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Validation of a novel protocol to differentiate striatal medium spiny neurons from small-molecules neural progenitor cells

Validazione di un nuovo protocollo per la differenziazione di neuroni spinosi medi striatali da cellule progenitrici neurali ottenute tramite small molecules

Supervisor:

Prof. Ilaria Maria Morella

Co-supervisor:

Dott.ssa Cecilia Zuglian

Experimental thesis by:

Fatima Kresht

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ABSTRACT

Medium spiny neurons (MSNs) constitute approximately 90-95% of striatal cells and represent the primary integrative and output component of the striatum.

Due to their extensive involvement in cortico-striatal circuits, MSNs play an essential role in coordinating motor, cognitive and behavioural processes. Therefore, alterations in MSNs function and striatal network organization have been strongly linked to a broad spectrum of neurological conditions.

Growing evidence indicates that dysfunction within the striato-cortical tracts is a key feature of several neurodevelopmental disorders (NDDs), which are characterized by impairments in social interaction, cognition, reward processing and attention.

Despite their functional importance, the molecular and developmental mechanisms underlying MSNs specification and maturation remain partially understood. MSN populations originate from transient embryonic regions known as ganglionic eminences (GEs), which also give rise to diverse interneuron populations across multiple brain regions. The transient nature of these structures, their complex developmental dynamics and interspecies differences in brain composition, present major limitations for accurately modelling human striatal development using conventional experimental systems.

Efforts to recapitulate MSNs development in vitro have largely relied on human stem cell-based systems, aiming to mimic key aspects of striatal maturation and patterning. These approaches rely on the temporal and dose-dependent modulation of key developmental signalling pathways, such as Sonic Hedgehog (SHH), Wingless-related integration site (WNT), and BMP (Bone Morphogenic Proteins)-related mechanisms, often implemented through small molecule-mediated modulation to achieve ventral telencephalic identity. However, these strategies often require prolonged differentiation timelines and complex culture conditions and remain limited by the generation of heterogeneous neuronal populations with variable differentiation efficiency.

In this study, Activin A was investigated as a potential regulator of MSNs development, using human derived small molecule neuronal progenitor cells (smNPCs) cultured in N2B27 media as starting cell population. An initial one-week differentiation was performed to identify the most suitable conditions for striatal MSNs maturation. smNPCs were exposed to increasing concentrations of Activin A, in the presence or absence of RB5, a cell penetrating peptide that

potentiates ERK signalling pathway, crucial for cell survival, proliferation and differentiation. Early differentiation outcomes were assessed using the MSN-early markers CTIP2 and FOXP2. Among the tested concentrations, treatment with 100ng/mL Activin A in the absence of RB5 resulted in the highest FOXP2 expression, suggesting enhanced early MSNs specification. This optimal condition was subsequently applied in a four-week differentiation protocol, to evaluate neuronal maturation over time. Prolonged Activin A exposure promoted progressive maturation, with the highest DARPP-32 expression detected at week 4. In addition, morphological observations revealed enhanced neurite outgrowth under this long-term Activin A treatment condition.

Collectively, these findings suggest that Activin A is promising regulator of MSNs differentiation from human smNPCs, promoting sustained neuronal maturation, supporting the development of in vitro models for studying human striatal development, disease mechanisms, and potential discovery approaches for neurological disorders.

RIASSUNTO

I neuroni spinosi medi (MSN) costituiscono circa il 90–95% delle cellule dello striato e rappresentano i principali neuroni di proiezione di questa struttura. Essi svolgono un ruolo centrale nell'integrazione e nella trasmissione delle informazioni all'interno dei circuiti cortico-striatali, contribuendo in modo determinante al controllo dei movimenti volontari, dei processi cognitivi e dei comportamenti motivazionali. Alterazioni della funzione degli MSN e dell'organizzazione della rete striatale sono state associate a numerose patologie neurologiche. Evidenze crescenti indicano che la disfunzione delle connessioni striato-corticali rappresenta una caratteristica comune di diversi disturbi del neurosviluppo (NDD), caratterizzati da compromissioni dell'interazione sociale, della cognizione, dei meccanismi di ricompensa e dell'attenzione.

Nonostante la loro rilevanza funzionale, i meccanismi molecolari e i processi di sviluppo che regolano la specificazione e la maturazione degli MSN non sono stati ancora completamente chiariti. Tali neuroni originano da regioni embrionali transitorie denominate eminenze ganglioniche (GE), dalle quali derivano anche diverse popolazioni di interneuroni distribuite in differenti aree cerebrali. La natura temporanea di queste strutture, la complessità della loro dinamica di sviluppo e le differenze nella composizione cerebrale tra specie rappresentano importanti limitazioni per una modellizzazione accurata dello sviluppo striatale umano mediante sistemi sperimentali convenzionali.

I tentativi di ricapitolare in vitro lo sviluppo degli MSN si sono prevalentemente basati su sistemi derivati da cellule staminali umane, con l'obiettivo di riprodurre gli aspetti fondamentali della maturazione e dell'organizzazione regionale dello striato. Tali protocolli si fondano sulla modulazione temporale e dose-dipendente di vie di segnalazione cruciali per lo sviluppo embrionale, tra cui SHH (Sonic Hedgehog), Wingless-related integration site (WNT) e BMP (Bone Morphogenetic Proteins), spesso mediante l'impiego di small molecules al fine di ottenere un'identità telencefalica ventrale. Tuttavia, queste strategie richiedono tempi di differenziamento prolungati e condizioni di coltura complesse. Un ulteriore limite di queste strategie è rappresentato dalla generazione di popolazioni neuronali eterogenee, che avviene con efficienza variabile.

Nel presente studio, la proteina ricombinante Activina A è stata valutata come potenziale regolatore del differenziamento degli MSN, utilizzando cellule progenitrici neuronali umane come popolazione cellulare di partenza, ottenute mediante modulazione con small molecules e coltivate in mezzo N2B27. È stato inizialmente effettuato un protocollo di differenziamento della durata di una settimana al fine di identificare le condizioni più idonee per maturazione striatale. Le cellule sono state esposte a concentrazioni crescenti di Activina A, in presenza o in assenza di RB5, un peptide in grado di potenziare la via di segnalazione ERK, fondamentale per la sopravvivenza, la proliferazione e il differenziamento cellulare. Gli esiti precoci del differenziamento sono stati valutati mediante l'analisi dei marcatori CTIP2 e FOXP2. Tra le condizioni testate, il trattamento con 100 ng/m Activina A in assenza di RB5 ha determinato la più elevata espressione di FOXP2, suggerendo una maggiore efficienza nella specificazione precoce degli MSN. Tale condizione è stata successivamente applicata in un protocollo di differenziamento della durata di quattro settimane per valutare la maturazione degli MSN nel tempo. L'esposizione prolungata ad Activina A ha promosso una maturazione progressiva, dei progenitore neurali in MSN, con la massima espressione di DARPP-32 osservata alla quarta settimana. Inoltre, le osservazioni morfologiche hanno evidenziato un incremento dell'estensione dei neuriti in seguito al trattamento prolungato.

Nel complesso, questi risultati suggeriscono che l'Activina A rappresenta un promettente regolatore del differenziamento degli MSN a partire da cellule progenitrici neuronali umane, favorendo una maturazione neuronale sostenuta e supportando lo sviluppo di modelli in vitro per lo studio dello sviluppo striatale umano, dei meccanismi patologici e di possibili strategie terapeutiche per i disturbi neurologici.

1. INTRODUCTION

1.1 The Basal Ganglia

The basal ganglia consist of a coordinated ensemble of interconnected nuclei located deep within the forebrain, where they participate in the regulation of movement, influencing motor learning, higher-order executive processes, behavioural regulation, and emotional functions (Lanciego et al., 2012).

These nuclei interact closely with cortical, thalamic, and brainstem regions, thereby supporting the integration of motor, cognitive, and limbic information (Haber, 2016).

At the functional level, the basal ganglia provide a fundamental computational framework for behavioural control and action selection. They facilitate the initiation of suitable motor actions while simultaneously suppressing competing or maladaptive alternatives, thereby enabling appropriate voluntary actions. The basal ganglia also contribute to the acquisition of goal-directed behaviours and gradual habits consolidation (Mink, 2018).

At the anatomical level, the basal ganglia and their associated nuclei can be organized into three major groups: output, intrinsic and input nuclei (Figure 1).

The output nuclei, consisting of the internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr), represent the final processing stage within the basal ganglia.

The intrinsic nuclei comprise the external segment of the globus pallidus (GPe), the subthalamic nucleus (STN), and substantia nigra pars compacta (SNc), which are anatomically positioned to mediate intra-basal ganglia signal integration (Gerfen & Surmeier, 2011; Lanciego et al., 2012).

The input nucleus of the basal ganglia is the striatum, which is formed by the caudate nucleus, putamen and nucleus accumbens (NAc). Within the striatum, glutamatergic afferents from cortical and subcortical regions converge with dopaminergic projections arising from midbrain nuclei, conferring to this structure a central role in learning and reward-guided behaviours.

The striatum is predominantly composed of medium spiny neurons (MSNs), which account for approximately 95% of the neuronal population in both the dorsal and ventral part of the striatum, whereas interneurons represent only a minor fraction (5%). The MSNs integrate complex incoming signals and transmit processed information through the basal ganglia pathways (Cerovic et al., 2013) .

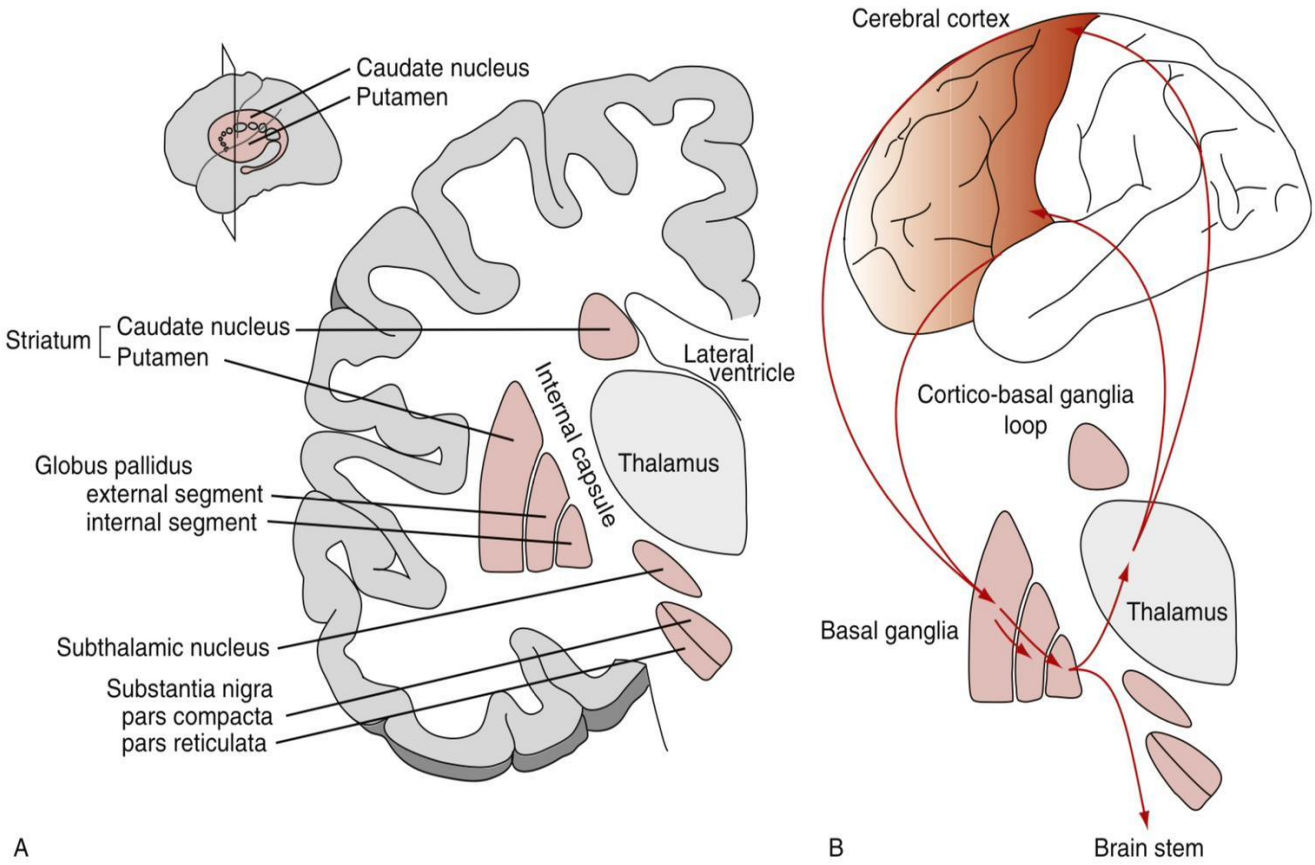

Figure 1. Basal ganglia organization (Adapted from Nambu, 2017).

1.2 The Striatum

1.2.1 Structural organization and cellular architecture of the striatum

Within the basal ganglia, the striatum constitutes the most prominent subcortical component, reaching an approximate volume of 10 cm^3 in the human brain. It represents a highly heterogenous region that functions as a central integrative hub, receiving widespread projections from the cerebral cortex and different subcortical areas, thereby contributing to the coordination of a broad range of complex behaviours (Basile et al., 2021; Lanciego et al., 2012). At the anatomical level, the striatum is commonly divided into two major regions: the dorsal striatum (DS) and ventral striatum (VS) (Helie et al., 2013).

In primates, the dorsal striatum consists of the caudate nucleus and the putamen, two structures that are anatomically separated by the internal capsule (Haber, 2016) (Figure 2).
The ventral striatum largely corresponds to the nucleus accumbens, which can be further subdivided into core and shell compartments (Cerovic et al., 2013).

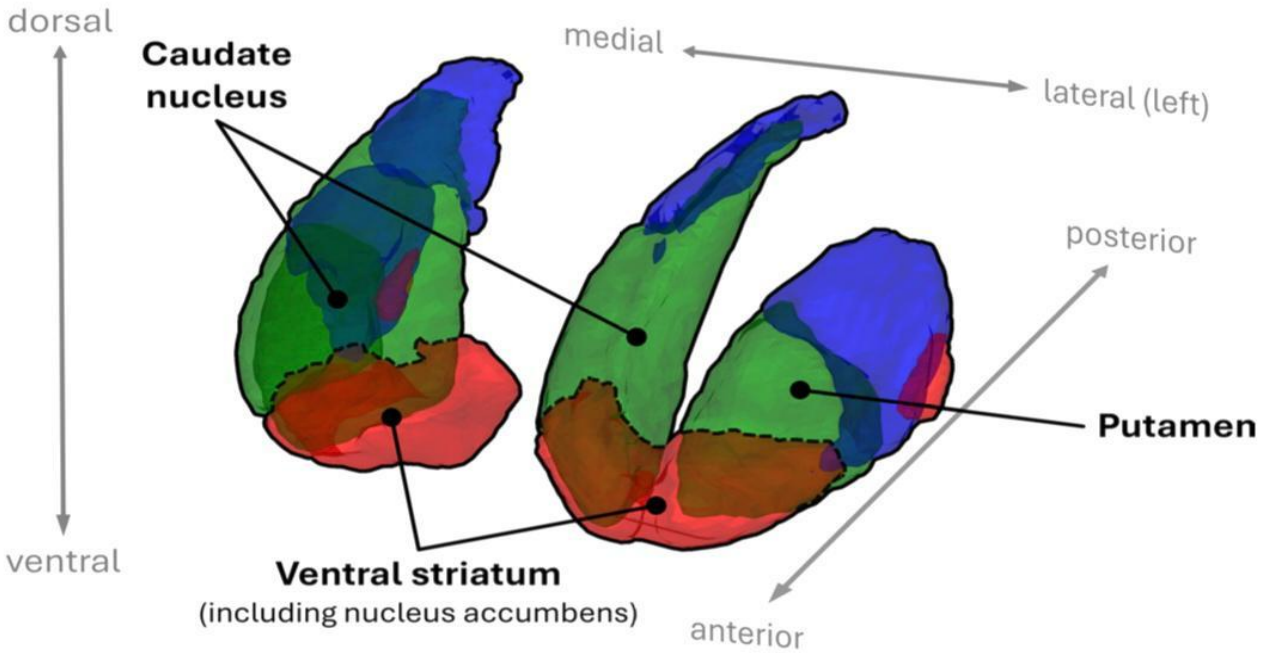

Figure 2. Macro anatomical organization of the striatum (Adapted from Lichte et al., 2025).

