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Proteom nervových kmenových buněk a jeho studium v průběhu cílené diferenciace

The study of neural stem cell proteome in the course of targeted differentiation

Dizertační práce

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Abstrakt

Neurologická onemocnění postihují velké množství lidí po celém světě a jejich počet každoročně stoupá. V této dizertační práci jsem se zaměřila na neurodegeneraci a nervové kmenové buňky, které představují důležitý experimentální model v neurobiologii a také nadějný prostředek pro léčbu poruch nervové soustavy. Aby bylo možné kmenové buňky v regenerativní terapii bezpečně používat, je nutné je přesně charakterizovat a určit nejen jejich diferenciační potenciál, ale také čistotu buněčné populace použité pro transplantaci. K tomu lze použít dobře definované buněčné markery. V této práci detailně popisuji vývoj cílené hmotnostně-spektrometrické metody Selected Reaction Monitoring (SRM) pro charakterizaci nervových kmenových buněk během jejich diferenciace. Tuto metodu založenou na kvantifikaci skupiny známých proteinových markerů lze použít pro rychlé určení diferenciačního stádia nervových kmenových buněk. V další části práce se věnuji podrobné charakterizaci povrchového proteomu nervových kmenových buněk v průběhu diferenciace pomocí hmotnostní spektrometrie a chemického značení živých buněk metodou Cell Surface Capturing (CSC). Tento přístup umožňuje nabohacení povrchových proteinů ve vzorku, jejich podrobnou analýzu a do budoucna také možnost účinnějšího třídění buněk. V přehledových studiích se věnuji hledání kandidátních proteinových markerů, které by mohly sloužit jednak pro efektivnější monitorování diferenciačního potenciálu nervových kmenových buněk a pro nabohacení subpopulace buněk vhodných pro transplantaci, ale také pro sledování rozvoje neuropatologie Huntingtonovy choroby.

Klíčová slova: nervové kmenové buňky, neurodegenerace, diferenciace, buněčná terapie, proteomika, hmotnostní spektrometrie

Abstract

Neurological diseases affect millions of people worldwide with growing incidence every year. In this work, I focused on neurodegeneration and neural stem cells which represent an important experimental model for neurobiology, and also a promising tool for the treatment of nervous system disorders. To ensure a safe regenerative therapy, the neural stem cells need to be precisely characterized before transplantation, and their differential potential and cell population purity must be verified. Here, I describe in detail the development of a targeted mass spectrometry method based on Selected Reaction Monitoring (SRM) demonstrating that this method is capable of characterizing neural stem cells upon differentiation. Via measuring well-defined cell markers, the method quantifies selected proteins and allows to determination of the differentiation stages of neural stem cells in a fast and reliable manner. Then, we aimed to perform a detailed proteome analysis of differentiating neural stem cells focused on cell surface proteins. I describe the results obtained from mass spectrometry-based analysis of proteins isolated by the technology called Cell Surface Capturing (CSC). The CSC method enriched cell surface proteins by chemical tagging of living cells. Using this approach, we were able to analyze in detail neural stem cells upon differentiation which has brought neural stem cells a step closer to more effective sorting strategies. Last but not least, I provided a list of candidate markers that could be either used to effectively monitor the differentiation potential of neural stem cells, or to enrich a preferable subpopulation of the cells for transplantation experiments. I also provided the list of candidate biomarkers for monitoring the development of Huntington's disease neuropathology.

Key words: neural stem cells, neurodegeneration, differentiation, cell-based therapy, proteomics, mass spectrometry

Seznam zkratek

AD	Alzheimerova choroba (z angl. *Alzheimer's Disease*)
ALP	Alkaline Phosphatase
ALS	amyotrofická laterální skleróza
BDNF	Brain-Derived Neurotrophic Factor
CD	Cluster of Differentiation
cDNA	komplementární DNA (z angl. *Complementary DNA*)
CNS	centrální nervová soustava
CNTF	Ciliary Neurotrophic Factor
CSC	Cell Surface Capture
CXCL1	Chemokine C-X-C motif ligand 1
DCX	Doublecortin
DDA	na datech závislá analýza (z angl. *Data-Dependent Acquisition*)
DIA	na datech nezávislá analýza (z angl. *Data-Independent Acquisition*)
EGF	Epidermal Growth Factor
ELISA	Enzyme-Linked Immuno Sorbent Assay
ESC	embryonální kmenové buňky (z angl. *Embryonic Stem Cells*)
FACS	Fluorescent Activated Cell Sorting
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
FGF-2	Fibroblast Growth Factor-2
GALC	Galactocerebrosidase
GDNF	Glial cell line-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
HD	Huntingtonova choroba (z angl. *Huntington's disease*)
HSC	hematopoetické kmenové buňky (z angl. *Hematopoietic Stem Cells*)

HTT	Huntingtin
ICAM1	Intercellular Adhesion Molecule 1
IF	imunofluorescence
IGF-1	Insulin-like Growth Factor-1
iPSC	indukované pluripotentní kmenové buňky (z angl. *Induced Pluripotent Stem Cells*)
iRT	indexovaný retenční čas (z angl. *Indexed Retention Time*)
LAMP1	Lysosome-Associated Membrane Glycoprotein 1
LC	kapalinová chromatografie (z angl. *Liquid Chromatography*)
MAP2	Microtubule-Associated Protein 2
MS	hmotnostní spektrometrie (z angl. *Mass Spectrometry*)
MSC	mezenchymové kmenové buňky (z angl. *Mesenchymal Stem Cells*)
Mw	molekulová hmotnost (z angl. *Molecular Weight*)
NES	Nestin
NGF	Nerve Growth Factor
NPC	nervové prekurzorové buňky (z angl. *Neural Precursor Cells*)
NSC	nervové kmenové buňky (z angl. *Neural Stem Cells*)
NT-3	Neurotrophin-3
OCT4	Octamer-binding transcription factor 4
OLIG1	Oligodendrocyte transcription factor 1
PD	Parkinsonova choroba (z angl. *Parkinson's Disease*)
PDGF-AA	Platelet-Derived Growth Factor-AA
PRM	monitorování paralelních reakcí (z angl. *Parallel Reaction Monitoring*)
SILAC	Stable Isotope Labeling by Amino Acids in Cell Culture
SRM	monitorování vybraných reakcí (z angl. *Selected Reaction Monitoring*)
TUBB3	Tubulin beta-3 chain
TOF	analyzátor doby letu (z angl. *Time Of Flight*)
VEGF-A	Vascular Endothelial Growth Factor-A

Obsah

1 Úvod

Neurologické poruchy jsou heterogenní skupina nemocí postihující centrální i periferní nervový systém. Příčiny mohou být genetické, ale i způsobené vnějšími faktory[1,2] či mechanickým poškozením[3]. Tato onemocnění postihují miliony lidí po celém světě, jejichž léčba je ve většině případů založena pouze na tlumení projevů nemoci[4]. Ve své dizertační práci se zaměřuji na neurodegeneraci a charakterizaci nervových kmenových buněk (NSC, z angl. *Neural Stem Cells*) v průběhu diferenciace.

NSC slouží v neurobiologii jako důležitý experimentální model regulace buněčných procesů a představují také slibný prostředek pro léčbu onemocnění nervové soustavy. Cílem buněčné terapie je náhrada poškozené tkáně a indukce regenerace prostřednictvím sekrece specifických proteinů[5]. Protokoly pro kultivaci, namnožení a diferenciaci NSC ale obvykle poskytují heterogenní populace buněk[6]. Velkou snahou je proto získání lépe definované bezpečné populace buněk, které lze snadno namnožit a udržet jejich diferenciační potenciál.

Pomocí studia proteinového složení jsem usilovala o podrobnou analýzu NSC a jejich monitorování v průběhu diferenciace. S využitím moderních metod založených na hmotnostní spektrometrii (MS, z angl. *Mass Spectrometry*) popisuji regulaci NSC na úrovni proteinových markerů dlouhodobě používaných pro sledování těchto buněk a jejich diferenciačního potenciálu. Dále se v této práci věnuji analýze povrchových proteinů NSC, které mohou najít uplatnění při charakterizaci diferencujících buněk, ale také při nabohacování subpopulace buněk vhodných pro transplantaci. Neméně důležitou součástí této práce je hledání nových kandidátních markerů, které by bylo výhodné monitorovat v průběhu diferenciace NSC a při rozvoji neuropatologie Huntingtonovy choroby (HD, z angl. *Huntington's disease*).

1.1 Kmenové buňky

Kmenové buňky jsou přítomné v těle po celý život jedince a dávají vzniknout diferencovaným buňkám, které tvoří základní stavební kameny tkání a orgánů. Zatímco v embryonální fázi představují kmenové nediferencované buňky většinu hmoty organismu, v dospělosti se nachází převážně v nikách kmenových buněk, kde vytváří mikroprostředí udržující rovnováhu mezi sebeobnovou a diferenciací.

