNs (Aii) expressing phTH-GFP and loaded with single Qdots and FM4-64. Colored arrowheads indicate the positions of single SVs in corresponding fluorescence images, and white or purple arrowheads indicate single Qdot-loaded SVs in GFP-positive or -negative processes, respectively. Scale bar = 5 μm. (B): Cumulative distributions of the latency of first fusion conducted by Qdot-labeled single SVs shows no significant difference regardless of GFP fluorescence or origin (MDNs or EDNs). Six fields of view of each group; all p > .05. (C): From the same images, FRF ratios during 1-minute 10-Hz electric stimulation showed no significant difference. All p > .05. (D): The fractions of SVs conducting various rounds of FRF were not different, regardless of GFP fluorescence or origin (MDNs or EDNs). All p > .05. (E): Sample traces of Qdot photoluminescence changes during 30-Hz imaging. (F): Although the correlations between pore opening time and SV reacidification time constant (τ) of single SVs were similar among all four groups of synapses, the averages of both variables were significantly smaller in GFP-positive synapses of MDNs and EDNs. Five fields of view of each group. ∗, p < .05. (G): The degree of SV opening (i.e., the deacidification of SV lumen), reflected by the increase of single Qdot photoluminescence, was significantly less in GFP-positive synapses of both MDNs and EDNs. ∗, p < .05; ∗∗, p < .01. Abbreviations: EDN, embryonic stem cell-derived neuronal culture; FRF, fast reversible fusion; GFP, green fluorescent protein; MDN, midbrain-derived neuronal culture; Qdot, quantum dot.
but larger than the fusion pore (supplemental online Fig. 7), which led to one Qdot per vesicle and limited Qdot release to only full-collapse fusion (FCF). In addition, their photoluminescence is pH-sensitive and compatible with GFP and FM4-64 (supplemental online Fig. 8). In the presence of 90-mM K+ and 0.8-nM Qdots, we achieved a sparse loading of Qdots in FM4-64-defined synapses (Fig. 4A). Based on quantal analysis (supplemental online Fig. 9A, 9B) and single Qdot-blinking measurements, we estimated that most loaded synapses had single Qdot-labeled SVs. Following prolonged perfusion to remove cell surface Qdots, we applied 60-second, 10-Hz field stimulation and detected single-vesicle exocytotic events based on the change of Qdot photoluminescence (supplemental online Fig. 9C, 9D). The latency of first fusion showed no significant difference between GFP-positive and -negative FM4-64 puncta in both MDNs and EDNs (Fig. 4B), indicating no difference in SV release probability. We then examined the modes of SV release, namely, FCF and fast reversible fusion (FRF), based on the distinct Qdot signals [27] (supplemental online Fig. 9C, 9D). Side-by-side comparison of FRF ratios between dopaminergic and nondopaminergic terminals in both MDNs and EDNs showed no significant difference during field stimulation (Fig. 4C). Consistently, we did not observe any difference in FRF incidents per SVs (Fig. 4D). Collectively, these results suggest that the release and retrieval of SVs in DA neurons were similar to those of non-DA neurons. Alternatively, the slower FM4-64 unloading in EDNs or MDNs may reflect incomplete release of SV content because of limited opening of fusion pores, especially during FRF [28]. This was particularly intriguing because FRF dominated the initial phase of the release (Fig. 4C) when the difference in FM4-64 unloading was the most obvious (Fig. 3E). Thus, we increased the imaging rate to 30 Hz to better resolve the duration of fusion pore opening and the time constant for SV reacidification (Fig. 4E) during FRF. We indeed found a significantly shorter duration of SV opening accompanied by faster SV reacidification in FRF events (Fig. 4F), supporting the idea of restricted fusion pore opening. In addition, we were able to detect a correlated reduction in Qdot photoluminescence increase in DA neurons during FRF (Fig. 4G). Notably, those differences were more significant in EDNs due to a larger number of GFP-positive synapses.

**CONCLUSION**

Our data demonstrate that a simple extension of the inductive step significantly improves the yield of DA neurons differentiated from mESCs. Moreover, such preparation is better for the mechanistic investigation of dopaminergic transmission in vitro because it provides many synaptically matured DA neurons. Using both EDN and MDN, we illustrate that the axon terminals of DA neurons have smaller and more dispersed pools of releasable vesicles compared with nondopaminergic presynaptic terminals, and that DA synaptic vesicles likely release their contents partially during transient and reversible fusion. These characteristics of dopaminergic synapses possibly reflect a presynaptic adaptation for handling cytotoxic dopamine and satisfy the unique needs of dopaminergic transmission [29]. We believe that our EDN preparation is an ideal platform for in vitro studies, especially those regarding the distinct molecular mechanisms regulating presynaptic dopamine release.

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**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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**AUTHOR CONTRIBUTIONS**

H.G.: conception/design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; R.M.L.: collection and/or assembly of data, data analysis, manuscript writing; D.K.: provision of study material or patients, collection and/or assembly of data; L.I.: provision of study material or patients; Q.Z.: conception/design, collection and/or assembly of data, data analysis and interpretation, manuscript writing.

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