At the cellular level, the striatum displays a highly specialized neuronal composition, being predominantly populated by the MSNs. In addition to their inhibitory γ-aminobutyric acid (GABA) phenotypic nature, they synthesize and release specific neuropeptides, including enkephalin, substance P, and dynorphin (Rocha et al., 2023).
The remaining neuronal population consists of interneurons, which account for a minor fraction (5 %) of striatal neurons and are characterized by smooth, aspiny dendrites. These interneurons comprise a heterogenous group that can be classified based on their morphological characteristics and neurochemical profile. One prominent class is represented by large cholinergic interneurons, commonly referred to as tonically active neurons (TANs), which have a pattern of spontaneous firing activity. Additional interneuron subtypes include fast-spiking neurons (FSI), marked by parvalbumin expression, as well as interneurons expressing

calretinin or synthesizing nitric oxide. Notably, both TANs and FSIs strongly modulate the activity of MSNs and are themselves subject to dopaminergic regulation, together forming a complex intrastriatal microcircuit (Lanciego et al., 2012; Matamales et al., 2009).

1.3 The Medium Spiny Neurons (MSNs)

1.3.1 Morphological Organization of the MSNs

The MSNs are GABAergic projection neurons, characterized by a well-organized morphological architecture that combines compact somatic dimensions with an extensive dendritic arborization densely populated with dendritic spines (Bicanic et al., 2017; C. J. Wilson & Groves, 1980).

Quantitative analyses have shown that the surface area of the soma ranges between 160 and 180 μm², from which five to six primary dendrites emerge. Each neuron contains approximately 40 dendritic segments, generating a radially organized and branched dendritic tree. The total dendritic length is approximately 2,100 μm, with an average length around 420 μm per individual dendrite. Segment length varies according to dendritic location, with intermediate segments averaging ~ 22 μm, terminal segments about ~ 100 μm and incomplete segments ~ 65 μm (Bicanic et al., 2017) (Figure 3).

Most of the dendritic arbor is confined to a limited spatial range, with many branches located within 20–70 μm from the soma. MSNs dendrites are densely covered with spines, whose density increases toward distal segments, thereby expanding the postsynaptic surface for synaptic integration. Consistent with their architecture, each MSN is estimated to receive thousands of excitatory synaptic contacts, ranging from 5,000 to 15,000 cortical synapses, most of which are formed on individual dendritic spines (Mink, 1996). In addition to their dendritic organization, the MSNs projects axons that give rise to extensive local collaterals, which may either overlap the dendritic field or extend over distances greater than 1 mm, thus supporting intrastriatal inhibitory interactions (Bicanic et al., 2017; Mink, 1996).

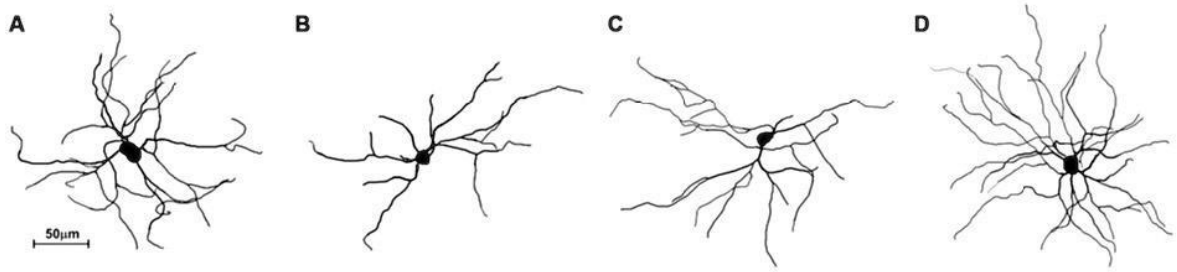

Figure 3. 3D reconstructions of mouse MSNs showing somato-dendritic variability (Adapted from Bicanic et al., 2017).

1.3.2 Striatal Output Pathways mediated by the MSNs

The functional organization of the basal ganglia is classically described by two major pathways originating in the striatum: the direct and the indirect pathways (Figure 4).

Functionally, these two circuits exert opposite effects on motor behaviour, and any disruption of this coordinated activity is thought to underlie both hyperkinetic and hypokinetic conditions (Gerfen, 2023).

Direct pathway-MSNs (dMSNs) are activated from cortical glutamatergic inputs and project directly to the internal segment of globus pallidus (GPi) and the substantia nigra pars reticulata (SNr). This reduces their inhibitory output to the thalamus, resulting in thalamic disinhibition and enhanced thalamocortical excitation, thereby facilitating movement initiation.

Indirect pathway-MSNs (iMSNs) project first to the external segment of the globus pallidus (GPe), where they exert inhibitory control. This decreases GPe-mediated inhibition of the subthalamic nucleus (STN), leading to increased excitatory input to the GPi and SNr, strengthening thalamic inhibition. Through this cascade, the iMSNs suppress motor output by limiting cortical activation (Albin et al., 1989; Franz et al., 2023).

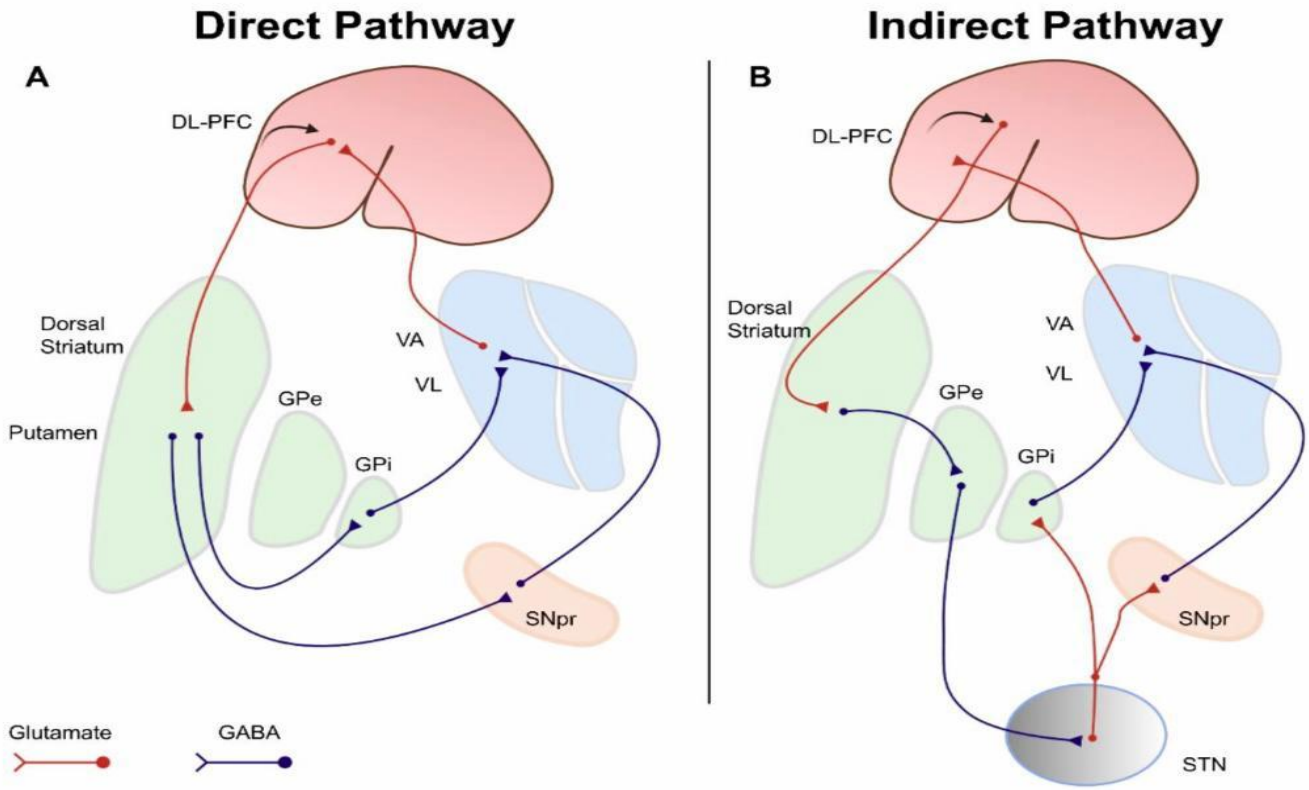

Figure 4. Graphical representation of direct and indirect pathways of the basal ganglia. (Adapted from Acharya & Kim, 2021).

1.3.3 Dopaminergic modulation of the MSNs

Dopaminergic modulation of the MSNs is mediated predominantly by Dopamine D1 and Dopamine D2 receptors, which are differently expressed across MSN populations and exert opposing effects on intracellular signalling. D1 receptors are expressed by the MSNs of the direct pathway, whereas D2 receptors characterize the MSNs of the indirect pathway (Calabresi et al., 2014).

Both D1 and D2 receptors belong to the family of G protein coupled receptors (GPCRs) that regulate neuronal activity through second-messenger cascades (Fernandez et al., 2006). Activation of D1 receptors stimulates adenylyl cyclase through *Gs/olf* proteins, leading to increased intracellular cAMP levels and promoting protein kinase A (PKA) activation. In contrast, D2 receptors are coupled to *Gi/o* proteins and inhibit cAMP production, thereby reducing PKA signalling (Figure 5). Through these opposing mechanisms, dopamine tunes

MSNs excitability via regulation of cAMP-PKA pathways. This signalling mechanism is spatially organized, as dendritic PKA modulates ion channels and glutamatergic receptors, whereas somatic signalling influences transcriptional regulators such as CREB and DARPP-32. Together, these mechanisms enable dopamine to shape striatal output (Nagai et al., 2016).

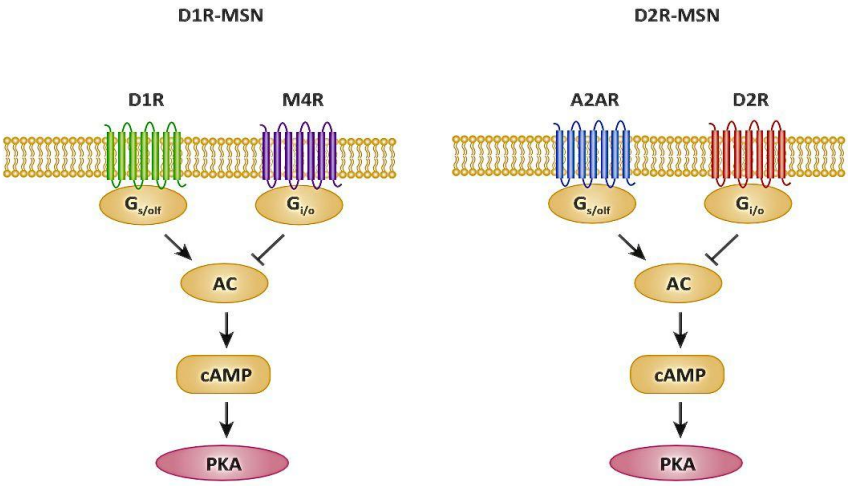

Figure 5. Dopaminergic modulation of cAMP–PKA signalling in striatal MSNs (Adapted from Nagai et al., 2016).

Although MSNs were long considered homogenous in their somato-dendritic morphology and physiological properties, more recent evidence indicates a meaningful structural and functional differences between D1 and D2 expressing neurons. Particularly, D1 MSNs exhibit a more extensive dendritic arborization with greater length, a higher number of primary dendrites, and increased dendritic complexity compared with D2 MSNs. This enhanced dendritic organization suggests a greater capacity for integrating glutamatergic synaptic inputs, highlighting how morphological specialization may contribute to functional divergence between the two populations (Gertler et al., 2008).

In addition to D1 dopamine receptors, also muscarinic M4 receptors are expressed in the direct pathway. M4 receptors are coupled to *Gi/o* proteins and negatively regulate adenylyl cyclase activity, leading to reduction in cAMP levels (Figure 5). Through this mechanism, M4 receptor activation counteracts D1 receptor-dependent cAMP-PKA signalling, thereby providing a cholinergic brake on the excitatory modulation mediated by D1 dopamine receptors (Jeon et al., 2010).

The adenosine A2A receptors are *Gs*-coupled receptors (GPCRs) with distinctive structural and functional properties. They operate by forming a stable complex with *Gs* proteins and contain a long C-terminal tail that contributes to resistance to agonist-induced internalization. Importantly, A2A receptors form heteromeric complexes with other GPCRs, particularly the D2 receptors, leading to functional antagonism by interfering with D2-*Gi/o* coupling (Figure 5). This interaction underlies the opposing effects of adenosine and dopamine signalling in the striatum, with important implications for motor control and Parkinson's disease (Zezula & Freissmuth, 2008).

1.4 Developmental Origin of the Striatal MSNs

1.4.1 Development of the ventral telencephalon

The earliest stages of brain development are characterized by the progressive compartmentalization of the neural tube into distinct anatomical and functional domains. During this process, the anterior forebrain region, or prosencephalon, undergoes further subdivision into diencephalon and the telencephalon, thereby representing a critical step in forebrain spatial organization (Leung et al., 2022).

The telencephalon specifically derives from cells located at the rostral margin of the neural plate and initially appears as a simple neuroepithelium positioned at the anterior end of the neural plate. As development proceeds, the telencephalon undergoes morphological elaboration to form paired telencephalic vesicles, which later give rise to some of the most complex structures of the vertebrate central nervous system (Hébert & Fishell, 2008; S. W. Wilson & Rubenstein, 2000).

In all vertebrates, the telencephalon is subdivided into two principal domains, the pallium (dorsal telencephalon) and the subpallium (ventral telencephalon), both of which originate from the dorsal plate of the neural tube (Moreno et al., 2009). While the pallium mainly contributes to cortical structures, the ventral telencephalon is subdivided into different progenitor domains, including the medial, lateral and caudal ganglionic eminences (MGE, LGE and CGE). These ventral progenitor domains generate neural populations of the basal ganglia and contribute to the formation of limbic system structures, such as the amygdala and the nucleus accumbens.

1.4.2 Formation of the ganglionic eminences

Following the establishment of dorsoventral patterning in the embryonic telencephalon, the subpallial domain gives rise to a set of highly proliferative germinal zones termed ganglionic eminences (GEs) (Figure 6) (Pelkey et al., 2017).

The GEs are transient subcortical grey matter structures that arise in the ventral telencephalon during early embryonic development, becoming morphologically evident around the fifth week of human gestation, corresponding to approximately embryonic day 9-10 (E9-10) in mice. They appear as prominent proliferative bulges along the floor of the telencephalic vesicles and protrude into the primitive lateral ventricles.

During mid-gestation, the GEs function as major germinal zones, generating large populations of GABAergic projection neurons and interneurons that subsequently migrate to populate the basal ganglia as well as distant brain regions, including the neocortex, hippocampus, thalamus, olfactory system, and the globus pallidus (Figure 6). As neurogenesis declines and cortical and subcortical circuits progressively mature, the GEs undergo a gradual regression and are largely no longer identifiable as distinct anatomical structures by late gestation, disappearing around the 35th week of human development (Boitor-Borza et al., 2020).

The GEs are subdivided into three major domains: the Medial Ganglionic Eminence (MGE), the Lateral Ganglionic Eminence (LGE) and the Caudal Ganglionic Eminence (CGE). The latter arises from the posterior fusion of MGE and LGE.

The MGEs is the earliest ganglionic eminence to emerge during development and represents the principal source of GABAergic neurons in the forebrain. Progenitors within the MGE generate a wide variety of interneurons subtypes, broadly classified into parvalbumin (PV)-expressing and somatostatin (SST)-expressing populations, with additional subpopulations expressing neuropeptide Y (NPY). MGE-derived neurons contribute to both the basal ganglia and the cerebral cortex, accounting for approximately 50-60% of cortical interneurons in mice (Gelman & Marín, 2010; Hébert & Fishell, 2008; Turrero García & Harwell, 2017).

The LGE represents the principal progenitor domain responsible for striatal development, as its transcriptionally and morphologically heterogeneous embryonic progenitor populations predominantly differentiate into striatal MSNs. In addition, a smaller subset of LGE-derived progenitors matures into the olfactory bulb interneurons (Knowles et al., 2021; Turrero García & Harwell, 2017).

Although the CGE was initially regarded as a minor contributor to cortical interneuron populations, accumulating evidence now demonstrates that it generates approximately 30-40% of all cortical interneurons, thereby constituting the second largest source of cortical interneurons. CGE-derived interneurons give rise to distinct GABAergic subtypes characterized by the expression of serotonin receptor 3c (5-HTR3c), reelin (RLN), calretinin (CR), and/or vasoactive intestinal peptide (VIP) (Pelkey et al., 2017; Turrero García & Harwell, 2017).

The cellular and functional heterogeneity of the GEs is closely linked to their developmental origin and molecular identity.