Embryonální kmenové buňky (ESC, z angl. *Embryonic Stem Cells*) vznikají z vnitřní buněčné masy blastocysty při vývoji embrya. Jsou pluripotentní, tedy schopné diferencovat do jakéhokoliv typu buněk (kromě buněk odvozených z trofoblastu), a mají neomezenou schopnost sebeobnovy. ESC jsou charakteristické přítomností nebo zvýšenou expresí transkripčních faktorů NANOG (*homeobox protein NANOG)*, OCT4 (*Octamer-binding transcription factor 4*) a SOX2 (*SRY-box transcription factor 1 a 2*), nebo proteinů ALP (*Alkaline phosphatase*), CD9 a dalších[7,8]. Studium časné diferenciace umožňují také indukované pluripotentní kmenové buňky (iPSC, z angl. *Induced Pluripotent Stem Cells*), které v roce 2006 vytvořil tým vědců z Japonska z myších fibroblastů[9] a o rok později z lidských fibroblastů[10]. Jedná se o somatické buňky reprogramované *in vitro* do pluripotentního stavu indukovanou expresí čtyř transkripčních faktorů OCT3/4, SOX2, c-Myc a Klf4[9]. Tyto buňky vykazující velmi podobné vlastnosti jako ESC. Pluripotentní ESC a iPSC nachází široké uplatnění při studiu celé řady (pato)fyziologických stavů[11] a představují nadějný zdroj pro buněčné terapie[12]. Použití lidských ESC v buněčné terapii s sebou však nese etické problémy kvůli jejich původu. Tento aspekt překonávají iPSC[13].

V postnatálním a dospělém období jsou nástrojem k opravě tkáňového poškození částečně diferencované kmenové buňky. Tyto buňky jsou multipotentní a diferencují pouze do tkáňově specifických buněk. Většina tkáňově specifických kmenových buněk se v nikách nachází v latentní fázi a při poranění a regeneraci tkáně jsou aktivovány specifickými stimuly[13]. Příkladem tkáňově specifických kmenových buněk jsou NSC, ze kterých v embryonální fázi vzniká centrální a periferní nervový systém procesem neurogeneze. Příkladem úspěšně aplikované terapie kmenovými buňkami je nyní už rutinní transplantace kostní dřeně s využitím hematopoetických kmenových buněk při léčbě např. leukémie nebo poruch imunity.

Velkou pozornost si také vysloužil tzv. falešný obchod s nadějí, který prostřednictvím svých klinik nabízí aplikaci kmenových buněk na nejrůznější více či méně závažná onemocnění,

ale také k léčbě život ohrožujících stavů. Odborníci před takovými aplikacemi varují, protože klinicky neověřená léčba nejen, že nemusí být účinná, ale může být také velmi nebezpečná. Vkládáním důvěry do nesprávné léčby se může promeškat čas na vhodnou léčbu. Výsledkem aplikace nedostatečně charakterizovaných kmenových buněk může navíc po čase docházet ke vzniku nádorů. Tyto praktiky diskreditují seriózní výzkumy a potenciál využití kmenových buněk v regenerativní medicíně.

1.2 Neurogeneze

1.2.1 Embryonální vývoj centrální nervové soustavy

Nervový systém má svůj původ ve vnějším zárodečném listu – ektodermu a nervové ploténce. Notochord (struna hřbetní) vysílá do ektodermu signály, čímž indukuje jeho vývoj v neuroektoderm a následně nervovou ploténku. Ta se ohýbá, uzavírá a vytváří neurální trubici. Jak neurony, tak gliové buňky pocházejí z ventrální části neurální trubice. Zatímco neuronální buňky opouštějí ventrikulární zónu v postmitotickém stadiu, prekurzory gliových buněk si udržují schopnost se dělit. Z okrajů nervové ploténky vzniká také neurální lišta. Buňky neurální lišty migrují do mnoha míst a diferencují do mnoha typů buněk. Dávají vzniknout také periferním nervovému systému.

Proces neurogeneze je charakterizován migrací nezralých neuronů z místa jejich vzniku. V okamžiku, kdy neurony dosáhnou své cílové pozice v mozku, začnou rozpínat své axony a dendrity, což jim umožní komunikovat s okolními neurony skrze synapse. Synaptická komunikace mezi neurony pak vede k ustavení funkčních nervových okruhů.

Centrální nervová soustava (CNS) obsahuje neurony a gliové buňky. Gliové buňky jsou nejhojnějším typem buněk v CNS a patří mezi ně astrocyty, oligodendrocyty, Schwannovy buňky, satelitní buňky, ependymové buňky, mikroglie a radiální glie. Všechny tyto buňky jsou důležité pro správné využití mozkových funkcí. Astrocyty poskytují podporu neuronům. Mohou také uvolňovat gliotransmitery (např. glutamát) a tím vysílat signály sousedním neuronům. Kromě toho astrocyty prostřednictvím svých výběžků rozšiřují nebo zužují cévy a kontrolují tím tok živin a kyslíku do mozku. Samotné astrocyty představují více než 50 % všech buněk v mozku a jsou 10krát hojnější než neurony. Oligodendrocyty těsně obklopují neuronální axony v CNS a vytvářejí myelinovou pochvu zajišťující rychlejší přenos vzruchu a udržení integrity axonů. Myelinovou pochvu v periferním nervovém systému

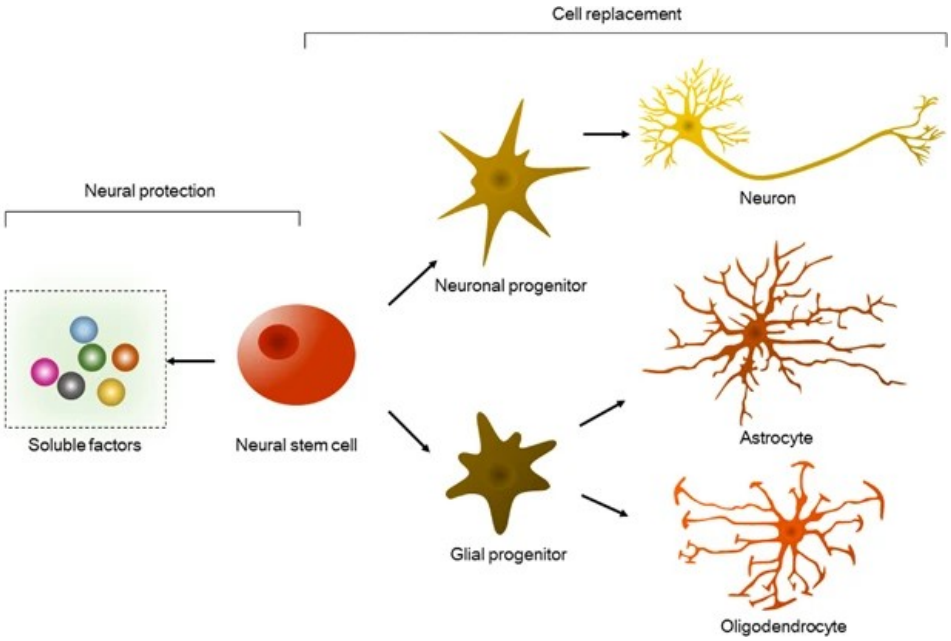
pomáhají vytvářet Schwannovy buňky. Metabolickou úlohu zastávají satelitní buňky a ependymové buňky. Ependymové buňky tvoří epitelovou výstelku dutin a hrají klíčovou roli v homeostáze mozkomíšního moku. Mikroglie hrají roli ve vývoji a údržbě neuronových sítí a při opravě poranění. Na rozdíl od ostatních glií nepocházejí z neuroektodermu[14] a mají imunitní vlastnosti, protože dokážou detekovat molekuly uvolňované z poškozených neuronů a produkovat v reakci na to zánětlivé mediátory[15,16]. Radiální glie jsou bipolární buňky se dvěma dlouhými výběžky. V největším počtu se vyskytují v počátečním období vývoje CNS, kdy slouží jako progenitory nových neuronů, astrocytů a oligodendrocytů a jako vodící skelet pro transport do místa určení takových buněk[17].

1.2.2 Neurogeneze v dospělém mozku

Lidský mozek byl dlouhou dobu považován za neschopný regenerace. V současnosti však už víme, že v dospělém centrálním nervovém systému se nachází kmenové buňky s potenciálem vytvářet neurony, astrocyty a oligodendrocyty. Taková neurogeneze je proces začínající dělením kmenové buňky a končící přítomností zralého, funkčního, integrovaného neuronu[18].

Nervové kmenové buňky jsou multipotentní buňky s neomezenou schopností sebeobnovy, které dávají vzniknout všem složkám CNS během embryogeneze. Udržují také určitou úroveň neurogenní aktivity po celý dospělý život jedince. Multipotentní progenitorové buňky jsou proliferující buňky s omezenou schopností sebeobnovy, které dávají vzniknout několika málo diferencovaným buněčným liniím. Liniově specifické prekurzory nakonec směřují pouze do jedné buněčné linie - neuronální, astrogliální nebo oligodendrogliální (shrnuto v [18]).

NSC se v dospělém savčím mozku nacházejí ve dvou oblastech, v oblasti hippokampu zvané gyrus dentatus, konkrétně v jeho subgranulární zóně, a dále v subventrikulární zóně postranních komor předního mozku, odkud putují podél rostrálního migračního toku do čichového laloku[19].



Obr. 1. Diferenciace NSC a jejich terapeutická úloha. NSC uvolňují rozpustné růstové a neurotrofní faktory chránící NSC před poškozením. NSC diferencují do neuronů, astrocytů a oligodendrocytů skrze stádia buněčných progenitorů. Podpora a náhrada buněk pak přispívají k regeneraci při léčbě neurologických poruch (převzato z [20]).

Dělení NSC v dospělém savčím mozku probíhá dvěma způsoby - symetricky a asymetricky[20]. V případě symetrického dělení dochází ke vzniku dvou stejných buněk, buď dvou diferencovaných buněk, nebo dvou dceřiných kmenových buněk, které zůstávají uvnitř ventrikulární zóny. V případě asymetrického dělení vzniká jedna kmenová buňka, která zůstává ve ventrikulární zóně, a jedna diferencovaná buňka, která putuje do výše umístěné intermediární zóny.

NSC v subventrikulární zóně preferují symetrické dělení, přičemž asi 80 % buněk se dělí na diferencované buňky a jen 20 % buněk má za úkol sebeobnovu. Toto rozložení významně snižuje zásoby NSC v subventrikulární zóně, ale zároveň produkuje velké množství prekurzorových buněk[20]. Při průchodu subventrikulární zónou mohou dát NSC vznik neuronálním prekurzorovým buňkám, zatímco při poškození míchy mohou diferencovat do astrocytů, které vytváří gliovou jizvu[21]. Na druhé straně, NSC v subgranulární zóně se dělí převážně asymetrickým způsobem, kdy vzniká stejný počet diferencovaných buněk jako kmenových[19].

1.2.3 Neurodegenerativní choroby

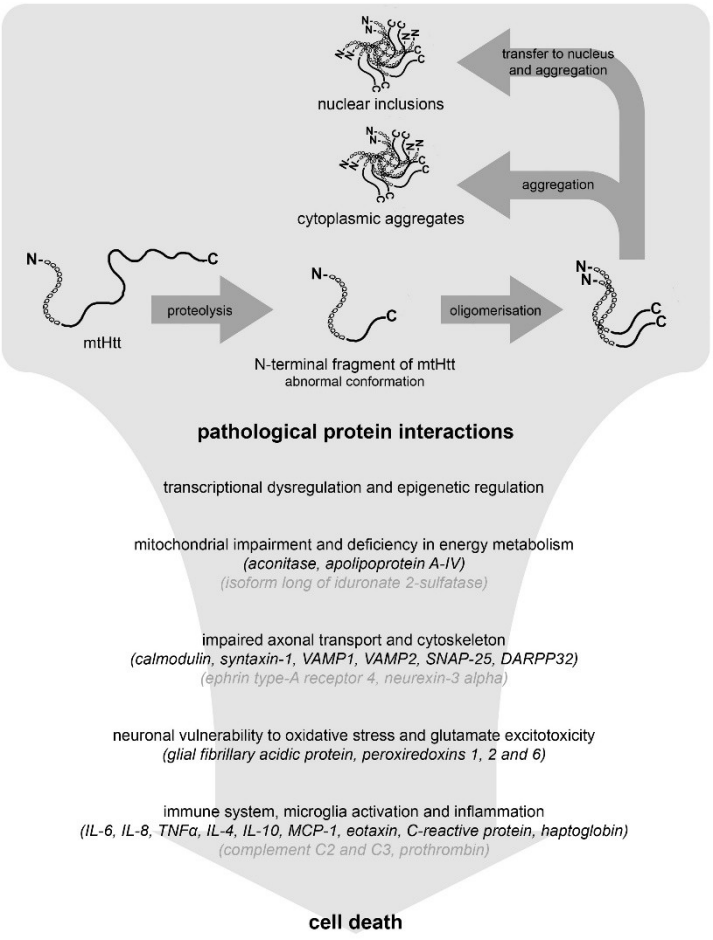
Onemocnění nervového systému představují ve stárnoucí populaci vyspělých zemí enormní zátěž pro zdravotnický a sociální systém. V této práci se zaměřuji na neurodegenerativní choroby ovlivňující životy milionů lidí po celém světě. Přesto, že příčiny a klinické projevy neurodegenerativních chorob jsou rozmanité, tato onemocnění jsou v zásadě charakterizována postupnou ztrátou nervových buněk, primárně neuronů, což se projevuje zhoršenou funkcí nervového systému. Mezi nejznámější nemoci patří Alzheimerova choroba, Parkinsonova choroba, Huntingtonova choroba nebo amyotrofická laterální skleróza.

Alzheimerova choroba (AD, z angl. *Alzheimer's disease*) patří mezi nejčastější příčiny demence ve vyšším věku. AD je charakterizována extracelulárním hromaděním plaků amyloidu beta a vzniku neurofibrilárních spletí Tau proteinu, v důsledku čehož dochází k poškození nervových synapsí a k smrti neuronů především v mozkové kůře[22]. AD se projevuje poruchami paměti, změnami chování a ztrátou kognitivních funkcí[23]. U vzácnější familiární formy AD hrají roli hlavně genetické faktory, zatímco u sporadické formy AD, která je mnohem častější a její projevy přichází až v pozdějším věku, jsou klíčové vnější faktory a běžné opotřebení tkání[22].

Parkinsonova choroba (PD, z angl. *Parkinson's disease*) je známá svými motorickými projevy, především nekontrolovatelnými pohyby a třesem. Projevuje se ale také zhoršením kognitivních funkcí, depresemi nebo poruchami spánku[24]. Toto onemocnění je charakterizováno úbytkem neuronů produkujících dopamin v části mozku zvané substantia nigra[25]. Nesprávně složené oligomery alfa-synukleinu tvoří intracelulární Lewyho tělíska, která způsobují oxidační stres a mitochondriální dysfunkce v dopaminergních neuronech[25]. Smrt těchto neuronů má za následek pokles produkce neurotransmiteru dopaminu, který je nezbytný pro regulaci pohybů. Při vývoji PD hrají roli především genetické faktory (zejména mutace genu pro alfa-synuklein), i když environmentální vlivy jsou také dobře popsány[25,26].

Amyotrofická laterální skleróza (ALS) je způsobena degenerací bulbárních, kortikálních a míšních neuronů vedoucí v konečném důsledku k jejich ztrátě. Většina případů ALS jsou sporadické a pouze přibližně 10 % má rodinnou anamnézu spojenou s mutacemi v řadě genů, jako je SOD1, TDP43 a FUS. Klinické projevy onemocnění jsou v obou případech velmi podobné a jsou charakterizovány nevratnou paralýzou či poruchami řeči, polykání i dýchání (shrnuto v [27]).

Huntingtonova choroba je autozomálně dominantně dědičné onemocnění způsobené mutací genu pro protein huntingtin (HTT), konkrétně expanzí CAG tripletu v 1. exonu tohoto genu. Výsledkem je produkce mutovaného huntingtinu nesoucího prodloužený polyglutaminový řetězec. Počet CAG tripletů je hlavní faktor určující počátek prvních projevů nemoci a délku života. HTT se vyskytuje v několika isoformách a jeho funkce a aktivitu regulují převážně jeho postranslační modifikace a interakce s jinými proteiny. Přítomnost mutovaného huntingtinu (mHTT) má za následek jeho proteolytické štěpení a hromadění fragmentů HTT zejména ve formě agregátů v jádře a cytoplazmě, což vede k narušení buněčných procesů (např. transkripce, autofágie, jaderného transportu nebo synaptického přenosu), které má za následek odumírání GABAergních neuronů zejména ve striatu a jeho atrofii (obr. 2, shrnuto v [28]). Vzhledem k rozmanitému působení HTT ve většině buněk těla jsou u pacientů s HD často zasaženy vedle CNS i další tkáně, např. kosterní svalstvo a srdce, varlata nebo krevní buňky[29]. Mezi klinické projevy patří hlavně mimovolní pohyby těla, poruchy řeči, poruchy kognitivních funkcí, deprese, ztráta paměti[30].



Obr. 2. Neurotoxické procesy a navazující patologie HD. Příklady proteinů nalezených v proteomových studiích jako regulované. Nevalidované proteiny jsou zobrazeny šedě (převzato z publikace *Challenges of Huntington's disease and quest for therapeutic biomarkers*[28]*)*.

1.3 Modely neurogeneze a neurodegenerativních onemocnění

Z praktických a etických důvodů se pro neurovědecký výzkum hojně využívají buněčné kultury a zvířecí modely. Zatímco díky zvířecím modelům se podařilo popsat celou řadu funkcí CNS, průlom v neurobiologii přineslo vytvoření linií lidských ESC a iPSC. Díky dalšímu rozvoji je možné v *in vitro* podmínkách vytvořit téměř jakýkoliv buněčný typ včetně nervových buněk a buňky v kultuře lze pozorovat, podrobně analyzovat a také kontrolovaně manipulovat[15].