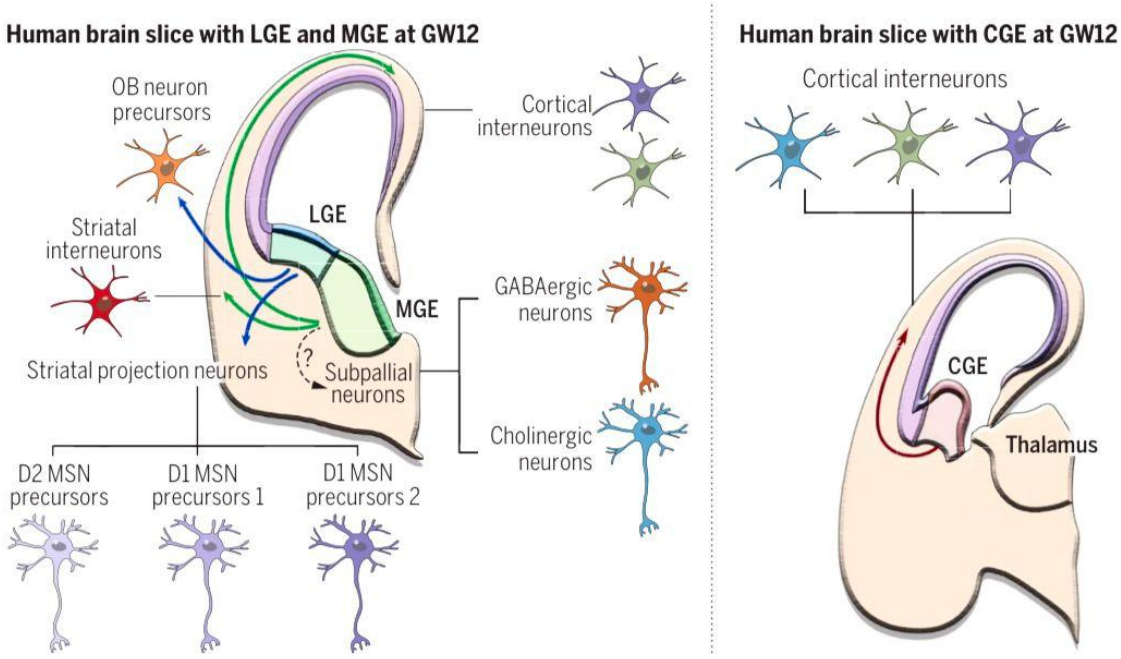

Figure 6. Developmental trajectories of the neuronal populations arising from the human GEs (Adapted from Shi et al., 2021).

1.4.3 The LGE: Progenitors Identity and MSNs Generation

The LGE is subdivided into two molecularly and spatially different progenitor domains, the dorsal (dLGE) and ventral (vLGE) domains, with distinct developmental potential (Tucker et al., 2008). Based on the different gene expression, the dLGE primarily generates interneurons destined for the olfactory bulb and the amygdala, whereas the vLGE give rise to the MSNs.

One of the earliest molecular markers of the LGE is *Gsx2* (formerly *Gsh2*), a homeobox transcription factor, essential for specification and development of its neuronal subtypes.
Between E12.5 and E18.5, *Gsx2* and *Glcci1*, another molecular marker of the LGE, exhibit a graded expression pattern within the ventricular zone (VZ), with high dorsal and low ventral levels, thereby contributing to the molecular subdivision of the LGE. In contrast, other ventral telencephalic genes such as Achaete-scute homolog 1 (*Ascl1*) and Distal-Less Homeobox 2 (*Dlx2*) are expressed more uniformly across both vLGE and dLGE (Kohli et al., 2018).
The homeobox genes *Gsx1* and *Gsx2* (formerly *Gsh1* and *Gsh2*) exert complementary and stage-dependent roles in progenitor regulation, while *Gsx2* is required for early LGE specification and temporal generation of neuronal subtypes, *Gsx1* promotes progenitor maturation and progression toward neurogenesis (Pei et al., 2011).
Together with *Ascl1*, *Dlx1/2* and Notch signalling, these transcriptional regulators orchestrate progenitor behaviour and ensure the orderly production of striatal neurons. Downstream transcription factors (TFs), including *Ebf1*, *Isl1*, and *Sp9*, further refine the temporal generation of MSNs (Knowles et al., 2021).
The LGE is also characterized by the expression of *Dlx1*, *Dlx2*, *Dlx5* and, within specific subdomains, *Isl1*, *Gsh2*, *ER81*, and *Pax6*, while notably lacking the expression of *Nkx2.1* in the VZ. The dorsal boundary of the LGE corresponds to the ventral pallium, whereas its ventral border is characterized by strong expression of *Nkx2.1*, which distinguish the LGE grom the MGE (Moreno et al., 2009).
In addition to these early progenitor markers, transcription factors such as Nolz1 (Zinc Finger protein 503; *ZNF503*), CTIP2 (*Bcl11b*), FOXP1, and FOXP2 contribute to MSNs fate specification. FOXP2 is strongly associated with striatal identity, whereas FOXP1 is expressed from early progenitor stages and persists into adulthood, marking the MSN lineage from specification through maturation and serving as a robust lineage marker (Arber et al., 2015).
FOXP1 expression precede the expression of dopamine-and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32, also known as PPP1R1B), a marker for mature MSNs. In both mouse and human striatum, FOXP1 colocalize with CTIP2 and DARPP-32, highlighting its role as a key marker of MSN development and identity (Arber et al., 2015; Precious et al., 2016).
Compartmental specialization is further refined by differential transcriptional signatures, with FOXP2 enriched in striosomes neurons and FOXP2 predominating in matrix neurons. Consistent with their lateral subpallium origin, LGE-derived MSNs lack expression of the MGE marker NKX2.1 (Precious et al., 2016).

The LGE constitutes the main embryonic source of MSNs. While studies in mice demonstrated that MSNs generation begins around E10.5 and proceeds progressively until birth (E19.5) (Pei et al., 2011), transcriptomic analyses in human LGE progenitors provided additional insights into lineage diversification. For instance, clusters enriched for ISL1, EBF1, and TAC1 correspond to D1 MSNs, whereas PENK marks D2 MSNs, and additional D1 sublineages defined by TSHZ1 and PDYN expression (Shi et al., 2021).

Single-cell transcriptomic profiling of Activin A-patterned induced pluripotent stem cells (iPSC)-derived striatal cultures showed a temporally ordered progression from forebrain/LGE progenitors to mature MSN subtypes. While early stages were enriched for LGE progenitors, nascent MSNs and dMSN/iMSN precursors expressing *BCL11B*, *MEIS2*, and *FOXP1/2* emerged by 18-24 days in vitro (DIV), mature MSN subtypes were detectable between 24-40 DIV. These findings were supported by abundant *FOXG1*$^{+}$ and *GSX2*$^{+}$ progenitors and the appearance of BCL11B^{+}, DARPP-32^{+}, FOXP1^{+}, and FOXP2^{+} MSNs from 24 DIV onward (Fjodorova et al., 2025).

Pseudotime trajectory analysis showed that MSNs arise from LGE progenitors and diverge into dMSN and iMSN lineages at 18-24 DIV, with dMSNs emerging before iMSNs, consistent with in-vivo developmental trajectories (Fjodorova et al., 2025).

Projection onto foetal (7-11 and 9-18 post-conception weeks (PCW)) and adult human striatal single-cell atlases demonstrated that iPSC-derived neurons span the developmental continuum from LGE progenitors to mature dMSN/iMSN subtypes. Reverse projection further confirmed a strong correspondence between in vitro-derived cells and their in vivo counterparts (Fjodorova et al., 2025).

Integrated transcriptomic analysis identified conserved developmental markers across datasets, including LGE progenitor genes (*GSX2*, *ASCL1*) and nascent MSN markers (*MEIS2*, *BCL11B*, *FOXP1/2*). Canonical dMSN genes (*EBF1*, *TAC1*, *ZFHX3*, *GAP43*) and iMSN markers (*SIX3*, *ZFHX4*) were similarly preserved. Differential gene expression analysis further distinguished developmental populations, confirming the expression of LGE progenitor genes (*FABP7*, *VIM*, *SALL3*, *ASCL1*), nascent MSN genes (*BCL11B*, *SP8/9*, *GAD1/2*), and mature subtype markers (*ARPP21*, *SIX3*, *ZFHX3/4*, *GAP43*). In addition, *NRGN* and *SYNPR* were identified as candidate markers of MSN precursors, whereas *NRN1* was enriched in dMSNs (Fjodorova et al., 2025).

The acquisition of striatal identity is characterized by the upregulation of the lineage-associated transcription factors CTIP2, GSX2 and FOXP2. Mature MSNs are defined by the expression DARPP-32 (Arber et al., 2015).

CTIP2 plays a key role in MSNs differentiation. It is first detected in the LGE at E12.5, persists throughout MSN neurogenesis, and remains expressed during adulthood. During development, CTIP2 is expressed from early postmitotic stages and colocalizes with doublecortin (DCX)-positive migratory neurons. In the adult striatum, CTIP2 is present in all DARPP-32-positive cells, confirming its expression across the entire MSN population. Loss of CTIP2 results in impaired differentiation, reduced expression of key MSN markers such as DARPP-32, FOXP1, *chrm4*, Reelin, MOR1, glutamate receptor 1, and plexin-D1, and disruption of striosomes-matrix organization (Knowles et al., 2021; Stenman et al., 2003).

1.5 Extrinsic Signalling Pathways in Ventral Telencephalic Specification

1.5.1 Sonic Hedgehog (SHH) and Wingless-related integration site (WNT) antagonism in dorsoventral patterning of neural tube

Morphogens are extracellular, secreted signalling molecules acting at distance in a graded, concentration-dependent manner to confer positional information and induce distinct cell fates during embryonic development. Among these, Sonic Hedgehog (SHH) represents a prototypical morphogen governing dorsoventral patterning of the central nervous system (CNS), and is essential for the specification of ventral progenitor identities, including the formation of ventral telencephalic structures (Hébert & Fishell, 2008; Ulloa & Martí, 2010).

In the developing forebrain, SHH promotes the differentiation of ventral telencephalic ganglionic eminences, which give rise to the basal ganglia. Loss of SHH signalling results in a reduced telencephalon accompanied by the absence of ventral cell types (Brady & Vaccarino, 2021; Hébert & Fishell, 2008).

Importantly, the specification of ventral progenitor populations is highly dependent on both concentration and duration of SHH signalling. Specifically, lower SHH levels preferentially promote LGE fate, whereas higher concentrations drive progenitors toward a MGE identity (Fjodorova et al., 2015; Memi et al., 2018).

The specification of GE progenitors is tightly regulated by the interplay between SHH and Wingless-related integration site (WNT) signalling pathways, which together govern the establishment of the LGE and MGE (Hunt et al., 2023).

Dorsoventral patterning of the developing neural tube is regulated by opposing morphogen gradients (Figure 7), in which dorsally expressed WNT and bone morphogenetic protein (BMP) signalling pathways promote dorsal cell identities, whereas SHH acts as the principal ventralizing morphogen and functionally antagonizes these dorsal cues (Brady & Vaccarino, 2021; Ulloa & Martí, 2010).

The antagonistic interaction between WNT and SHH signalling is largely mediated by the transcription factor GLI3. In this context, GLI3 acts as a potent inhibitor of the SHH pathway, thereby restricting the SHH-mediated ventral specification and contributing to dorsoventral patterning of neural progenitors (Li et al., 2009).

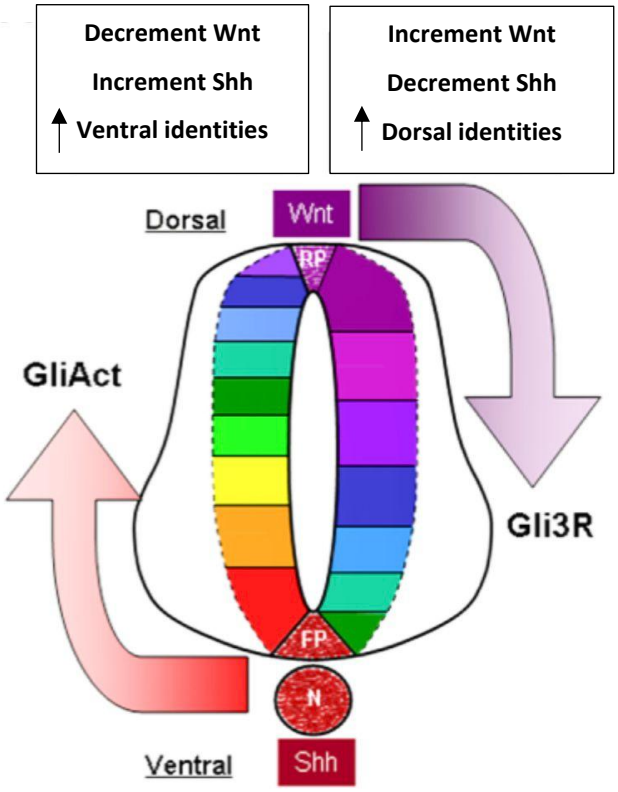

Figure 7. Antagonistic regulation of dorsoventral patterning of the neural tube by WNT and SHH signalling gradients (Adapted from Ulloa & Martí, 2010).

1.5.2 The role of Activin A signalling in MSNs generation

Activins are multifunctional cytokines belonging to the transforming growth factor-β (TGF-β) superfamily, which comprises more than 30 members in mammals, including TGF-βs, BMPs, Activins and growth differentiation factors (GDFs) (Olsen et al., 2020).

Structurally, activins are homo- or heterodimeric proteins composed of β subunits, giving rise to three main isoforms: Activin A (βAβA), Activin B (βBβB), and Activin AB (βAβB).
Unlike TGF-β, which is secreted as an inactive precursor requiring subsequent activation, Activin A is released in an intrinsically active form (Loomans & Andl, 2014).
Activin A plays a crucial role in neural development by regulating the balance between progenitor self-renewal and differentiation, while also directing neuronal subtype specification. Particularly, it promotes the acquisition of lateral forebrain characteristics by guiding neural progenitors toward a LGE fate.
Activin A supports the generation of mature DARPP-32-positive neurons and promotes the development of GABAergic neurons with MSNs characteristics. Furthermore, Activin A biases the fate of forebrain progenitors, particularly FOXG1-positive cells, toward a striatal lineage, thereby contributing to the precise control of neural lineage commitment during telencephalic development (Arber et al., 2015; Cambray et al., 2012).
Activin A signalling pathway starts with the binding of the active ligand to type II Activin receptors (ACVR2A or ACVR2B) present on the surface of target cells (Figure 8).
Upon ligand binding, type II receptor recruits type I receptor, especially Activin receptor-like kinase (ALK4), forming a heteromeric serine/threonine kinase receptor complex.
The type II receptor subsequently phosphorylates and activates the type I receptor. Activated ALK4 then induces the phosphorylation of intracellular mediators SMAD2 and SMAD3, which associate with the common mediator SMAD4, forming a transcriptional complex. This SMAD2/3-4 complex translocates into the nucleus, where it binds to specific DNA sequences, such as the SMAD-binding elements (e.g., CAGA motifs), thereby regulating the transcription of downstream target genes (Loomans & Andl, 2014; Wijayarathna & De Kretser, 2016).
The requirement of Activin A-mediated effects on MSNs specification was demonstrated by pharmacological inhibition of ALK4/5. Treatment of forebrain progenitors with the ALK4/5 inhibitor SB431542 resulted in the complete loss of key LGE markers, including CTIP2 and GSH2.
Similarly, selective TGF-β/Activin pathway inhibitors, such as SB525334 (SB5) and LY2109761 (LY), which target ALK4/5 signalling while sparing BMP-associated receptors (ALK2/3/6), abolished the Activin-driven upregulation of CTIP2, despite its strong induction under normal conditions (Fjodorova et al., 2020).
In addition to the canonical pathway, Activin A can also signal through non-canonical, SMAD-independent pathways, including the PI3K/AKT, MAPK/ERK, and WNT/β-catenin cascades, which regulate diverse cellular processes through alternative intracellular mechanisms.

Notably, activation of MAPK pathway, involving kinases such as ERK1/2, JNK, and p38, represent a key non-canonical route through which Activin A modulates gene expression (Wijayarathna & De Kretser, 2016).

As previously discussed, BMP is crucial for promoting dorsal neural identity and contributes to the dorsoventral patterning by antagonising SHH.
Inhibition of BMP signalling has been shown to enhance Activin A-induced upregulation of LGE-specific genes, likely by suppressing dorsomedial telencephalic fates and thereby favouring a shift toward a ventrolateral LGE-like identity (Arber et al., 2015; Fjodorova et al., 2020).