1.3.1 Buněčné modely

Přesto, že lze u zvířecích modelů simulovat mnoho neurologických onemocnění, ne vždy tyto modely věrně napodobují průběh nemoci u lidí na molekulární úrovni. V některých případech pro studium mechanismů, které jsou podstatou onemocnění, lépe poslouží kmenové buňky izolované z nemocné tkáně nebo kmenové buňky geneticky modifikované tak, aby exprimovaly mutantní proteiny.

iPSC představují pokročilý *in vitro* model nejrůznějších onemocnění. Přeprogramované somatické buňky lze pomocí specifických faktorů diferencovat do neuronů či gliových buněk a u takto připravených buněk může být zkoumán jak mechanismus vzniku a průběhu onemocnění, tak mohou sloužit pro vývoj a efektivní testování nových léčiv, jejich účinnosti i toxicity[15,31]. Nesmírnou výhodou je, že iPSC mohou být připraveny přímo z buněk pacienta a obsahují tak jeho konkrétní genotyp, včetně specifických mutací vedoucích k onemocnění. Patofyziologie daného onemocnění se pak v *in vitro* podmínkách může projevovat bez dalších vnějších zásahů.

Ve zkumavce lze za specifických podmínek s využitím speciálních kmenových buněk vytvořit malé části nazývané organoidy, tj. zmenšené verze orgánů jako je například mozek[32]. Protože tkáně jsou tvořeny buňkami propojenými mezibuněčnou hmotou ve struktuře, která ovlivňuje jejich funkci, tuto organizaci lze úspěšně rekapitulovat s využitím organoidů, ve kterých si buňky vytváří specifické 3D mikroprostředí pro růst a vývoj[32]. Experimenty s využitím organoidů přináší nové neobjevené možnosti studia neurobiologie[33–35] a do budoucna jistě také etické otázky s nutností nastavit pravidla pro používání těchto modelů s obrovským potenciálem.

1.3.2 Zvířecí modely

Zvířecí modely jsou velmi cenné jak pro studium etiopatogeneze neurodegenerativních onemocnění, tak pro výzkum v oblasti terapeutických přístupů. Obecně lze zvířecí modely rozdělit na geneticky modifikované a indukované (farmakologicky, toxicky).

Historicky se u většiny lidských chorob nejvíce využívají myší modely, které představují důležitý nástroj pro zkoumání a pochopení nejrůznějších patologických stavů. Jejich výhodou je krátká doba života s možností brzkého vývoje fenotypu, velká reprodukční kapacita a nízké ekonomické náklady na chov. Pro plnohodnotné modelování lidského onemocnění mají však myší modely určitá omezení. Jsou zde zejména limity v důsledku malé velikosti experimentálního zvířete a z toho vyplývající omezené možnosti použití zobrazovacích metod, chirurgické intervence apod. Omezená je také možnost testování kognitivních funkcí a behaviorálních projevů[28].

Tyto limity je možné překonat s využitím modelů velkých zvířat: transgenní ovce, primáti a (mini)prasata. Vzhledem k délce jejich života umožňují věrnější pozorování progrese choroby. U velkých zvířecích modelů lze použít neurochirurgické procedury a neinvazivní zobrazovací metody podobné těm používaným v humánní klinické diagnostice[36]. Díky mnohým anatomickým, fyziologickým a metabolickým podobnostem se pro studium lidských chorob s výhodou využívá modelu miniprasat, která jsou pro své sofistikované kognitivní a motorické schopnosti ideální také pro dlouhodobé studie učení, paměti a chování[37].

1.4 Buněčná terapie

V současnosti existují desítky variant seriózní buněčné terapie s využitím různých typů buněk vkládaných do různých orgánů a tkání za různých podmínek. Jejich působení lze charakterizovat dvěma základními principy. Zaprvé, buňky vložené do určeného místa působení nahrazují poškozenou tkáň. Zadruhé, transplantované buňky generují v místě působení růstové faktory, cytokiny nebo chemokiny, které podporují vlastní opravu poškozených tkání[38,39].

1.4.1 Buněčná terapie kmenovými buňkami

Lidské pluripotentní kmenové buňky mají schopnost neomezené sebeobnovy a diferenciace do téměř všech typů zralých buněk. Výhoda iPSC spočívá v zamezení etických obav spojených s použitím lidských ESC a dláždí cestu k personalizované medicíně. K buněčné terapii lze využít multipotentní kmenové buňky, mezi něž patří nejčastěji používané hematopoetické kmenové buňky (HSC, z angl. *Hematopoietic Stem Cells*), epidermové kmenové buňky, NSC a mezenchymové kmenové buňky (MSC, z angl. *Mesenchymal Stem Cells*)[11].

Transplantace NSC v léčbě neurodegenerativních chorob a mechanických poškození nervové soustavy má velký potenciál, především díky jejich schopnosti produkovat neurotrofní faktory, působit protizánětlivě, zvyšovat neuronální plasticitu a nahrazovat odumřelé buňky[5]. Některé z těchto pozitivních efektů probíhají skrze buněčný kontakt, jiné skrze sekreci cytokinů, růstových faktorů, jednoduchých metabolitů a proteinů důležitých pro buněčnou komunikaci[5].

Pro terapeutický účinek v CNS je rozhodující schopnost transplantovaných buněk migrovat z místa vpichu do vzdálenějších oblastí nebo alespoň v postižených místech. V dospělé mozkové tkáni totiž chybí difúzní radiální glie účastnící se navádění NSC do vhodné cílové oblasti. Migrace a integrace transplantovaných NSC do hostitelského mozku může dále záviset na stádiu diferenciace dárcovských buněk, což ovlivňuje úspěch přihojení (shrnuto v [5]). Bylo publikováno několik experimentů ukazujících, že k transplantaci nejsou vhodné nezralé NSC ani terminálně diferenciované neurony, ale spíš neurony ve střední fázi diferenciace[40]. Důležitý je dostatek naváděcích signálů[5].

Úspěšná integrace neuronů odvozených od NSC do hostitelské nervové sítě vyžaduje, aby transplantované neurony byly schopné prodloužit své axony na dlouhé vzdálenosti, což lze podpořit pomocí genetické manipulace lidských NSC směrem k vyšší produkci transkripčních faktorů zodpovědných za růst axonů[41]. Další možností je ošetření dárcovských NSC speciálními látkami podporujícími růst axonů ještě před transplantací nebo modifikace vnitřního prostřední CNS k větší propustnosti pro migraci buněk a axonální růst[41].

Zvýšení terapeutického účinku NSC je možné také prostřednictvím modulace exprese vybraných růstových faktorů. Všeobecně NSC se zvýšenou expresi neurotrofinů BDNF (z angl. *Brain-Derived Neurotrophic Factor*), GDNF (z angl. *Glial cell line-Derived Neurotrophic Factor)*, NT-3 (*Neurotrophin-3*), IGF-1 (z angl. *Insulin-like Growth Factor-1*)

nebo NGF (z angl. *Nerve Growth Factor*) vykazují lepší schopnost přežívat a mají zvýšené proliferační a neuroprotektivní vlastnosti[5,42].

1.4.2 Klinické studie

Pro klinické studie buněčné terapie neurologických onemocnění se nejčastěji využívají MSC, a to jednak z důvodu jejich snadné dostupnosti z kostní dřeně či tukové tkáně, ale také protože jsou schopné integrace do místa poškození a mají imunomodulační efekt. Navíc jsou imunitně privilegované, takže je menší pravděpodobnost jejich odhojování po alogenní transplantaci (shrnuto v [38]). V posledních letech vrůstá také zájem o terapii neurologických onemocnění, především mozkové obrny a hypoxické ischemické encefalopatie, pomocí HSC. HSC získané z kostní dřeně pacienta nebo pupečníkové krve jsou tradičně využívané v terapii nádorových onemocnění krve nebo onemocnění imunitního systému[38].

Terapie neurologických poruch pomocí NSC se zaměřují především na mrtvici, roztroušenou sklerózu, míšní poškození nebo ALS, všechny se zatím vyskytují v klinické fázi I nebo II (shrnuto v [38]). Nervové kmenové buňky není možné získat přímo od pacienta, proto se využívají buď lidské ESC pro alogenní transplantace, nebo iPSC a MSC, které lze pomocí specifických protokolů diferencovat na NSC pro autologní transplantace[43]. Působení NSC v nemocné tkáni je založeno na produkci neurotrofních faktorů a na jejich schopnosti diferencovat se do neuronů a gliových buněk, a tedy nahradit poškozené nebo odumřelé buňky[38].

Míšní poškození

Terapie míšního poškození se nachází ve většině případů v I. fázi klinického testování. Jejím cílem je pomocí NSC nebo MSC transplantovaných do místa poškození překlenout mezeru v míšních drahách vzniklou poraněním a dodat do míchy nové buňky, které pomohou přenášet signál z mozku do místa za poraněním. Existuje několik mechanizmů, jakými působí kmenové buňky na míšní lézi. Především je to remyelinizace demyelinizovaných vláken, podpora pučení axonů, angiogeneze, imunosupresivní efekt či sekrece neurotrofních faktorů, které mohou vést k funkčnímu zlepšení[44]. Současný stav klinických studií nedovoluje vzhledem k malému počtu pacientů s míšním poraněním v jednotlivých studiích a heterogenitě souborů vyvodit jednoznačné závěry ve smyslu funkčního významu aplikace,

zatím lze pouze vyvodit jednoznačně pozitivní závěry ve smyslu bezpečnosti transplantace kmenových buněk[45].