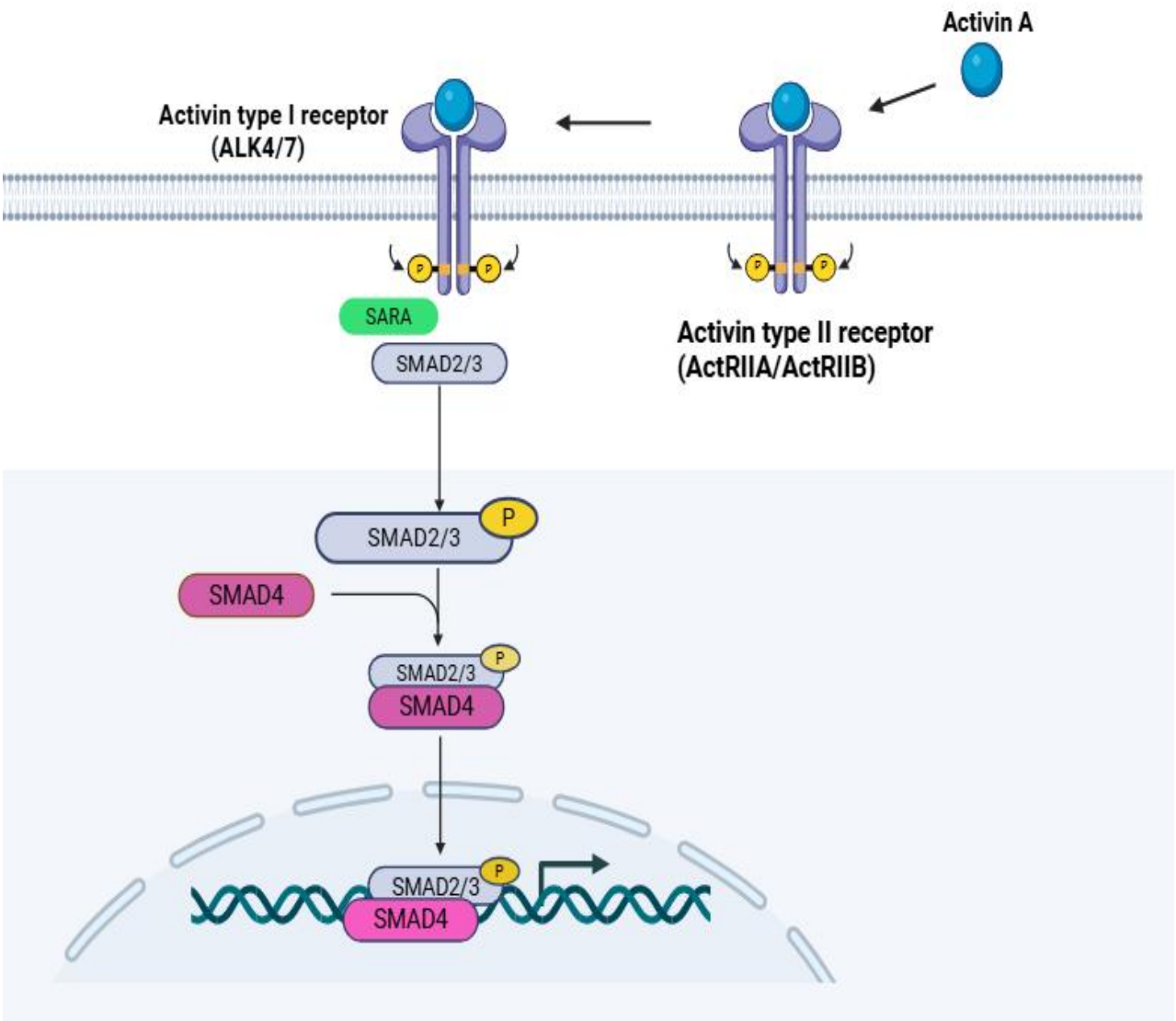

Figure 8. Schematic representation of the canonical Activin A signalling pathways (Created by BioRender).

1.5.3 The Ras-ERK signalling pathway

Extracellular signal-regulated kinases ERK1 and ERK2, encoded by the *MAPK3* and *MAPK1*, genes located within the 16p11.2 and 22q11.2 genomic regions respectively, are key components of the Ras-Raf-MEK-ERK signalling cascade (Leone et al., 2024; Sun & Nan, 2017).

During embryogenesis, ERK1/2 regulates essential processes such as cell proliferation, differentiation, and fate determination (Sun & Nan, 2017). In the adult brain, ERK1/2 is critically involved in synaptic plasticity, a process underlying learning and memory, including short-term and long-term memory formation, retrieval, reconsolidation, and persistence. Overall, ERK1/2 signalling acts as a key integrator of intracellular signalling events that supports higher-order cognitive functions (Medina & Viola, 2018; Sun & Nan, 2017).

ERK1/2 signalling is activated by extracellular stimuli that trigger upstream signalling molecules such as Ras, leading to activation of Raf kinases. Raf subsequently phosphorylates MEK1/2, which in turn activates ERK1/2. Once activated, ERK1/2 translocate to the nucleus, where it regulates gene expression through the activation of transcription factors, thereby modulating diverse cellular responses (Sun & Nan, 2017; Wiegert & Bading, 2011).

RB5 is a novel cell penetrating peptide derived from the N-terminal region (amino acids 7-38) of human *MAPK3* (ERK1), fused to TAT sequence to facilitate cellular uptake, and designed to selectively modulate ERK signalling (Indrigo et al., 2023).

RB5 relies on exploits the structural differences between ERK1 and ERK2, particularly within their N-terminal domains. Under physiological conditions, ERK1 preferentially interacts with the importin α1 protein KPNA2.

KPNA2 belongs to the importin α family of nuclear transport adaptors, which, in association with importin β, allow the translocation of cargo proteins through the nuclear pore complex. This preferential binding of ERK1 to KPNA2, promotes its nuclear translocation, thereby restricting ERK2 entry into the nucleus.

RB5 disrupts the ERK1-KPNA2 interaction by binding to the ERK1 N-terminal domain, thereby facilitating binding of ERK2 to KPNA2 and promoting its nuclear translocation. This

shift results in a selective enhancement in global ERK signalling, predominantly driven by ERK2-mediated nuclear activity, which is strongly associated with neuronal survival and plasticity (Indrigo et al., 2023).

Experimental evidence supporting this mechanism in acute striatal slices, show that RB5 induces a dose-dependent increase in ERK activation and significantly enhances nuclear ERK signalling.

In vivo, RB5 administration enhances ERK phosphorylation and nuclear signalling markers in wild-type mice. Furthermore, RB5 increases ERK activity in both wild-type and Huntington's disease striatal cells, supporting its functional relevance across physiological and pathological contexts (Indrigo et al., 2018, 2023).

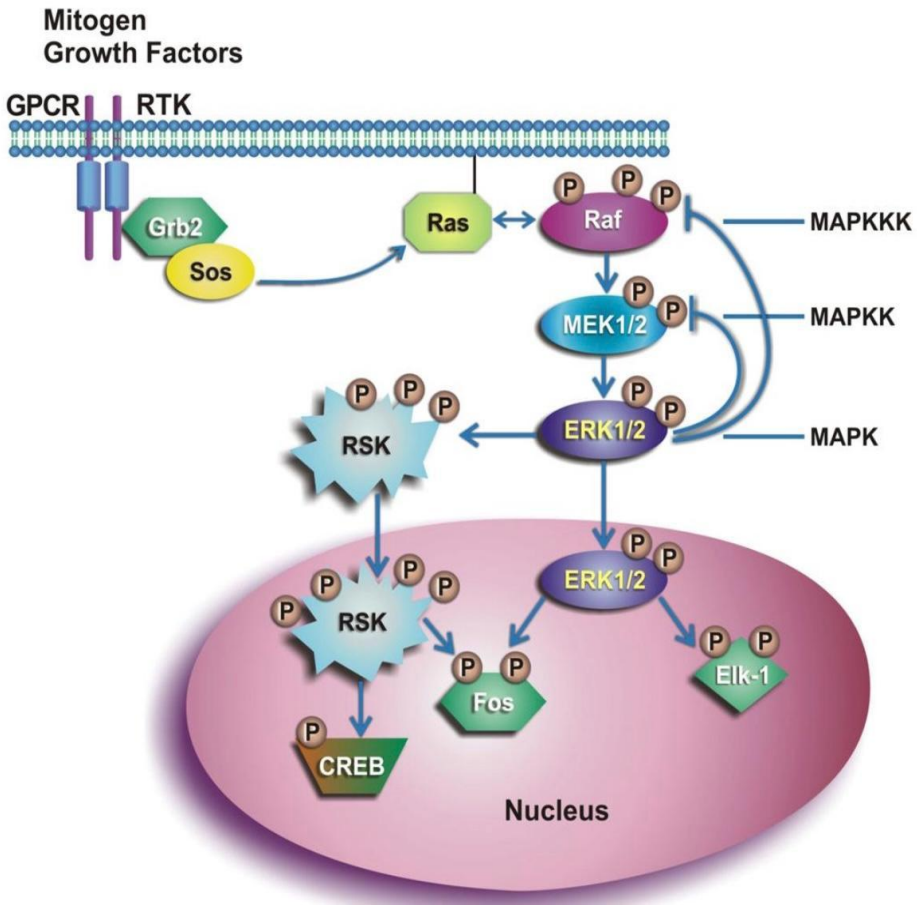

Figure 9. Canonical Ras–Raf–MEK–ERK signalling and nuclear transcriptional outputs (Adapted from Mebratu & Tesfaigzi, 2009).

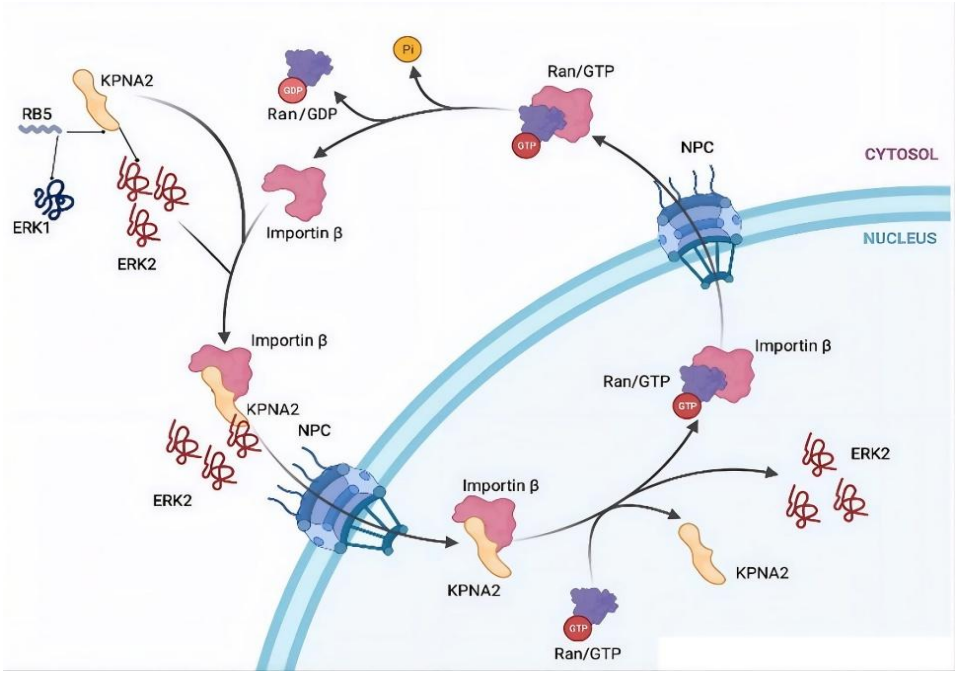

Figure 10. Potential model of RB5 effects on ERK2 nuclear translocation (Created with BioRender).

1.6 MSNs dysfunctions in neurodevelopmental and neurodegenerative disorders

As the principal neuronal population of the striatum, the MSNs play a critical role in the regulation of motor and cognitive functions. Accordingly, dysfunction of striatal MSNs has been widely implicated in a broad spectrum of neurological disorders, including neurodegenerative and neurodevelopmental disorders (Mattis & Svendsen, 2017; Mei et al., 2025).

Neurodevelopmental disorders (NDDs) comprise a heterogenous group of pathological conditions affecting a significant proportion of the paediatric population and are characterized by diverse genetic origins and overlapping clinical features. These disorders arise from alterations in brain development occurring at both cortical and subcortical levels during prenatal and early postnatal stages. Although highly variable in their presentation, NDDs

commonly include conditions such as autism spectrum disorder (ASD), intellectual disability (ID), and attention-deficit/hyperactivity disorder (ADHD), with schizophrenia also increasingly considered within this spectrum, often accompanied by additional psychiatric and non-psychiatric abnormalities (Leone et al., 2024).

Among NDDs, ASD is characterized by persistent impairments in social communication and interaction, together with restricted and repetitive patterns of behaviour, with symptoms emerging during early developmental stages.

The striatum plays a critical role in the pathophysiology of ASD, as it integrates widespread cortical inputs and regulates complex behavioural functions. Disruptions within dorsal and ventral striatal circuits have been strongly associated with main ASD symptoms, including restricted and repetitive behaviours, underscoring the striatum's central involvement in this pathology (Evans et al., 2024).

Individuals with ASD show pronounced structural abnormalities within the striatum, including increased caudate volume and alterations in the organization of striatal compartments. An increased matrix-to-striosome ratio suggests disrupted developmental organization of MSN populations. At the molecular level, elevated expression of dopamine receptor D2 in caudate nucleus and putamen further indicates altered dopaminergic signalling within striatal circuits (Evans et al., 2024; Waugh et al., 2025).

Recent work has demonstrated that 16p11.2 copy number variants (CNVs), strongly associated with NDDs, disrupt MSN neurogenesis and differentiation kinetics. Using human iPSC-derived striatal models, reciprocal shifts in cell-cycle dynamics were observed in 16p11.2 deletion and duplication lines, altering the timing and magnitude of MSN production. Specifically, 16p11.2 deletion cultures showed increased proliferative fractions and prolonged total cell-cycle duration, consistent with delayed neurogenic commitment and an accumulation of $GSX2^{+}$ progenitors, accompanied by reduced generation of mature $NeuN^{+}$ neurons. In contrast, duplication lines exhibited reduced progenitor tools and comparatively accelerated neuronal output. These divergent striatal neurogenesis deficits mirror phenotypes reported in CNV carriers and experimental models, indicating that early-stage disruptions in proliferation and neuronal maturation represent a convergent mechanism contributing to NDD pathophysiology (Fjodorova et al., 2025).

Neurodegenerative disorders, are characterized by the progressive loss of neuronal structure and function over time, ultimately leading to impaired neural connectivity, circuit activity and clinical manifestations. Within this context, early synaptic degeneration, particularly in the

striatum, has been associated with the initial stages of disorders such as Parkinson's disease and Huntington's disease (Galli et al., 2021).

Huntington's disease (HD) is a fatal neurodegenerative disorder caused by expansion of polyglutamine repeats in the Huntingtin (*HTT*) gene, leading to progressive motor dysfunction, cognitive impairment and psychiatric abnormalities (Mattis & Svendsen, 2017).

HD predominantly targets the striatum, where MSNs undergo early dysfunction followed by progressive degeneration. MSNs degeneration in HD is not uniform across subpopulations, as neurons of the indirect pathway have traditionally been considered more vulnerable. However, emerging evidence suggest that the MSNs of the direct pathway exhibit functional impairment at earlier stages of the disease. Consistent with this widespread neuronal dysfunction, the expression of DARPP-32 is markedly reduced in both human patients and experimental models, reflecting the profound disruption of MSNs identity and function (Ehrlich, 2012).

At the molecular level, transcriptional regulators, such as CTIP2, have been implicated in HD pathogenesis. Reduced CTIP2 expression has been observed in both human and experimental models prior to the onset of MSN degeneration, and its deficiency is associated with structural striatal abnormalities and cognitive impairments, highlighting its critical role in HD neurodegeneration (Fjodorova et al., 2019).

Parkinson's disease (PD) is a neurodegenerative disorder primarily characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra, leading to a marked reduction in dopamine levels within the striatum and resulting in the hallmark motor symptoms of tremor and bradykinesia (Witzig et al., 2020; Zhang et al., 2017).

PD is associated with pronounced structural alterations in striatal MSNs, including a substantial reduction in dendritic complexity, characterized by decreased dendritic length and significant loss of dendritic spine across different striatal regions such as putamen and caudate nucleus. These morphological changes have been consistently observed in both mouse models and patients, with reports indicating up to a 50% reduction in spine density (Witzig et al., 2020; Zhang et al., 2017).

Overall, these findings highlight the critical role of MSNs in maintaining striatal function, as their dysfunction or loss can disrupt neural circuitry and contribute to the onset of multiple neurological disorders. Consequently, the generation of MSNs from iPSCs has emerged as a promising strategy to model disease mechanisms and develop potential therapeutic approaches.

1.7 In vitro specification of MSNs from human pluripotent stem cells (hPSCs)

There is a growing need to establish reliable and reproducible in vitro strategies for generating striatal neurons, not only to better understand the mechanisms underlying their development and maturation under physiological conditions, but also to provide strong platforms for investigating disease mechanisms, drug screening, and the development of regenerative therapies for neurodegenerative disorders such as Huntington's disease and others (Conforti et al., 2022; Delli Carri et al., 2013).

In this context, in vitro differentiation approaches aim to recapitulate key molecular and cellular aspects of neuronal development under controlled conditions. These systems allow the sequential progression through defined stages of neural differentiation, thereby providing a framework for generating specific neuronal approaches from hPSCs (Noakes et al., 2015).

hPSCs, including embryonic stem cells (ESCs) and induced pluripotent stem stem cells (iPSCs), are characterized by self-renewal and pluripotency, enabling the generation of diverse neural populations with defined phenotypes. They represent a powerful platform for modelling human brain development, investigating the pathogenesis of neurological disorders, and developing potential cell-based therapeutic strategies (Amimoto et al., 2021).

Transplantation of fetal striatal tissue has shown preliminary success in restoring striatal function such as in Huntington's disease patients. However, its clinical application remains severely limited by ethical concerns, restricted tissue availability, and challenges related to graft safety and control of neural cell proliferation. Furthermore, although stem cell-derived MSN-like neurons has been transplanted in animal models, their functional integration into host neural circuits remains limited (Aubry et al., 2008; Reddington et al., 2014).

These limitations highlight the need for more reliable strategies for generating clinically relevant neuronal populations (Aubry et al., 2008; Reddington et al., 2014).

An alternative approach involves direct reprogramming, or induced neuron (iN) technology, which enables the conversion of post-mitotic somatic cells into functional neurons through the forced expression of lineage-specific factors. Although this strategy provides a useful model for studying neuronal identity and fate specification, it presents significant limitations, as it bypasses critical developmental stages and does not allow the generation or expansion of neural progenitor populations, thereby limiting its applicability for developmental studies and large-scale applications (Reddington et al., 2014).

Following these limitations, extensive efforts have been directed toward the development of in vitro differentiation protocols aimed at efficiently generating striatal MSNs from hPSCs through the controlled modulation of signalling pathways. These approaches aim to recapitulate key stages of embryonic telencephalic development, including neural induction, and ventralization (Amimoto et al., 2021; Conforti et al., 2022).

SHH-mediated ventralization promotes the induction of striatal lineage factors like ASCL1, while suppressing dorsal markers like PAX6, thereby promoting the production of GABAergic MSN-like neurons. Nevertheless, these strategies still produce heterogenous neuronal populations and often fail to achieve complete specification toward an authentic MSNs identity (Fjodorova et al., 2015).

To overcome the limitations associated with recombinant proteins, including high costs, variable bioactivity, and limited stability, recent differentiation strategies have increasingly relied on synthetic small molecules to achieve more controlled and reproducible outcomes. These approaches enable precise modulation of key developmental signalling pathways involved striatal development. Efficient multi-step protocols have been established in which telencephalic identity is induced through inhibition of WNT and SMAD signalling using XAV939, LDN193189, and A38-01, followed by SHH pathway activation via Purmorphamine (PMA) to promote ventral specification, and subsequent neuronal maturation through inhibition γ-secretase and FGF signalling pathways, ultimately yielding functional GABAergic MSNs (Amimoto et al., 2021).

Despite these improvements, current MSNs differentiation strategies still face important challenges, including low MSNs yield, heterogenous neuronal populations, and the limited reproducibility observed across other protocols (Fjodorova et al., 2020; Le Cann et al., 2021).

Over recent years, in vitro differentiation protocols have increasingly incorporated Activin A, as a key signalling factor to generate striatal MSNs from hPSCs. Components of Activin signalling are endogenously expressed during striatal development, and their application has been shown to effectively induce LGE fate in hPSC-derived forebrain progenitors, highlighting the important role of Activin A signalling (Noakes et al., 2015). Importantly, this effect is mediated through direct induction of LGE identity rather than through increased progenitor proliferation.

Activin A regulates the identity of telencephalic neural precursors through inhibition of the mitogenic SHH pathway and concomitant enhancement of retinoic acid (RA) signalling, thereby promoting neuronal differentiation (Cambray et al., 2012; Noakes et al., 2015).

Consistently, Activin A signalling rapidly induces LGE-specific gene expression, with upregulation of key markers observed as early as 24 hours, supporting a direct regulatory role in lineage specification. This effect is associated with selective induction of transcription factors, including *BCL11B*, *NOLZ1*, and *FOXP2*, together with pan-ganglionic eminence markers such as *GSX2* and *DLX2* (Fjodorova et al., 2015; Noakes et al., 2015).

This specification process ultimately leads to the generation of functional GABAergic neurons expressing DARPP-32 under defined conditions, commonly employing Activin A at a concentration of 25ng/mL. Similar outcomes have been achieved using Alantolacatone, an Activin A agonist, typically applied at 250 nM, further supporting the central role of TGF-β signalling in driving MSN differentiation and specification (Arber et al., 2015; Fjodorova et al., 2015, 2020; Noakes et al., 2015)

Two widely used in vitro differentiation strategies for generating striatal MSNs from hPSCs are represented by protocols described in (Fjodorova et al., 2015) and (Stanslowsky et al., 2016), based on distinct experimental approaches.

In (Fjodorova et al., 2015), the authors employed a monolayer differentiation strategy in which hPSCs were sequentially guided through the controlled application of small molecules and signalling factors. Neural induction was achieved under defined N2B27 media supplemented with inhibitors of BMP/TGF-β pathways, followed by a critical patterning phase driven by Activin A. Subsequent maturation was supported by neurotrophic factors, including BDNF (Brain-Derived Neurotrophic Factor) and GDNF (Glial cell line-Derived Neurotrophic Factor), in combination with RA, enabling progression toward neuronal phenotype. Although this protocol is structured and reproducible, its efficiency remains limited, yielding relatively low proportions of neuronal cells (approximately 14-31% Tuj1-positive cells), with cultures predominantly composed of mixed neuronal populations enriched in interneuron-like identities rather than fully specified MSNs (Fjodorova et al., 2015; Le Cann et al., 2021).

In contrast, in (Stanslowsky et al., 2016), the protocol employed a distinct differentiation strategy based on three-dimensional embryoid bodies (EBs) formation, mimicking aspects of early embryonic development. In this protocol, hPSCs were initially cultured and subsequently aggregated in suspension to induce EB formation, followed by neural induction and regional specification through the application of small molecules, including ROCK inhibitor (Y-27632), Dorsomorphin, SB-431542, and IWP2. Ventral patterning was promoted through PMA-mediated activation of SHH signalling. Cells were then allowed to mature in the presence of neurotrophic and differentiation factors such as BDNF, GDNF, TGF-β3, and dbcAMP.

Despite its developmentally inspired design, this protocol also exhibited variable efficiency, yielding approximately 9.2-40.1% Tuj1-positive neurons, with cultures characterized by heterogeneous neuronal populations that were not fully committed toward MSN identity (Le Cann et al., 2021).

Taken together, current differentiation strategies for generating striatal MSNs from hPSCs, including both monolayer-based and embryoid body-based approaches, remain constrained by fundamental limitations.

Despite employing diverse developmental patterning cues, such as SHH signalling and small molecule-based modulation of signalling pathways, comparative analyses of available protocols reveal persistent challenges related to low MSN yield, heterogenous neuronal populations, and incomplete striatal specification. These findings suggest that the major challenge lies not only in protocol design but also in the intrinsic sensitivity of PSCs to the combination of factors, timing and the concentration values. Consequently, achieving robust and reproducible MSN specification will need more refined differentiation approaches capable of precisely recapitulating the spatiotemporal dynamics of striatal development.

2. AIM OF THE STUDY

The aim of this study is to establish an efficient protocol for generating human medium spiny neurons (MSNs) starting from neural progenitor cells (NPCs).

Medium Spiny Neurons, the principal projection neurons of the striatum, are the key regulators of its important functions, and are selectively vulnerable across a broad range of neurological disorders. Dysfunction within the cortico-striatal pathway has increasingly been associated with neurodevelopmental disorders (NDDs).

Consequently, *in vitro* generation of human MSNs from iPSCs has emerged as an essential platform for modelling disease mechanisms and advancing therapeutic strategies. However, existing differentiation protocols still fail to reliably recapitulate striatal development, often resulting in low and heterogeneous neuronal populations (Fjodorova et al., 2015, 2020).

Unlike conventional approaches based on iPSCs, the use of NPCs as a starting population provides a significant advantage, as they represent an already lineage-committed stage within neural development, thereby reducing variability and bypassing early differentiation steps. Moreover, NPCs are easier to maintain in culture, expand, and cryopreserve, enabling more consistent experimental conditions.

In this work, we performed a stepwise optimization of MSNs differentiation from smNPCs. In the first experiment, we evaluated the effect of increasing concentrations of Activin A, in the presence or absence of RB5, to identify the most suitable condition for later long-term differentiation. In the second experiment, the chosen condition (100ng/mL Activin A without RB5) was applied in a long-term differentiation paradigm extending up to four weeks to assess its ability to support MSN maturation over time.

Overall, this study aims to define and validate an optimized and scalable differentiation strategy for the efficient specification of human MSNs from smNPCs.

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3. MATERIALS AND METHODS

3.1 Culture and Maintenance of Neural Progenitor Cells (NPCs)

3.1.1 Human Neural Progenitor Cells

Human neural progenitor cells (hNPCs) of the *HMGU1* line were used in all experiments. The *HMGU1 cell line* is derived from human fibroblasts that were reprogrammed through a neural induction, resulting in a stable and expandable population of hNPCs. These NPCs were cultured under standardized and tightly controlled conditions to preserve progenitor identity. Cells were maintained at 37°C in a humidified incubator with 5% CO_2, ensuring physiological pH buffering of the culture medium (Reinhardt et al., 2013).

3.1.2 Plate Coating

All coating procedures were conducted under sterile condition in a laminar flow hood. Matrigel (Sigma-Aldrich) was stored at -80°C and handled on ice throughout preparation to avoid premature polymerization. For plate preparation, Matrigel was diluted 1:100 in Cold DMEM/F12 (Dulbecco's Modified Eagle Medium/Ham's F-12) and applied to culture wells (1mL per well in 6-well plates). Coated plates were incubated at 37 °C for 30-60 minutes to ensure complete matrix polymerization prior to cell seeding.

3.1.3 Thawing of Cryopreserved NPCs

Prior to thawing, SM+ medium (see Table 1) was prepared (Reinhardt et al., 2013).

Cryopreserved smNPCs vials were retrieved from liquid nitrogen and maintained on dry ice until processing. Cells were rapidly thawed in a 37 °C water bath until only a small frozen pellet was left. Cell suspension was transferred to 15 mL falcon tube containing pre-warmed DMEM/F12 and centrifuged at 800 g for 5 minutes. The supernatant was aspirated carefully, and the pellet was resuspended in SM+ medium.

Matrigel-coated plates were prepared in advance, the coating solution was removed, and fresh SM+ medium (1-1.5 mL per well) was added prior seeding. Cells were distributed according to pellet size and estimated density.

Table 1. SM+ media recipe

NPC, SM+ MEDIA COMPONENTS (50mL)			
Components:	*Final Concentration*	*Added Volume*	*Supplier*
DMEM/F12	48% of total volume	24 mL	Gibco
Neurobasal	48% of total volume	24 mL	Gibco
N2 Supplement	0.5x	250 µL	Gibco
B27 Supplement (without Vitamin A)	1x	500 µL	Gibco
GlutaMAX	0.5x	250 µL	Gibco
Penicillin-Streptomycin (PS)	1%	500 µL	Gibco
CHIR 99021	3 µM	3 µM	Cayman Chemical
PMA	500 nM	500 nM	TOCRIS
Ascorbic acid	150 µM	150 µM	Sigma Aldrich

3.1.4 Passaging of smNPCs

smNPCs cultures were routinely expanded when reaching approximately 70-80% confluency. At this stage, the culture medium was removed, and cells were washed with 1x phosphate-buffered saline (PBS). Accutase (0.5-1 mL per well) was added, and plates were incubated at 37°C for 3-5 minutes to allow enzymatic detachment. Enzymatic activity was stopped by adding an equal volume of DMEM/F12. The cell suspension was moved to a 15 mL sterile

Falcon tube and centrifuged at 800 g for 5 minutes. After aspiration of the supernatant, the cell pellet was resuspended in 1mL of SM+ medium (see Table 1) to obtain a single-cell suspension. Cells were seeded onto pre-coated Matrigel plates containing SM+ medium. Plates were returned to a humidified incubator maintained in proper conditions.

3.1.5 Cryopreservation and Storage of smNPCs

Cells were detached following the standard passaging procedure. After centrifugation, the supernatant was carefully removed, and the resulting cell pellet was resuspended in freezing medium (see Table 2) to obtain homogenous suspension. Cells were then transferred into a sterile cryovial and placed in a controlled-rate freezing container at -80°C overnight. For long-term preservation, cryovials were subsequently transferred to liquid nitrogen.

Table 2. Freezing media recipe

Freezing Media (6mL)		
Components	*Added Volume*	*Supplier*
KO Serum (KnockOut Replacement Serum)	60%	Life Technologies
SM+ Medium	30%	/
DMSO (Dimethyl sulfoxide bioreagent)	10%	Life Technologies

3.2 NPCs Differentiation into Medium Spiny Neurons

3.2.1 Activin A and RB5 treatment during induction of MSNs from NPCs

smNPCs were plated at a density of approximately 25,000 cells per well onto a sterile glass coverslip in Matrigel-coated 24-well plates.

Cells, cultured in N2B27 media (see Table 3), were exposed to increasing concentrations of Activin A (25, 50, 100 ng/mL), in the presence or absence of 50 μM of RB5, in order to assess

dose-dependent effects over a one-week differentiation period. Activin A and RB5 were freshly added 3 times a week during media change. Cells were maintained at 37°C in a humified atmosphere containing 5% CO_2.

3.2.2 Four-weeks differentiation of NPCs into MSNs with 100 ng/mL Activin A

50,000 cells at passage 19 were seeded into each well of Matrigel-coated 12-well plates containing sterile coverslips.

During each media change (3 times per week) treated cells received 100 ng/mL Activin A. Cells were then fixed at 5 different timepoints T0, week1, week 2, week 3 and week 4.

Table 3. N2B27 media recipe

N2B27 Differentiation Media Compositions			
Components	*Final Concentrations*	*Added Volume*	*Supplier*
DMEM/F12	48% of the total volume	24 Ml	Gibco
Neurobasal	48% of the total volume	24 mL	Gibco
N2 Supplement	0.5x	250 µL	Gibco
B27 Supplement (without vitamin A)	1x	500 µL	Gibco
GlutaMAX	1x	500 µL	Gibco
Penicillin-Streptomycin (PS)	1%	500 µL	Gibco
MEM NEAA	1x	500 µL	Gibco
β-Mercaptoethanol	0.1x	100 µL	Thermo Fisher

3.3 Immunofluorescence

Cells were fixed using 4% cold paraformaldehyde (PFA) for 15 minutes at room temperature, followed by three washes with PBS1x under gentle agitation. Cells were then incubated for 1 hour at room temperature in a blocking solution containing 10% normal goat serum (NGS) and 0.2% Triton X-100 in PBS1x to reduce non-specific binding and ensure cell permeabilization. Cells were subsequently incubated overnight at 4°C with primary antibodies diluted in PBS1x and blocking solution (1:1). For the first experiment (one-week differentiation), the following primary antibodies were used: anti-CTIP2 (rat, Abcam ab18465) and anti-FOXP2 (rabbit, Abcam ab16046) (see Table 4). For the second experiment (Four-weeks differentiation), cells were incubated with anti-NeuN (Mouse, Merck Millipore MAB377) and anti-DARPP32 (rabbit, Abcam ab40801) antibodies (see Table 4).

Following primary antibodies incubation, cells were rinsed three times with PBS1x on a shaker and then incubated with, fluorophore-conjugated secondary antibodies for 1-2 hours at room temperature under gentle agitation. For the first experimental set (one-week differentiation), Alexa Fluor® 488 goat anti-Rat IgG (H+L) and Alexa Fluor® 555 goat anti-Rabbit IgG (H+L) were used to detect CTIP2 and FOXP2, respectively. For the second experimental set (Four-term differentiation), Alexa Fluor® 488 goat anti-Rabbit IgG (H+L) and Alexa Fluor® 546 goat anti-Mouse IgG (H+L) were used to visualize DARPP-32 and NeuN, respectively.

Coverslips were subsequently mounted onto glass slides using Flouromount-G containing DAPI for nuclear counterstaining.