Amyotrofická laterální skleróza

Buněčná terapie ALS probíhá v mnoha klinických studiích za využití NSC i MSC. Ty mají za úkol chránit a vyživovat zbývající motorické neurony a případně opravit poškozené neurony, a to prostřednictvím sekrece růstových faktorů, imunomodulačním působením na aktivované astrocyty a mikroglie, nebo diferenciací do podoby funkční glie[46]. Ačkoliv mnohé experimentální práce dokládají pozitivní efekt buněčné terapie na průběh nemoci, dosud publikované studie mohou stejně jako v případě míšního poškození pouze potvrdit bezpečnost buněčné terapie, ať už pomocí NSC[47] nebo MSC[48].

Jedním z příkladů je transplantace autologních MSC indukovaných k vyšší expresi neurotrofních faktorů firmou Brainstorm-Cell Therapeutics. Transplantace těchto MSC do míchy pacienta v II. klinické fázi prokázala bezpečnost terapie, fáze III ale bohužel neprokázala klinicky významné zlepšení[48]. Další z klinických studií testovala transplantaci neurálních progenitorových buněk do spinální míchy, které se diferencovaly do astrocytů a pomocí zvýšené exprese GDNF podporovaly přežívání míšních motorických neuronů pacienta. Nicméně ani zde se nepodařilo prokázat víc než bezpečnost terapie[47]. Je nezbytné poznamenat, že v drtivé většině experimentálních studií je léčba zahájena v presymptomatické fázi nebo bezprostředně po objevení se příznaků. Vzhledem ke značné prodlevě od prvních symptomů k diagnóze nelze takovou situaci reprodukovat v klinických studiích a v kontextu rychle progredující neurodegenerace se pak jedná o výrazně pokročilejší patologický proces[49].

Přesto, že terapie pomocí kmenových buněk má velký potenciál a lékaři i pacienti do ní vkládají naděje, většina těchto buněčných terapií je doposud ve fázi klinického testování. Dosud jediný prokazatelný úspěch terapie kmenovými buňkami tedy stále představuje transplantace krvetvorných kmenových buněk při léčbě leukémie, selhání kostní dřeně, některých vrozených krevních onemocnění, těžkých poruch imunity a dědičných poruch metabolismu[50]. Cestou k efektivnější buněčné terapii pomocí NSC je zdokonalení protokolů pro jejich izolaci, kultivaci a diferenciaci poskytující co nejméně heterogenní buněčné populace. Dále je zapotřebí podrobnější charakterizace vnášených buněk a intenzivní vývoj podpůrných strategií pro úspěšnou migraci a integraci transplantovaných buněk.

1.5 Proteiny typické pro ESC, NSC a diferencované buňky

K cílené a bezpečné buněčné terapii je nezbytná přesná charakterizace buněk. Jednotlivá stadia diferenciace od ESC, NSC až po zralé neurony a gliové buňky lze charakterizovat mimo jiné podle hladiny intracelulárních, povrchových a sekretovaných proteinů.

1.5.1 Intracelulární proteiny

Nejznámějšími a nejlépe popsanými markery ESC a iPSC jsou transkripční faktory NANOG a OCT4, které jsou zodpovědné za udržení pluripotence a schopnosti sebeobnovy[51]. Indukce pluripotentních buněk do NSC probíhá skrze formaci embryoidních tělísek a jejich kultivaci v bezsérovém mediu s přídavkem růstových faktorů EGF (z angl. *Epidermal Growth Factor*) a/nebo FGF (z angl. *Fibroblast Growth Factor*) na plastiku potaženým lamininem, který vytváří specifické prostředí podobné extracelulární matrix v mozku[52]. Pro udržení nervových kmenových buněk v nediferencovaném stavu a zachování jejich schopnosti sebeobnovy jsou nezbytné transkripční faktory PAX6 *(Paired box protein Pax-6*), SOX1 a SOX2 [53,54]. SOX2 má svou úlohu jak při udržování pluripotence v ESC a iPSC, tak při diferenciaci do NSC, kde ale jeho hladina klesá[55]. Výskyt intermediálního filamentu Nestinu značí přeměnu v multipotentní neurální prekurzor s omezenou schopností sebeobnovy a regenerační kapacitou[56].

Diferenciace NSC do liniově specifických subpopulací je doprovázena přítomností růstových faktorů BDNF a/nebo GDNF směrujících NSC k neurálním prekurzorům, nebo PDGF-AA (z angl. *Platelet-Derived Growth Factor*), který směruje diferenciaci k oligodendrocytům (shrnuto v [5,26,57]). V průběhu neuronální diferenciace se začínají objevovat markery DCX (*Doublecortin*), MAP2 (z angl. *Microtubule-Associated Protein 2*) a ßIII Tubulin (TUBB3, z angl. *Tubulin beta- 3 chain*)[58,59]. Buňky mění svoji morfologii, formují axony a dendrity a vytváří propletené sítě vláken[60]. Prekurzory astrocytů jsou typické přítomností GFAP (z angl. *Glial Fibrillary Acidic Protein*) nebo S100B (*Protein S100-B*)[61].

1.5.2 Povrchové proteiny

Pro klinické aplikace a transplantační experimenty je nezbytné nabohatit a očistit specifické populace buněk, k čemuž může sloužit selekce na základě přítomnosti/nepřítomnosti proteinů. Mezi nejznámější povrchové markery lidských pluripotentních buněk patří SSEA-3, SSEA-4 (z angl. *Stage Specific Embryonic Antigen 3/4*), TRA-1–60 a TRA-1–81[62]. Selekce buněk negativních pro tyto markery může eliminovat přítomnost nediferencovaných buněk, a tedy tvorbu teratomů po transplantaci. Neurální prekurzory lze charakterizovat expresí prominin-1 (CD133), SSEA-1 (CD15) nebo FORSE-1[5,62]. Diferencované nervové buňky exprimují PSA-NCAM (CD56) a CD24, zatímco astrocytální prekurzory jsou charakterizované expresí A2B5, CD44 a oligodendrocytální expresí PDGFR (CD140a), O1 a O4 (shrnuto v [5]). Úroveň exprese proteinů může být ovlivněna experimentálními podmínkami, proto je pro zvýšení specifity separace nejvhodnější zaměřit se na kombinaci povrchových markerů (shrnuto v [26,63]).

1.5.3 Sekretované proteiny

Pozitivním efektem transplantace NSC je jejich schopnost snížit zánětlivou reakci[64]. NSC dále podporují přežívání a regeneraci endogenních neuronů tím, že produkují neurotrofní faktory jako jsou BDNF, GDNF, NGF, NT-3, CNTF (z angl. *Ciliary Neurotrophic Factor*), VEGF-A (z angl. *Vascular Endothelial Growth Factor-A*) and IGF-1 a 2 (shrnuto v [5]). Tyto faktory podporují axonální/dendritické spoje a zvyšují přežívání a přihojování transplantovaných NSC. Převážná většina studií se však spoléhá na nepřímou korelaci příčiny a účinku, aby prokázala, že transplantované buňky mají imunomodulační roli spíše, než aby zobrazovaly specifické molekulární mechanismy[65].

1.6 Proteomové studie

Proteom je soubor proteinů, které se nachází v určitém čase na určitém místě, například v buňce organismu či specificky v některé buněčné organele za působení konkrétních vnějších i vnitřních podmínek. Proteomika se pak zabývá studiem změn hladin nebo posttranslačních modifikací proteinů, nebo reakcí proteinů mezi sebou. V oblasti proteomiky je k dispozici široká paleta separačních a identifikačních metod, které umožňují provádět explorativní i cílené analýzy proteomu. Každá z dostupných metod má svá specifika, která předurčují její využití. Kombinace různých proteomických metod a technologií vymezuje řadu dobře definovaných proteomických přístupů[66].

1.6.1 Protilátkové metody

Detekce proteinů pomocí specifických protilátek vyniká vysokou citlivostí a jednoduchostí. Skutečný proteomický protilátkový přístup spočívá v dostupnosti technologií, které umožňují analyzovat v jednom vzorku desítky až stovky různých proteinů[66]. Nevýhodou protilátkových metod je, že specifita a selektivita některých protilátek není vždy dostatečně otestována, a tak mohou tyto metody někdy poskytovat falešně pozitivní nebo negativní výsledky[66].

Protilátkové čipy umožňují screeningové vyšetření vzorků. Slouží například k porovnání vzorků s cílem vybrat kandidátní proteiny pro konkrétnější studium. Na pevném podkladu je imobilizované široké spektrum protilátek, které na sebe vážou vybrané proteiny. Detekce poté probíhá navázáním sekundární protilátky se značkou[66].

Multiplexní analýza typu Luminex funguje na stejném principu jako protilátkové čipy, ale protilátky jsou imobilizované na kuličkách, nejčastěji magnetických. Výhodou této metody je vysoká citlivost a malá spotřeba vzorku. Naopak nevýhodou je možnost detekce omezeného množství proteinů během jedné analýzy v důsledku vzájemné reaktivity protilátek[66].