Table 4. Details of primary and secondary antibodies for immunofluorescence staining

Antibodies Used in the Immunostaining				
Type	*Target or Fluorophore*	*Species*	*Dilution*	*Supplier/Catalog Number*
Primary	CTIP2	Rat	1:1000	Abcam / ab18465
Primary	FOXP2	Rabbit	1:2000	Abcam / ab16046
Primary	DARPP-32	Rabbit	1:500	Abcam / ab40801
Primary	NeuN	Mouse	1:500	Merck Millipore MAB377
Secondary	Alexa Fluor® 488 goat anti-Rat IgG (H+L)	Goat	1:2000	Life Technologies
Secondary	Alexa Fluor® 555 goat anti-Rabbit IgG (H+L)	Goat	1:2000	Life Technologies
Secondary	Alexa Fluor® 488 goat anti-Rabbit IgG (H+L)	Goat	1:2000	Thermo Fisher Scientific (A11034)
Secondary	Alexa Fluor® 546 goat anti-Mouse IgG (H+L)	Goat	1:2000	Thermo Fisher Scientific (A11030)

3.4 Image acquisition and quantification

Fluorescence images were acquired using a Leica DM6B widefield microscope at 20x and 40x magnifications depending on experimental setup. In addition, complementary brightfield images were obtained using *EVOS™ XL Core* inverted light microscope to assess gross cell morphology and culture appearance. For each experimental condition, four to five images per coverslip were acquired from multiple independent coverslips. Following image acquisition, quantitative image analysis was performed using ImageJ software. For the first experiment,

fluorescence intensity of CTIP2 and FOXP2 was measured from individual cells and expressed as integrated intensity values. Regions of interest (ROIs) were defined for each cell based on the corresponding fluorescence signal, and measurements were collected at the single-cell level. For the second experiment, fluorescence signals were quantified using the same approach, and values were averaged across cells within the same coverslip to obtain representative measurements for each condition using Excel. All images belonging to the same experiment were acquired using identical microscope settings to ensure comparability between conditions. Quantified values were subsequently exported for statistical analysis and graph generation.

3.5 Statistical analysis

All data analyses and graph generation were performed using GraphPad Prism software. Data are presented as mean ± SEM. Normality and log normality of the data distribution was assessed prior to statistical testing. Depending on the dataset, comparisons between two groups were performed using either parametric or non-parametric t-tests, while comparisons among multiple groups were conducted using one-way ANOVA or Kruskal-Wallis. Statistical significance was defined according to standard p-value thresholds: * $0.01<p\leq 0.05$, ** $0.001<p\leq 0.01$, *** $0.0001<p\leq 0.001$, **** $p<0.0001$.

4. RESULTS

4.1 Short-term impact of Activin A and RB5 co-treatment on early MSN differentiation from smNPCs

Despite the availability of multiple strategies to generate MSNs from iPSCs, many differentiation protocols remain time-consuming and depend on complex combinations of patterning cues, often resulting in variable differentiation efficiency (Fjodorova et al., 2020).

Activin A has been identified as an important signalling factor involved in promoting MSNs differentiation, primarily through activation of SMAD2/3-related pathways (Loomans & Andl, 2014).

Considering that smNPCs already represent a developmentally committed neural population, our lab aimed to refine early differentiation conditions by focusing on intracellular signalling dynamics. Previous unpublished data from our lab showed that the cell penetrating RB5, that potentiates global ERK signalling (Indrigo et al., 2023), enhances cortical neuron differentiation. Based on these findings, we aimed to investigate whether RB5 could also promote Activin A-mediated MSNs differentiation.

A short-term differentiation protocol was established to assess the dose-dependent effects of Activin A (25,50, and 100 ng/mL), either alone or in combination with RB5, on the first week of differentiation (Figure 11). Early MSNs commitment was assessed through immunofluorescence analysis of CTIP2 and FOXP2 expression, which are two markers associated with early stages of striatal neuronal development (Arber et al., 2015; Knowles et al., 2021a) (Figure 12). This approach was designed to identify the experimental condition that could most effectively promoted early MSN specification and could therefore be used in long-term differentiation experiments.

Our results showed that CTIP2 expression did not significantly change across the experimental conditions after one week of differentiation (Figure 13), therefore, CTIP2 expression did not provide a clear indication of the most effective treatment condition. This may be partially explained by the fact that CTIP2 is not exclusively specific to MSN identity, as it is also expressed in cortical neuronal populations (Arlotta et al., 2005).

Regarding FOXP2 marker, the analysis showed that 100 ng/mL Activin A alone induced the highest FOXP2 expression. Surprisingly, co-treatment with RB5 did not enhance, but instead reduced FOXP2 expression compared to Activin A alone (Figure 14).

These findings suggest that RB5 do not cooperate with Activin A in promoting MSNs specification but may instead divert the striatal fate towards a cortical associated phenotype. This interpretation is consistent with the reduction of FOXP2 expression following Activin A +RB5 co-treatment, which decreased to levels closer to the control, suggesting the loss of Activin A effects in striatal fate induction. In addition, the relative increase observed in CTIP2 expression in RB5-treated conditions may suggest a shift toward cortical identity. Further experiments will be needed to clarify the molecular mechanisms underlying this effect.

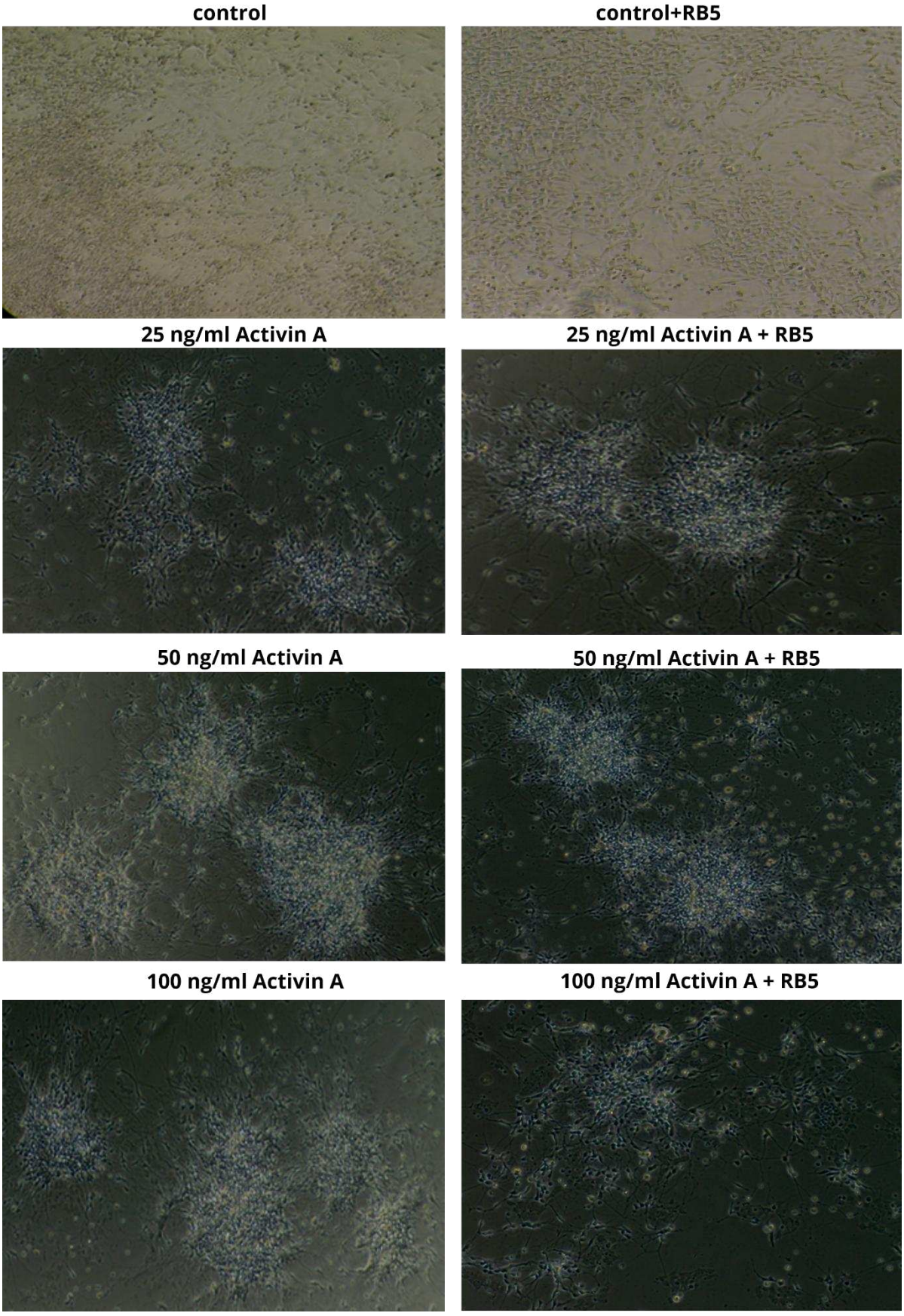

Figure 11. Representative brightfield images of Activin A treatment, in the presence or absence of RB5, after one week of differentiation.

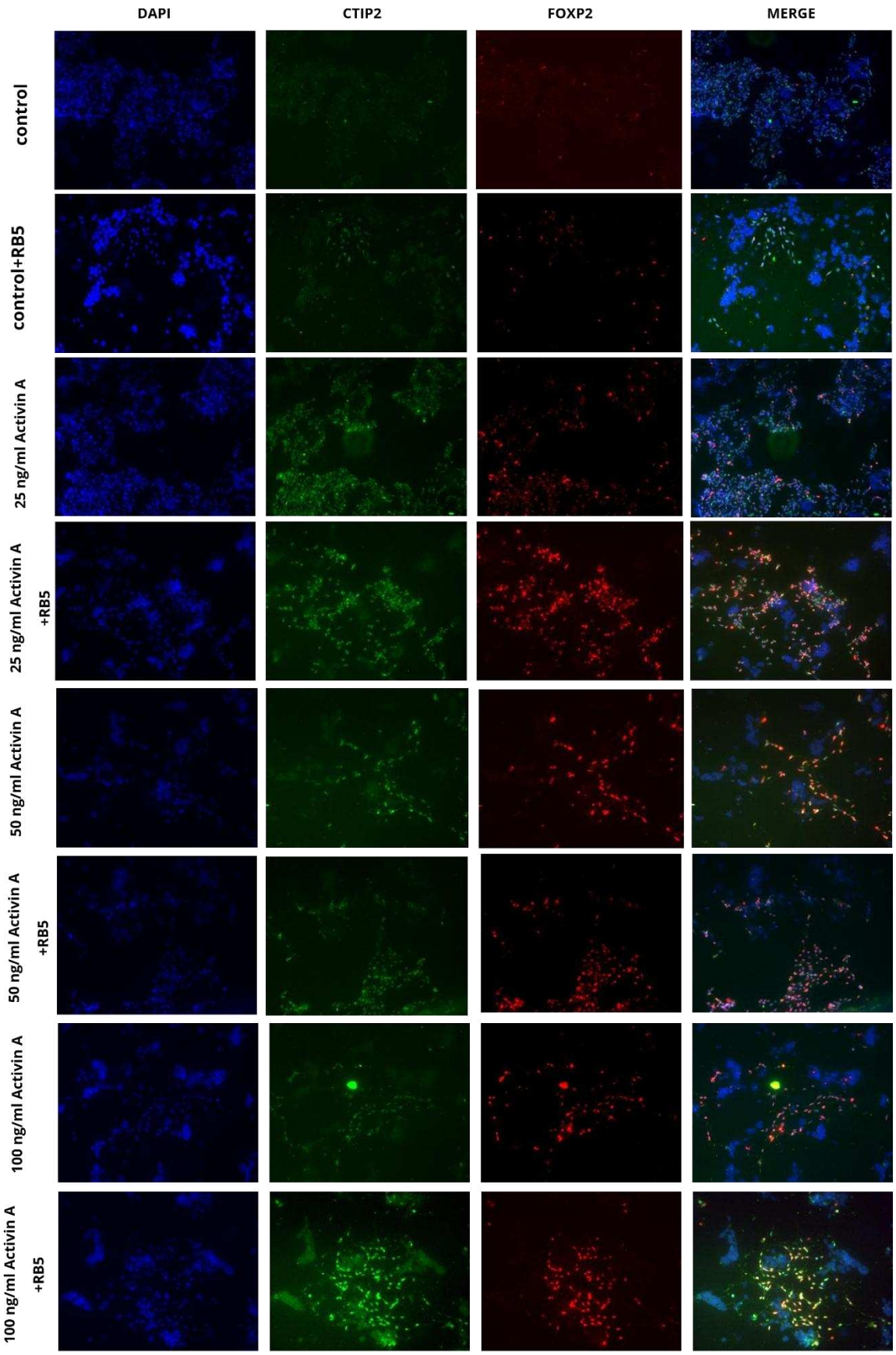

Figure 12. Representative immunofluorescence images showing CTIP2 and FOXP2 expression following treatment with increasing concentrations of Activin A, in the presence or absence of RB5.

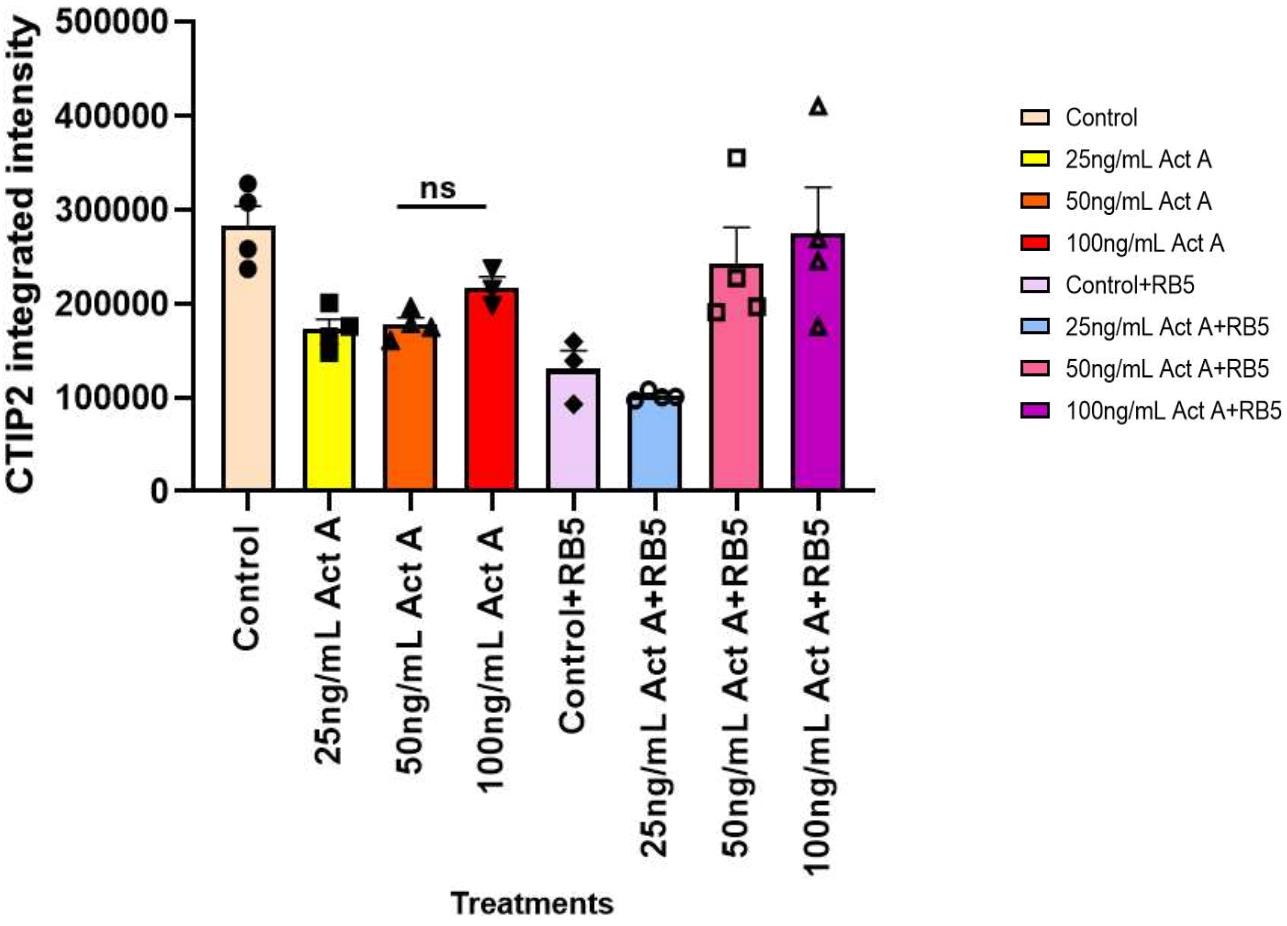

Figure 13. Quantification of CTIP2 integrated intensity following treatment with increasing concentrations of Activin A, in the presence or absence of RB5.

One-way ANOVA revealed no significant differences in CTIP2 integrated intensity across experimental conditions. Control n = 4; 25 ng/mL Activin A (Act A) n = 4; 50 ng/mL Act A n = 4; 100 ng/mL Act A n = 3; Control+RB5 n = 3; 25 ng/mL Act A+RB5 n = 4; 50 ng/mL Act A+RB5 n = 4; 100 ng/mL Act A+RB5 n = 4.

n = number of biological replicates (independent samples).

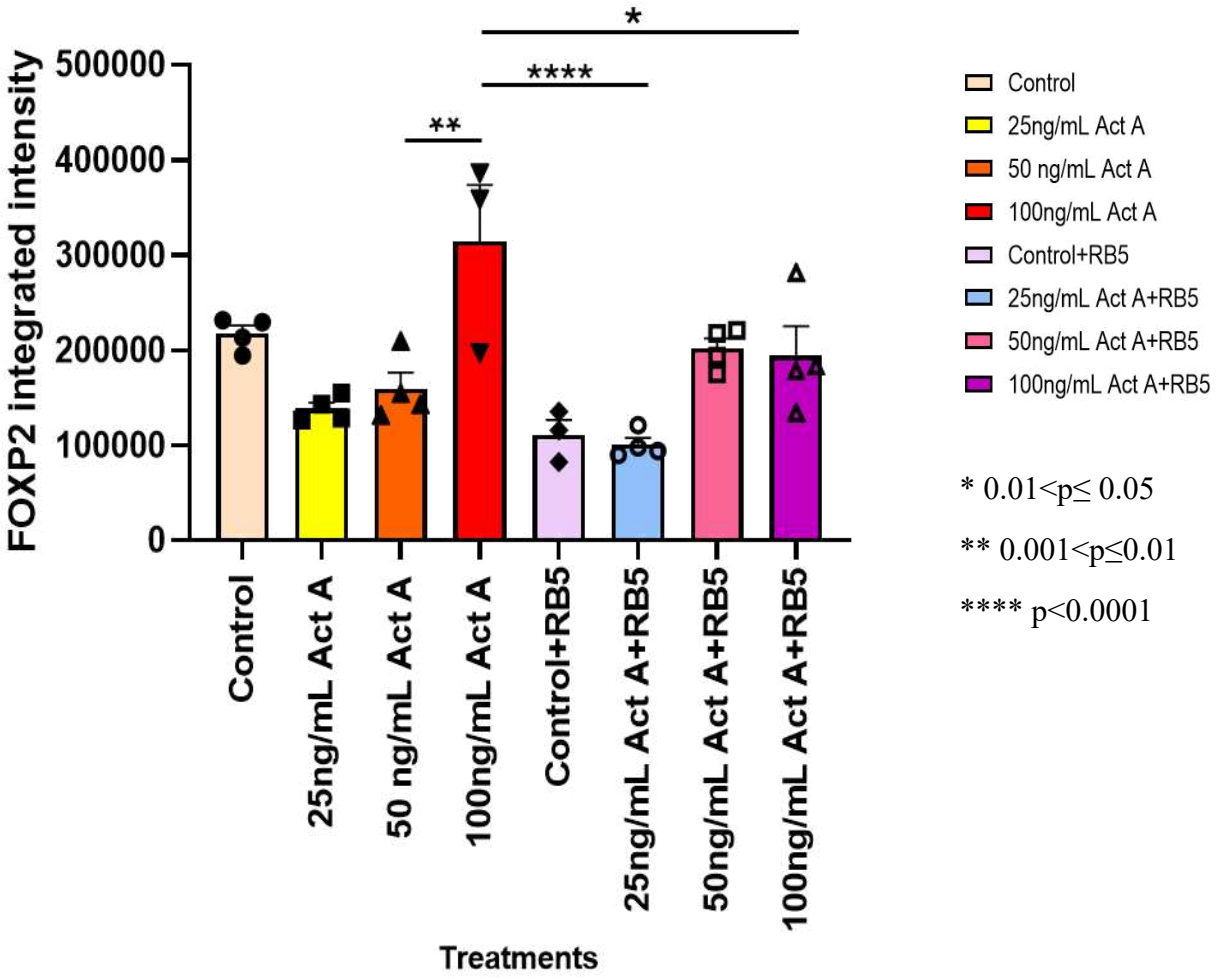

Figure 14. Quantification of FOXP2 integrated intensity following treatment with increasing concentrations of Activin A, in the presence and absence of RB5.

A pronounced increase in FOXP2 expression was observed at 100 ng/mL Activin A compared to all experimental conditions. One-way ANOVA statistical analysis revealed significant differences between 100 ng/mL Activin A and 100 ng/mL Activin A + RB5 (P-value = 0.0349), between 100 ng/mL Activin A and 25 ng/mL Activin A + RB5 (P-value < 0.0001), and between 50 ng/mL Activin A and 100 ng/mL Activin A (P-value = 0.0030).

Control n = 4; 25 ng/mL Act A n = 4; 50 ng/mL Act A n = 4; 100 ng/mL Act A n = 3; Control+RB5 n = 3; 25 ng/mL Act A+RB5 n = 4; 50 ng/mL Act A+RB5 n = 4; 100 ng/mL Act A+RB5 n = 4. n = number of biological replicates (independent samples).

4.2 Four-week assessment of Activin A-mediated differentiation and maturation of MSNs from smNPCs

Based on the previous experiment showing a significant induction of the early striatal marker FOXP2 after treatment with 100 ng/mL Activin A, we selected this dose to differentiate MSNs over four weeks.

Although early neuronal markers can provide initial information of MSN lineage commitment, they are not sufficient to fully evaluate neuronal maturation and the establishment of mature neuronal like phenotype.

Therefore, we performed a prolonged differentiation to further investigate whether sustained Activin A exposure could support the progression of MSN maturation.

smNPCs were differentiated in the presence of 100 ng/mL Activin A and analysed longitudinally from T0 (day 1 of treatment) to week 4 (Figure 15).

Immunofluorescence analysis was performed to evaluate MSNs differentiation using DARPP-32, a late-stage MSN marker (Arber et al., 2015) and NeuN, a post-mitotic neuronal marker (Kim et al., 2009) (Figure 16).

We observed that NeuN expression increased across differentiation (Figure 19), thus confirming efficient transition toward a post-mitotic neuronal state. Notably, DARPP-32 expression showed a marked and progressive enrichment with the highest levels observed at week 4 in the presence of Activin A (Figure 17 and 18), thus indicating strengthened commitment toward a striatal MSNs identity.

Collectively, these findings demonstrate that sustained Activin A exposure promotes a progressive and time-dependent maturation of smNPC-derived neurons.

Importantly, the increased expression of mature neuronal markers was paralleled by evident morphological changes. As shown in Figure 15 and 16, Activin A-treated cells displayed enhanced neurite extensions over time, clearly evident under a bright field microscope (Figure 15) from, week 3 onwards and by immunofluorescence staining (Figure 16).

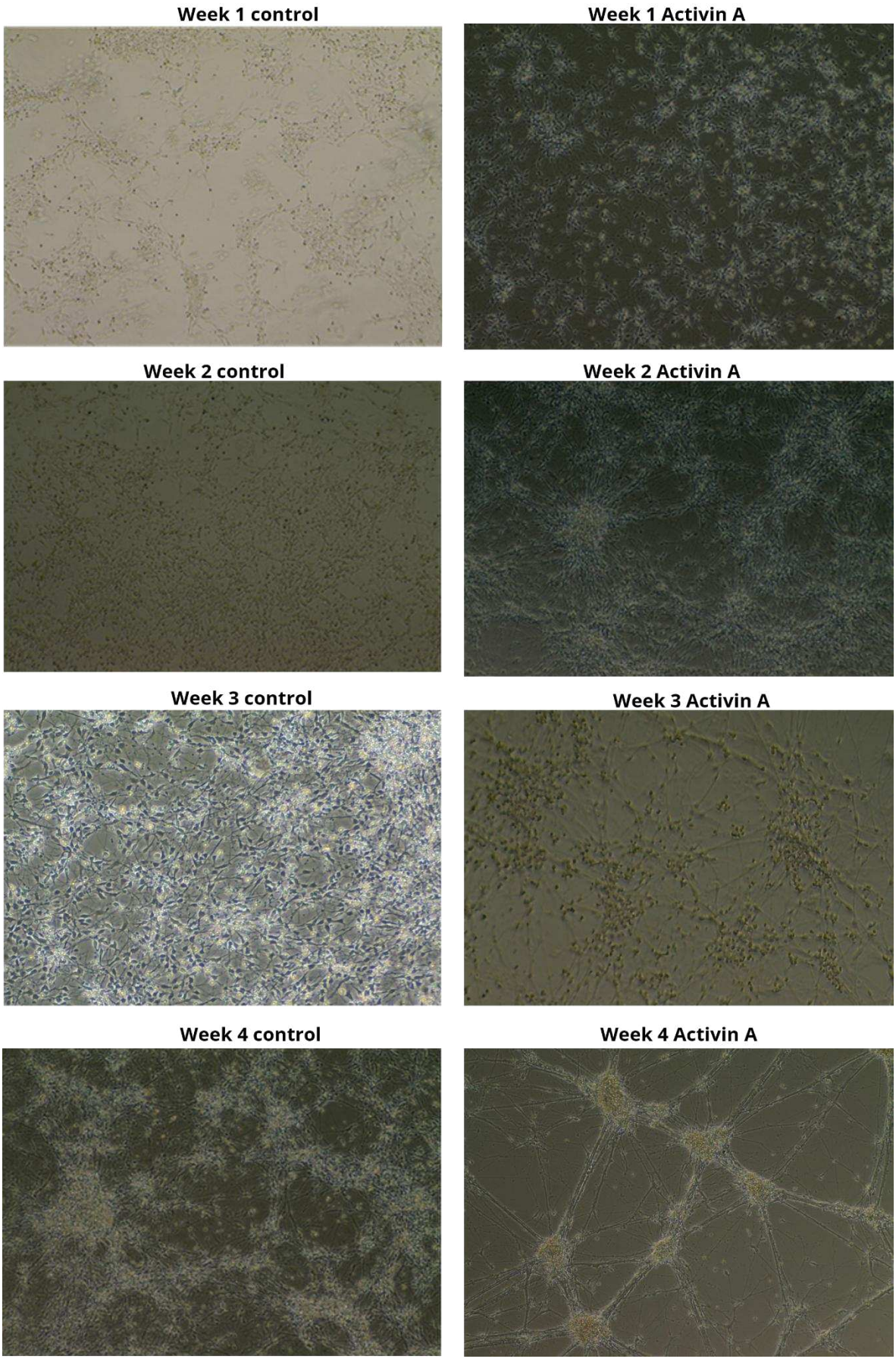

Figure 15. Representative bright-field images showing Activin A-treated cultures across four weeks of differentiation.

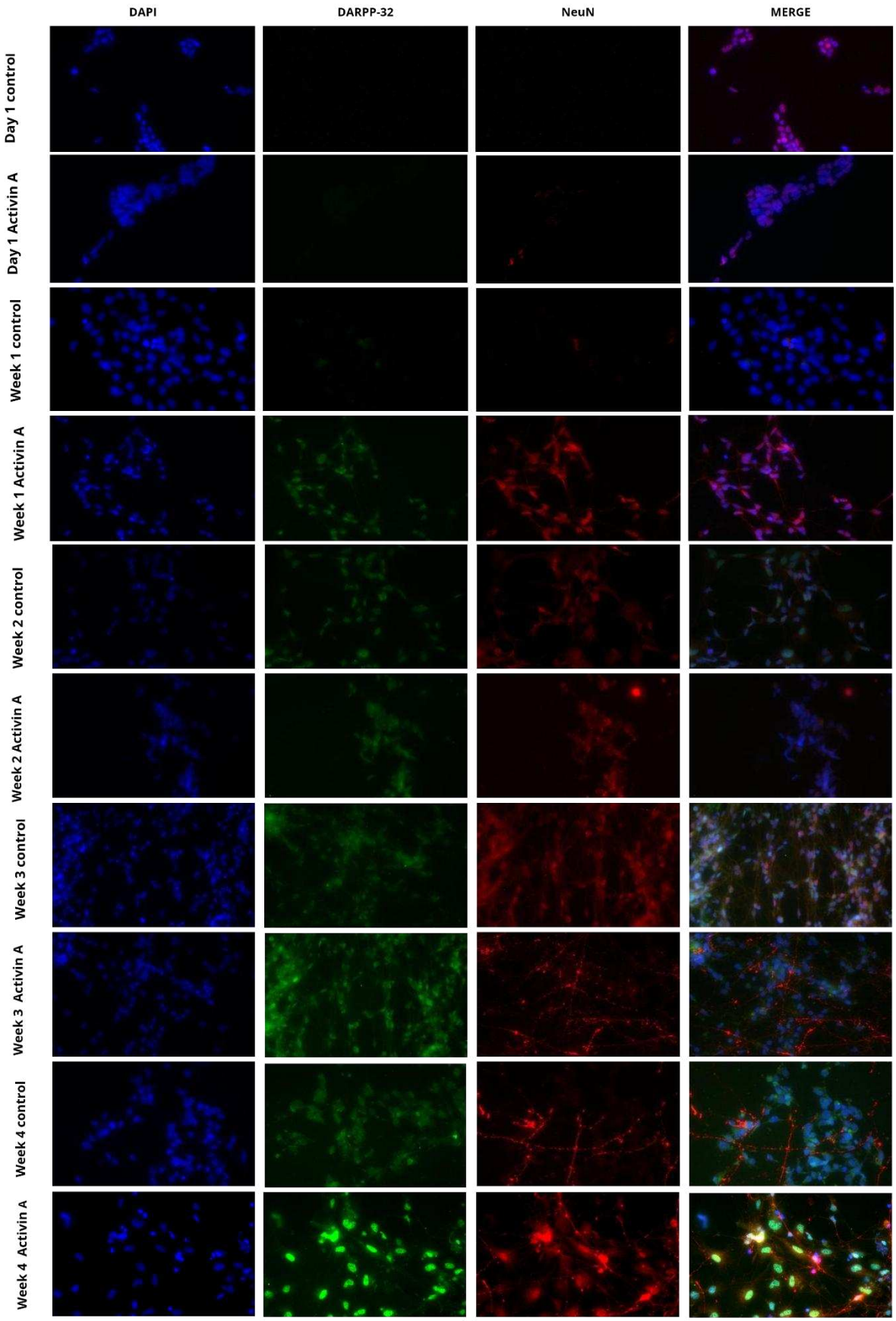

Figure 16. Representative immunofluorescence images of cultures differentiated over four weeks with 100 ng/mL Activin A treatment.

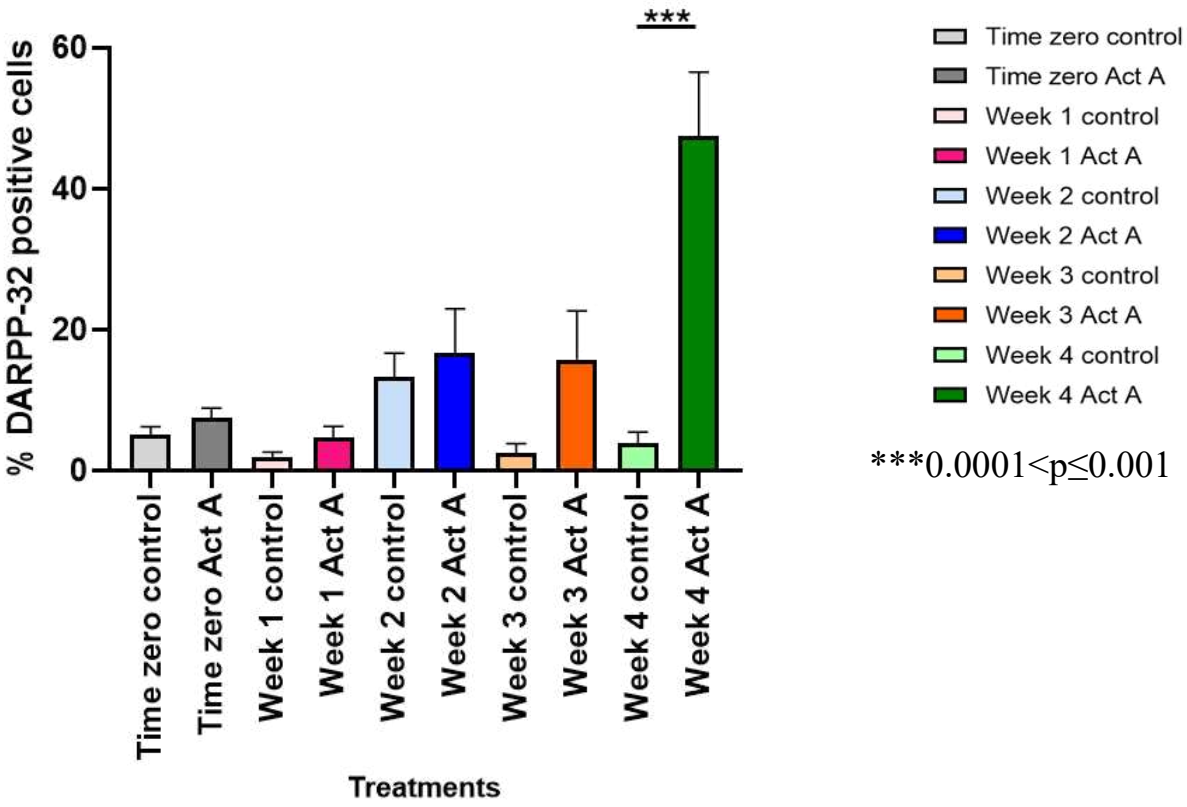

Figure 17. Percentage of DARPP-32-positive cells across four weeks of differentiation with 100 ng/mL Activin A.