Průtoková cytometrie umožňuje analýzu jednotlivých buněk, živých i fixovaných. Vzorek je vstřikován do průtokového cytometru a tenký proud tekutiny unáší jednotlivé buňky do místa, kam je namířen paprsek laseru. Při interakci s buňkami dochází k absorpci, fluorescenci a rozptylu světla. Parametry prošlého, vyzářeného a rozptýleného světla jsou charakteristické pro různé buňky a jejich části. Ačkoli průtoková cytometrie umožňuje analýzu velkého množství buněk za velmi krátký čas, spektrum parametrů, které lze hodnotit, je velmi malé

(shrnuto v [67]). Buňky mohou být během průtokové cytometrie také tříděny podle nastavených kritérií, tato metoda se nazývá FACS (z angl. *Fluorescent Activated Cell Sorting)*. Princip třídění je založen na elektrostatickém vychylování nabitých kapének nesoucích buňky na základě fluorescenční charakteristiky. Částice obsahující sledovaný fluorescenční znak je pozitivně nebo negativně nabita a poté dochází u kapiček obsahujících nabité částice k vyklonění z proudu a zachycení buněk do testovacích zkumavek[67].

Hmotnostní cytometrie kombinuje principy průtokové cytometrie s detekcí signálu pomocí MS s indukčně vázaným plazmatem a analyzátorem doby letu (TOF, z angl. *Time Of Flight*). Protilátky jsou značeny stabilními izotopy kovů, jednotlivé označené buňky jsou vaporizované, atomy kovů jsou ionizované a analyzované pomocí MS. Tento přístup umožňuje simultánní použití více stabilních isotopů kovů a analýzu většího množství parametrů, čímž je překonáno omezení dané spektrálním překryvem fluorescenčních značek. Neumožňuje však analýzu a třídění živých buněk (shrnuto v [68]).

1.6.2 Hmotnostně-spektrometrické metody

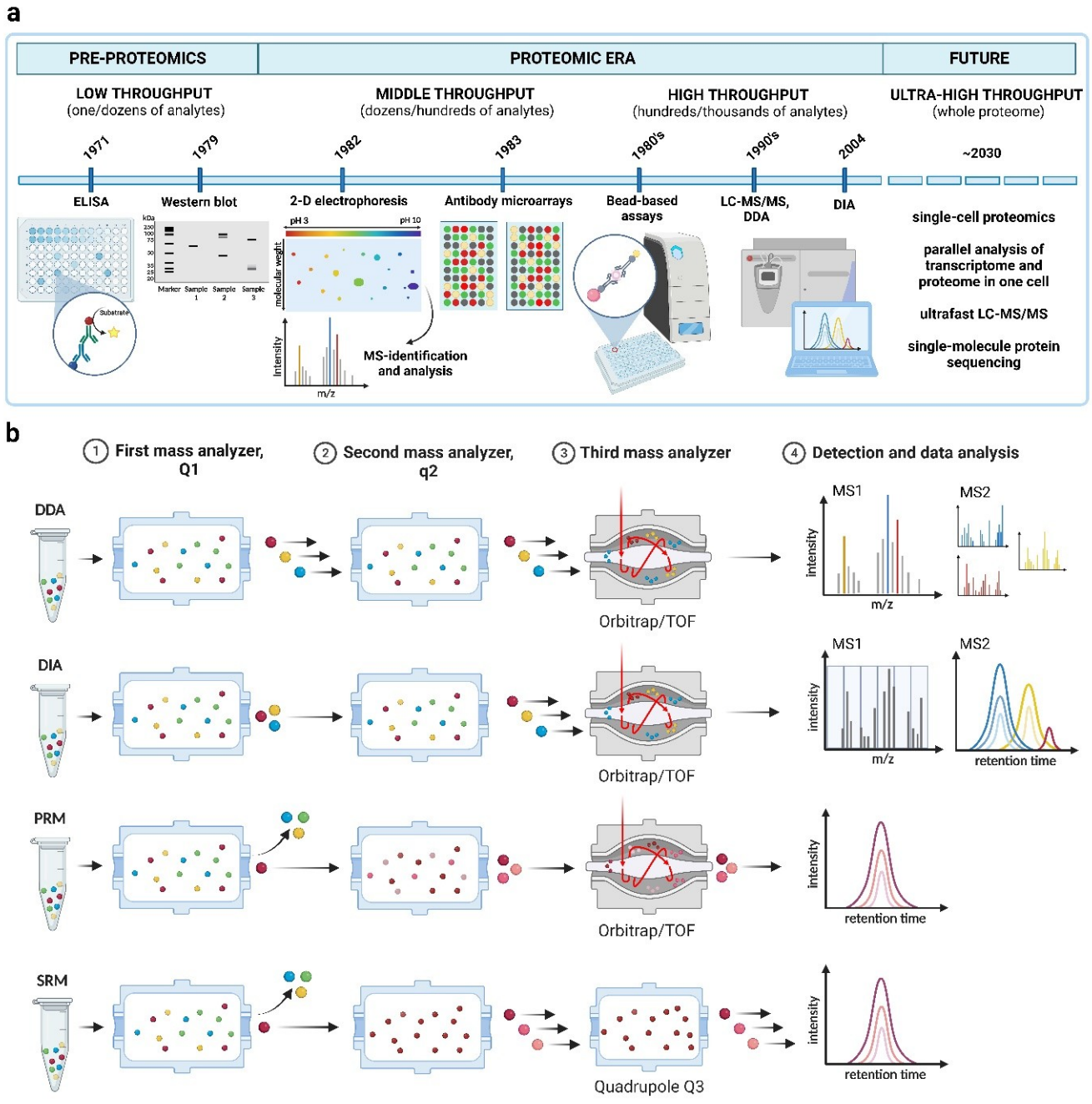
Hmotnostní spektrometrie je díky své přesnosti, citlivosti a všestrannosti nepostradatelným nástrojem pro analýzu proteinů. Může být použita ke stanovení aminokyselinových sekvencí peptidů a k charakterizaci široké škály posttranslačních modifikací, jako je fosforylace nebo glykosylace. MS lze v kombinaci s účinnou separací pomocí kapalinové chromatografie (LC, z angl. *Liquid Chromatography*) použít ke kvantifikaci tisíce proteinů z komplexních vzorků (shrnuto v [69]).

Obr. 3. Vývoj a princip proteomických přístupů.

a. Časová osa zobrazuje vývoj od počátečního přístupu ke studiu proteinů pomocí biochemických metod, které mají omezenou multiplexní kapacitu (imunoblot, ELISA), přes první skutečné proteomické metody (2-D elektroforéza s identifikací diferenčních proteinů pomocí MS, protilátkové čipy, multiplexní protilátková analýza na kuličkách), až po moderní metody založené na měření LC-MS/MS (DDA, DIA). Ve výhledu lze očekávat častější kombinaci s výsledky analýz transkriptomu a další rozvoj DIA ve smyslu vyšší rychlosti analýzy, citlivosti a rozlišení na úrovni jednotlivých buněk nebo molekul.

b. Při analýze založené na LC-MS/MS měření jsou proteiny štěpeny specifickou proteázou na peptidy, které jsou separovány kapalinovou chromatografií, ionizovány a analyzovány v tandemovém hmotnostním spektrometru. Analyty jsou filtrovány, fragmentovány a detekovány na základě velmi přesného stanovení poměru jejich hmotnosti a náboje (m/z). V případě globálních analýz DDA a DIA jsou cyklicky měřena kompletní spektra

prekurzorových iontů MS1. Při DDA je fragmentováno zvolené množství nejintenzivnějších iontů, jejichž produktové ionty jsou detekovány v MS2 s vysokým rozlišením (Orbitrap, TOF). Při DIA je MS1 spektrum rozděleno do definovaných oken a k fragmentaci jsou do MS2 poslány všechny prekurzorové ionty z tohoto okna. Cílené měření LC-MS/MS využívají metody PRM a SRM, které vyžadují znalost m/z prekurzorových iontů. Při PRM se zaznamenávají celá spektra MS2 ve vysokém rozlišení. Při SRM je nutné znát také m/z produktových iontů, které jsou před detekcí opět filtrovány, což umožňuje sledování pouze vybraných iontových přechodů. Zaznamenaná data jsou po integraci kvantifikována. DDA, Data-Dependent Acquisition; DIA, Data-Independent Acquisition; ELISA, Enzyme-Linked Immuno Sorbent Assay; LC, Liquid Chromatography; MS, Mass Spectrometry; PRM, Parallel Reaction Monitoring; SRM, Selected Reaction Monitoring; TOF, Time Of Flight. Převzato z [66].