DARPP-32-positive cells (over DAPI) progressively increase over time, with highest percentage observed after weeks of Activin A treatment. Kruskal Wallis analysis demonstrated a statistical significance between control and Activin A-treated groups at week 4 of differentiation (P-value = 0.0004). No other significant differences were detected between the other groups.

Time zero control n = 19; Time zero Act A n = 20; week 1 control n = 18; week 1 Act A n = 18; week 2 control n = 15; week 2 Act A n = 19; week 3 control n = 15; week 3 Act A n = 15; week 4 control n = 19; week 4 Act A n = 20.

n = number of technical replicates (images analysed).

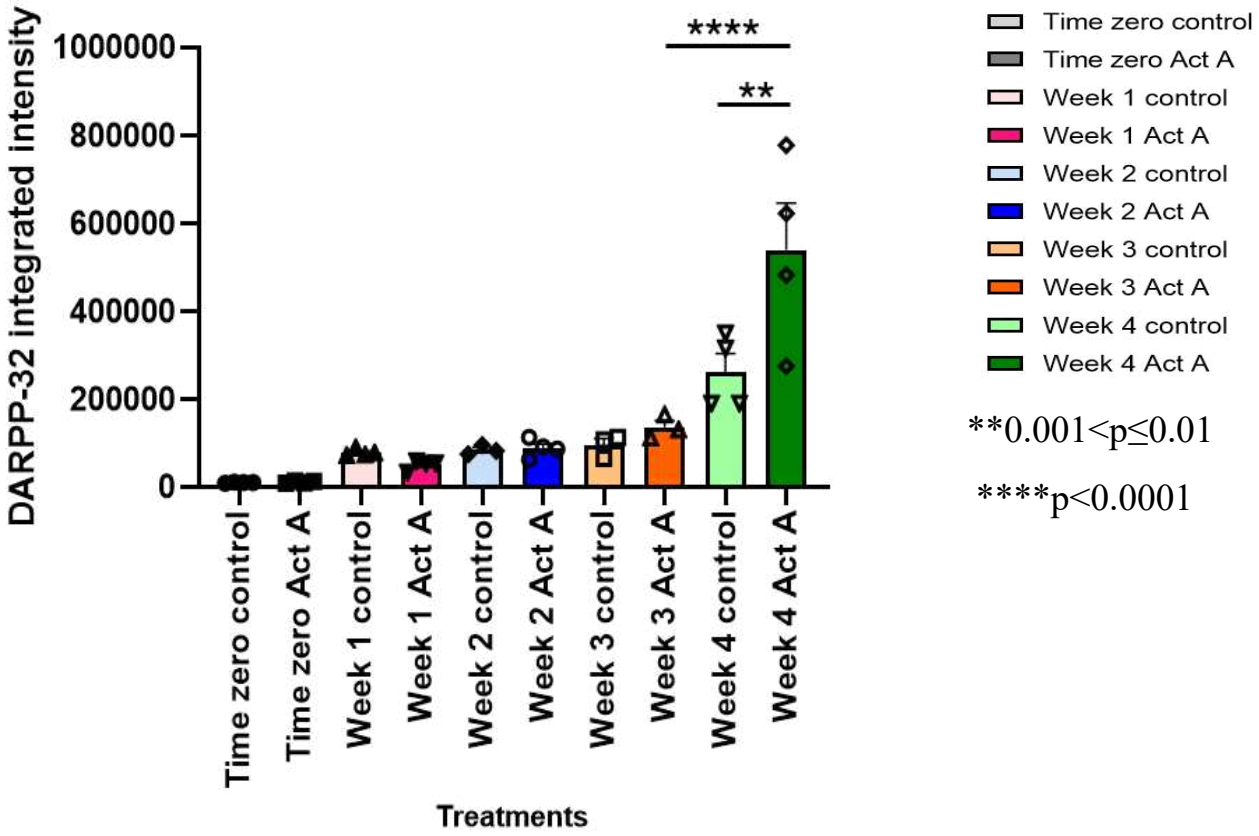

Figure 18. Quantification of DARPP-32 integrated intensity across 4 weeks of differentiation with 100 ng/mL Activin A.

One-way ANOVA revealed a statistically significant difference between control and Activin A-treated groups at week 4 of differentiation (P-value = 0.0012), and between week 3 and week 4 treated cells ($P < 0.0001$).

Time zero control n = 4; Time zero Act A n = 4; week 1 control n = 4; week 1 Act A n = 4; week 2 control n = 3; week 2 Act A n = 4; week 3 control n = 3; week 3 Act A n = 3; week 4 control n = 4; week 4 Act A n = 4.

n = number of biological replicates (independent samples).

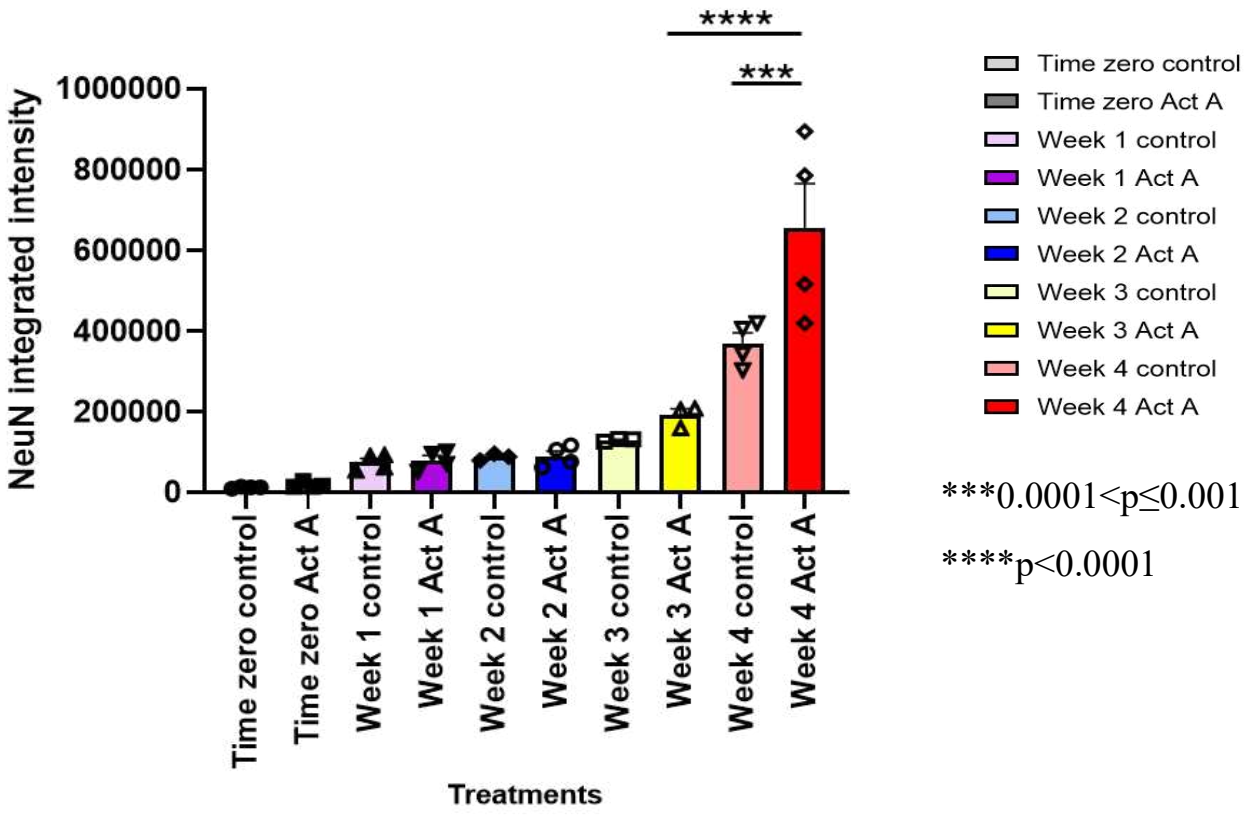

Figure 19. Quantification of NeuN integrated intensity during four-weeks differentiation with Activin A.

NeuN integrated intensity reached its maximum after 4 weeks of differentiation in the presence of Activin A. One-way ANOVA confirmed a significant difference between control and Activin A-treated group at week 4 (P-value = 0.0008). Furthermore, NeuN intensity at week 4 was significantly higher in Activin A-treated cells at week 4 compared to week 3 ($P < 0.0001$). Time zero control n = 4; Time zero Act A n = 4; week 1 control n = 4; week 1 Act A n = 4; week 2 control n = 3; week 2 Act A n = 4; week 3 control n = 3; week 3 Act A n = 3; week 4 control n = 4; week 4 Act A n = 4.

n = number of biological replicates (independent samples).

5. DISCUSSION

In this study, we investigated an Activin A-based strategy to promote the differentiation of human smNPCs toward MSN lineage.

Unlike other differentiation protocols that directly use induced pluripotent stem cells to produce MSNs (Arber et al., 2015; Noakes et al., 2015), our approach used small molecule-derived NPCs as the starting population, due to their already committed neural identity and reduced differentiation timeline.

Since MSN differentiation protocols frequently show variability in efficiency and maturation outcomes, this study aimed to optimize early differentiation conditions and to evaluate whether the modulation of ERK signalling pathway could influence MSN specification.

The NPCs used in this study were initially maintained under SM+ media (see Table 1), to preserve their neural progenitor characteristics and, proliferative capacity.

The Small Molecule protocol (Reinhardt et al., 2013) relies on the action of PMA, that activates the SHH pathway, necessary to induce progenitor proliferation and ventralization through LGE specification. In addition, the protocol employs CHIR99021, that activates canonical WNT/β-catenin signalling through GSK3β inhibition, thereby supporting neural progenitor self-renewal and survival (Reinhardt et al., 2013; Zink et al., 2021).

During early stages of neural development, SHH signalling is essential to preserve developmental competence and support the acquisition of ventral forebrain characteristics associated with MSN-generating regions (Ulloa & Martí, 2010).

As differentiation proceeds, the cellular requirements gradually shift from progenitor maintenance toward neuronal maturation. In this later phase, Activin A signalling becomes increasingly essential in promoting cell cycle exit and MSN lineage specification through activation of differentiation-associated pathways, particularly SMAD2/3 signalling, thereby facilitating progression toward a more mature neural phenotype (Loomans & Andl, 2014).

Consistent with these developmental procedures, several differentiation protocols have incorporated Activin A to promote MSN differentiation in vitro, from human pluripotent stem cell populations (Fjodorova et al., 2020).

Based on this approach, the first experiment in our study aimed to evaluate whether modulation of Activin A concentration could influence the early MSN differentiation efficiency in smNPCs cultures. NPCs were exposed to increasing concentrations of Activin A (25,50, and 100 ng/mL)

either alone or in combination with RB5 (50 μL), for one week, to determine the most effective condition. We assessed maturation using earliest MSN neuronal markers CTIP2 and FOXP2 (Figure 12), which are commonly expressed during initial stages of striatal neuronal development. Among all tested conditions, treatment with 100 ng/mL Activin A without RB5 showed the strongest FOXP2 expression (Figure 14), suggesting a more suitable condition for MSNs induction.

The interpretation of CTIP2 expression, however, was less clear across different experimental conditions (Figure 13). Although CTIP2 is widely used as an early MSN marker, its expression is not only restricted to striatal lineage. Previous studies have indeed shown that CTIP2 is also highly expressed in corticospinal and deep-layer cortical projection neurons during neurodevelopment (Arlotta et al., 2005). For this reason, FOXP2 expression was considered the most informative readout for evaluating early MSN differentiation in this study.

RB5, a newly generated cell penetrating peptide, was included in our study to investigate whether the enhancement of global ERK signalling could support Activin A-mediated MSN differentiation, based on previous unpublished results from our lab showing RB5-mediated enhancement of cortical neurons differentiation. However, RB5 treatment did not potentiate FOXP2 expression compared with Activin A treatment alone, suggesting that ERK signalling modulation does not support early MSN differentiation under these conditions.

One possible interpretation is that ERK signalling induced by RB5, may interfere with ventral transcriptional programs involved in ventral MSN specification. Since MSN differentiation depends on tightly regulated ventral telencephalic patterning (Arber et al., 2015; Ulloa & Martí, 2010), altered ERK activity could shift differentiation balance toward a more dorsal identity rather than strengthening the ventral pathway. Interestingly, previous observations obtained within our laboratory showed an increased tendency toward neuronal differentiation in RB5-treated cultures compared to controls, further supporting the hypothesis that RB5 may promote dorsalization-related mechanisms and antagonizes the activity of Activin A.

Based on the results obtained from the one-week differentiation experiments, 100 ng/mL Activin A was selected for a four-week differentiation protocol.

To further investigate whether prolonged Activin A exposure could support MSN maturation, smNPCs were differentiated and analysed using DARPP-32, as a late MSN-specific marker and NeuN, as a post-mitotic neuronal marker to assess neuronal differentiation and maturation throughout the culture period (Figure 16). DARPP-32 expression progressively increased over time, with the strongest and highest signal observed at week 4 in cultures treated with 100

ng/mL Activin A (Figures 17 and 18). NeuN increased expression overtime further supported the demonstrates neural commitment during long-term differentiation (Figure 19).
In addition, both bright-field and immunofluorescence images (see Figures 15 and 16) revealed more evident neurite outgrowth and neuronal processes in the treated group after 4 weeks of differentiation compared with earlier time points, where these morphological features appeared less pronounced.

Differentiation experiments in the present studies were performed using N2B27 medium as the basal neuronal differentiation condition. This serum-free medium is widely used to support neuronal survival and maturation. During prolonged differentiation, regular media replacement is essential to maintain optimal culture conditions, as it provides nutrients and growth factors, removes toxic metabolites and reduces accumulation of cellular stress that may negatively affect cell viability. Importantly, the N2B27 used in our experiments lacked Vitamin A supplementation. Several MSN differentiation protocols have similarly used B27 without Vitamin A including those described by (Delli Carri et al., 2013; Stanslowsky et al., 2016).
In contrast, other protocols introduced Vitamin A in N2B27 medium in differentiation protocols (Cambray et al., 2012; Hunt et al., 2023).
Since retinoic acid (RA) has been shown to promote caudalization of ventral forebrain progenitors and enhance CGE specification (Hunt et al., 2023), its inclusion in the differentiation media could influence regional identity within GE progenitors. Given that MSNs originate from LGE-like domain, excessive RA exposure might shift progenitor identity toward more caudal fates. Therefore, the use of B27 without vitamin A in our protocol media may have limited RA-driven effects, enabling a more specific evaluation of Activin A-mediated differentiation.
Despite the clear differentiation outcomes obtained in the present study, several limitations should be considered when interpreting the differentiation outcomes. MSN specification was primarily evaluated through immunofluorescence analysis of FOXP2, CTIP2, DARPP-32 and NeuN expression, without additional molecular validation approaches such as qPCR or transcriptomic profiling. Moreover, although FOXP2 showed clearer and more consistent MSN-associated expression patterns across experimental conditions, interpretation of CTIP2 expression remains more complex due to its broader developmental association with other neuronal populations, including cortical neurons.
Another limitation concerns the mechanistic interpretation of RB5-related effects. Although the present findings suggest that modulation of ERK signalling may counteract MSN

differentiation, the downstream molecular pathways involved in this process were not directly analysed. Consequently, the precise relationship between ERK signalling, ventral MSN specification, and potential dorsalization-related mechanisms remains to be fully understood.

Future studies may help to further optimize this differentiation strategy by investigating additional Activin A concentrations and longer maturation timelines to better characterize the MSN differentiation over time.

Incorporating complementary molecular approaches, such as qPCR and western blot analysis, together with functional assays such as electrophysiological recordings, may provide a more comprehensive evaluation of neuronal identity and maturation.

Future characterization of D1- and D2- associated MSN populations could also help define MSN subtypes specification. In addition, further experiments could be designed to enhance long-term culture conditions by testing whether specific supplements, such as Vitamin A, may be more beneficial when applied at specific maturation stages rather than throughout the entire protocol. In parallel, comparing different media-change regimens could also provide insights into the conditions that better support neuronal survival, neurite formation and neuronal maturation.

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