1.6.2.1 *Data-dependent acquisition*

Na datech závislá analýza (DDA, z angl. *Data-Dependent Acquisition*), také nazývaná shotgun MS, je přístup používaný k identifikaci proteinů bez nutnosti předchozích znalostí vzorků. Metoda vyžaduje použití tandemového hmotnostního spektrometru a probíhá v cyklech. Nejprve se provádí MS1 skenování prekurzorových iontů, poté jsou prekurzorové ionty postupně vybírány pro fragmentaci na základě intenzity jejich signálu. Fragmentové ionty jsou následně měřeny v MS2. Pomocí DDA lze identifikovat a kvantifikovat tisíce proteinů, včetně jejich posttranslačních modifikací. Nejčastěji se ke kvantifikaci používají label-free metody založené na počítání spekter pro konkrétní peptid nebo na měření intenzity píku prekurzoru na úrovni MS1. Hlavní slabinou této metody je fragmentace a měření pouze nejintenzivnějších prekurzorů, což může negativně ovlivnit kvantifikaci v případě porovnání vzorků, ve kterých je specifický protein zastoupen v řádově odlišné míře[70,71]. Stochasticitu DDA lze obejít značením a kvantifikací na úrovni MS2. Existuje celá řada možností tohoto značení, například metabolické značení pomocí aminokyselin se stabilními izotopy v buněčné kultuře (SILAC, z angl. *Stable Isotope Labeling by Amino Acids in Cell Culture*)[72], proteolytické značení $18O$[73], nebo chemické značení pomocí izobarických značek pro relativní a absolutní kvantifikaci (iTRAQ nebo tandem-mass-tags)[74,75].

Dynamický rozsah proteinů ve směsi je velkou výzvou proteomických analýz. Pokud chceme detekovat i proteiny s nízkou hladinou, je možné před LC-MS analýzou odstranit ze směsi proteinů ty majoritní s vysokou hladinou[76]. Tento přístup se využívá například při analýze tělních tekutin jako je krevní plazma, mozkomíšní mok apod. U vzorků buněk nebo tkání lze méně zastoupené proteiny nabohatit nejrůznějšími technikami jako je imunoprecipitace, izolace váčků (např. exosomy, synaptosomy), nebo třeba izolací proteinů s konkrétní modifikací. V této práci jsme využili nabohacení membránových proteinů pomocí technologie CSC (z angl. *Cell Surface Capture*) fungující na principu chemického značení živých buněk[77].

Přestože je DDA z podstaty měření méně vhodná pro kvantifikaci, je ideální metodou pro tvorbu knihovny spekter, která je klíčová pro vysoce spolehlivou a přesnou kvantifikaci pomocí cíleného měření.

1.6.2.2 *Cílená hmotnostní spektrometrie*

Kvantitativní omezení DDA překonávají cílené metody MS. Tyto metody umožňují kvantifikaci souboru proteinů s vysokou přesností a reprodukovatelností. Typicky se používají cílené techniky – analýza nezávislá na datech (DIA, z angl. *Data-Independent Acquisition*), monitorování vybraných reakcí (SRM, z angl. *Selected Reaction Monitoring*) a monitorování paralelních reakcí (PRM, z angl. *Parallel Reaction Monitoring*). Peptidy použité pro kvantifikaci musí být pečlivě vybírány a hodnoceny s ohledem na jejich jedinečnost a vhodnost pro kvantifikaci (např. je výhodné se vyhnout peptidům s interferencí nebo aminokyselinami s nekonzistentní strukturální modifikací jako je oxidace methioninu).

Monitorování vybraných reakcí (SRM)

Pomocí metody SRM lze kvantifikovat desítky předem vybraných proteinů v rámci jedné analýzy a díky širokému lineárnímu dynamickému rozsahu je tak možné stanovit proteiny obsažené v množství 41 až 1,3 × 10^6 kopií na buňku v jedné analýze[78]. Pro analýzu SRM se používá hmotnostní spektrometr s trojitým kvadrupólem (QQQ). Předem vybrané prekurzorové ionty s definovanou hodnotou m/z jsou s rozlišením 0,7 Daltonu filtrovány v prvním kvadrupólu (Q1), odeslány do kolizní cely (druhý kvadrupól – Q2) a fragmentovány. Fragmentové (produktové) ionty jsou poté vedeny do třetího kvadrupólu (Q3), ve kterém jsou předem vybrané fragmentové ionty s definovanou hodnotou m/z filtrovány a odeslány do detektoru. Specifický pár prekurzorových a fragmentových iontů jednotlivých hodnot m/z se nazývá přechod (tranzice). Měření pouze vybraných přechodů zaručuje vysokou specificitu a selektivitu metody SRM. Hlavní nevýhodou metody SRM je omezený počet peptidů, které lze monitorovat v jedné analýze, nutnost znalosti proteinů a potřeba vývoje metody (výběr vhodných peptidů a přechodů).

Monitorování paralelních reakcí (PRM)

Tato metoda je principiálně stejná jako SRM, jen místo hmotnostního filtru QQQ se používá kvadrupól s analyzátorem s vysokým rozlišením (Q-TOF, Q-Orbitrap). Předem vybrané prekurzorové ionty jsou filtrovány v kvadrupólu, odeslány do kolizní cely a poté jsou detekovány všechny fragmentové ionty[79].

V SRM i PRM lze pro vývoj metody využít peptidy značené stabilními izotopy, které se přidají do zájmového vzorku. Ze znalosti charakteristik tohoto „těžkého“ peptidu jsme následným porovnáním signálu MS schopni potvrdit identitu a porovnat množství

endogenního peptidu mezi vzorky. Při použití syntetických peptidů vysoké kvality je možná také absolutní kvantifikace proteinů a jejich PTM[80].

Data-independent aquisition (DIA)

Na rozdíl od metody DDA, která selektivně vybírá nejintenzivnější peptidy pro fragmentaci, DIA přístup fragmentuje všechny ionty v určitém rozsahu m/z[81]. Poskytuje tak informace o množství všech detekovaných proteinů bez předchozího výběru peptidů pro analýzu. Jednou z DIA metod je SWATH-MS (z angl. *Sequential Windowed Acquisition of all Theoretical Mass Spectra*). Kvadrupól Q1 funguje i zde jako hmotnostní filtr, který postupně propouští do kolizní cely soubory peptidů spadajících do oblasti m/z okna o šířce přibližně 25 Da. Všechny propuštěné ionty jsou pak fragmentovány v kolizní cele (Q2) a analyzovány pomocí analyzátoru TOF[82]. Okno pro výběr peptidů k fragmentaci je širší než v případě SRM, což sice snižuje selektivitu měření, ale citlivý a přesný koncový analyzátor (TOF nebo Orbitrap) umožňuje simultánní přesnou kvantifikaci mnohonásobně většího množství proteinů. Výhodou DIA je možnost pozdějšího využití naměřených dat při hledání nových kandidátních proteinů. Data je možné kdykoli znovu analyzovat, například pomocí nové knihovny spekter s hlubším pokrytím proteomu.

Výsledná fragmentační spektra jsou díky velkému množství paralelně měřených iontů poměrně složitá. K identifikaci a kvantifikaci peptidových prekurzorů se používají dva přístupy, založené na použití či nepoužití knihovny spekter. Metoda založená na použití knihovny analyzuje data pomocí předem vytvořené knihovny obsahující relativní intenzitu peptidových fragmentových iontů a retenční čas (RT, z angl. *Retention Time*). Přístupy bez knihovny využívají k analýze DIA-MS dat databázi proteinových sekvencí nebo predikované knihovny. Zatímco metody bez knihoven nabízejí větší flexibilitu, často vyžadují další kontrolu pro stanovení FDR (z angl. *False Discovery Rate*). Vybudování komplexní knihovny pomocí frakcionovaných dat DDA je stále klíčové pro analýzy DIA, zatímco malé knihovny se častěji nahrazují přístupem bez knihovny[81,83].

1.6.2.3 *Software pro vyhodnocení hmotnostně-spektrometrických dat*

Klíčové je v proteomice bioinformatické zpracování dat, které umožní z velkého množství naměřených dat získat relevantní výsledky. K dispozici je široká škála softwarů, ať už jde o volně dostupné nebo komerční verze softwarů. Pro zpracování dat z DDA metod lze využít volně dostupný software MaxQuant, který využívá také řada biologických laboratoří bez proteomického vybavení. Je navržený pro analýzu velkých souborů MS dat a umožňuje zpracování dat pro kvantifikaci pomocí několika metod značení (např. SILAC) i bez značení (label-free)[84]. Pro analýzu SRM dat je volně dostupný software Skyline. Tento software je vhodný jak pro vytváření a optimalizaci cíleného měření, tak pro kvantitativní analýzu dat[85]. Pro zpracování dat z DIA analýz s použitím spektrální knihovny lze použít volně dostupné softwary, např. Skyline[85], DIA-NN[86], EncyclopeDIA[87] nebo OpenSWATH[88]. MaxQuant a jeho MaxDIA umožňuje analýzu jak s využitím specifické knihovny spekter, tak s využitím *in silico* knihovny ze sekvencí FASTA[89]. Z komerčních softwarů je často využívaný např. Spectronaut od firmy Biognosys[90]. Pro analýzu dat bez použití spektrální knihovny lze použít například software DIA-Umpire[91] nebo GroupDIA[92]. Základní i pokročilou statistickou analýzu umožňuje prostředí R s využitím balíčků MSstats[93], DEP[94] či iQ[95].

2 Cíle práce

V této dizertační práci se zaměřuji na neurodegeneraci a neurální kmenové buňky v průběhu diferenciace. Tyto buňky klíčové pro správné fungování CNS představují důležitý model pro studium neurobiologie za fyziologických i patologických podmínek. Jsou také slibným zdrojem buněk pro regenerativní medicínu. Ve snaze přispět k získání lépe definované populace NSC jsem s využitím proteomických metod usilovala o podrobnou analýzu těchto buněk a jejich monitorování v průběhu diferenciace.

Specifické cíle této dizertační práce jsou:

1. podrobná charakterizace NSC v průběhu diferenciace pomocí cílené metody SRM
2. analýza povrchového proteomu NSC v průběhu diferenciace
3. identifikace nových biomarkerů pro monitorování diferenciace NSC
4. identifikace biomarkerů pro sledování progrese HD

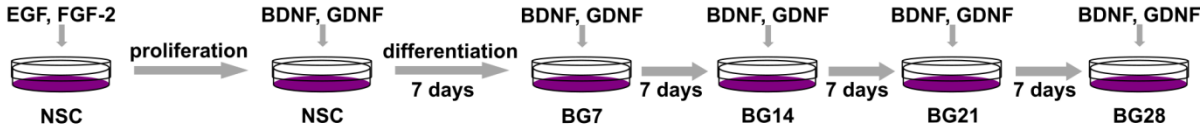
3 Metody

3.1 Kultivace NSC odvozených od H9 buněk

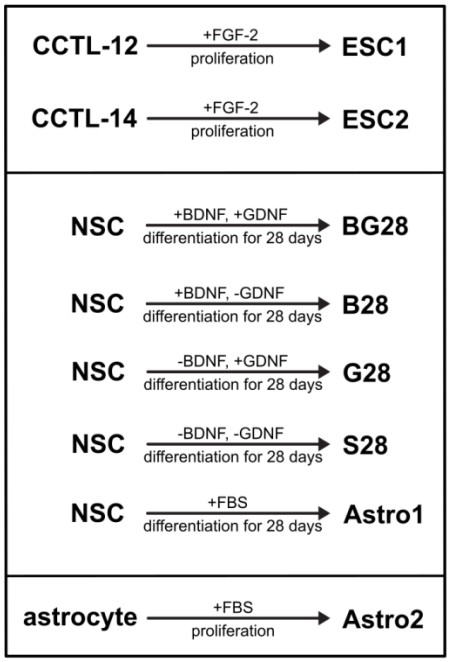
NSC odvozené od lidské ESC linie H9 (Thermo Fisher Scientific Inc.) byly kultivovány v mediu s přídavkem lidského rekombinantního FGF-2 (též označovaný jako bFGF, z angl. *Basic Fibroblast Growth Factor*) a EGF, jak je popsáno v [96].

Pro řízenou diferenciaci do neuronů bylo proliferační médium NSC vyměněno za neuronální diferenciační médium výměnou FGF-2 a EGF za lidský rekombinantní BDNF a lidský rekombinantní GDNF. Diferenciace NSC byla směrována do neuronů po dobu 7, 14, 21 a 28 dnů (podmínky BG7, BG14, BG21, BG28, obr. 4) a po dobu 1–8 dnů (podmínky BG1-8). Pro spontánní neuronální diferenciaci nebyl použit ani BDNF ani GDNF a NSC diferencovaly díky odstranění FGF-2 a EGF (stav S28). Další podmínkou bylo odebrání FGF-2 a EGF a výměna za samotné BDNF (B28) nebo GDNF (G28) (obr. 5).

Pro diferenciaci do astrocytů byly NSC kultivovány v mediu s přídavkem 1% FBS (z angl. *Fetal Bovine Serum*), čímž se diferenciace NSC směrovala do astrocytů po dobu 28 dnů (Astro1, obr. 5). Referenční lidské astrocyty (Gibco) byly kultivovány dle instrukcí výrobce v mediu s přídavkem 10% FBS (Astro2, obr. 5). Lidské ESC linie CCTL-12 (ESC1) a CCTL-14 (ESC2) byly kultivovány v přítomnosti mitoticky inaktivovaných primárních myších embryonálních fibroblastů v mediu s přídavkem FGF-2 (obr. 5).



Obr. 4. Schéma diferenciace H9 NSC do neuronů (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).



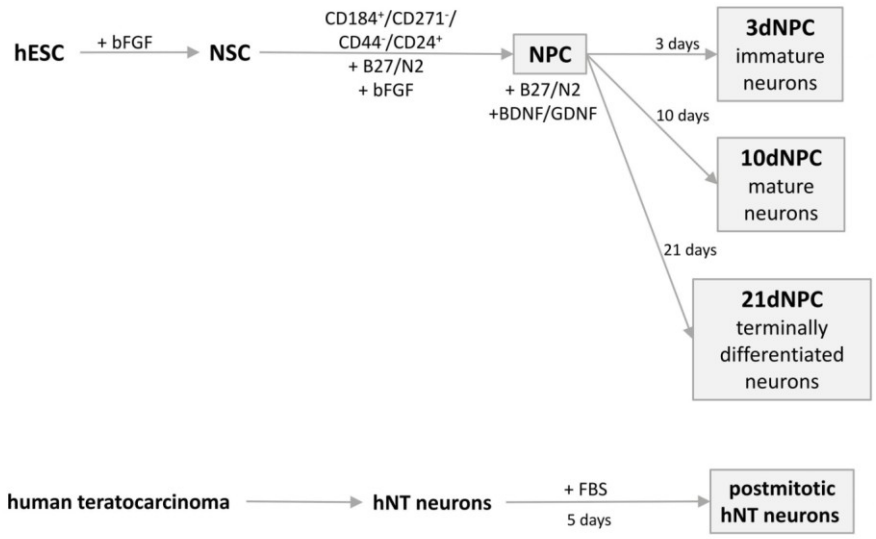
Obr. 5. Panel podmínek pro validaci metody SRM zahrnoval dvě referenční linie ESC (ESC1, ESC2), H9 NSC spontánně diferencované s BDNF i GDNF (BG28), pouze s BDNF (B28), pouze s GDNF (G28) nebo bez neurotrofní podpory (S28), diferenciaci NSC do astrocytů (Astro1) a referenční zralé astrocyty (Astro2) (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]*).*

3.2 Kultivace NSC odvozených od HUES7 buněk

Buňky HUES7 byly kultivovány, jak je popsáno v [63], na vyživovací vrstvě myších embryonálních fibroblastů v médiu obsahujícím FGF-2 (v publikaci označované jako bFGF). Neurální indukce byla spuštěna vytvořením embryonálních tělísek, z nichž byly izolovány NSC. NSC byly kultivovány v NSC proliferačním médiu obsahujícím FGF-2 (obr. 6).

Subpopulace $CD184^{+}/CD271^{-}/CD44^{-}/CD24^{+}$ NSC označená v dizertační práci jako NSC a v přiložené publikaci jako NPC (z angl. *Neural Precursor Cells*), byla vytříděna pomocí FACS ARIAII a kultivována v NSC proliferačním mediu. Pro neuronální diferenciaci bylo proliferační médium vyměněno za diferenciační médium výměnou FGF-2 za BDNF a GDNF (obr. 6).

Jako referenční populace terminálně diferencovaných neuronů byly použity postmitotické hNT, které jsou odvozené z lidské buněčné linie NTera-2[97,98]. Lidské neurony hNT byly kultivovány po dobu 5 dní v médiu s 10% FBS (obr. 6).



Obr. 6. Kultivační a diferenciační schéma HUES7 NSC a referenčních hNT neuronů (převzato z publikace *Surface N-glycoproteome patterns reveal key proteins of neuronal differentiation*[63]).

3.3 Imunocytochemie

Vybrané markery byly monitorovány v NSC v průběhu BG diferenciace a v astrocytech pomocí imunofluorescenčního (IF) zobrazení. Buňky byly vysazeny na komůrková sklíčka Nunc Lab-Tek a po fixaci a permeabilizaci byly barveny pomocí primárních protilátek proti vybraným proteinům. Primární protilátky byly detekovány pomocí fluorescenčně značených sekundárních protilátek (Alexa Fluor 488). DNA byla obarvena pomocí DAPI. Fluorescenční snímky byly zachyceny pomocí invertovaného fluorescenčního mikroskopu DMI6000 B (Leica Microsystems) a sestaveny v softwaru ImageJ.

3.4 Průtoková cytometrie

Přibližně 2 x 10^5 HUES7 NSC a NSC diferencujících po dobu 21 dnů bylo použito k analýze průtokovou cytometrií pomocí imunocytochemického barvení povrchového proteinu ICAM1 (z angl. *Intercellular Adhesion Molecule 1*). Primární protilátka anti-ICAM1 konjugovaná s R-fykoerythrinem (monoklonální myší protilátka) byla inkubována s buňkami.

Následně byly buňky inkubovány s propidium jodidem k označení mrtvých buněk a analyzovány cytometrem FACS ARIAII (Becton Dickinson).

3.5 Měření genové exprese

Relativní genová exprese byla analyzována pomocí kvantitativní polymerázové řetězové reakce v reálném čase (RT-qPCR). Celková RNA byla izolována z H9 NSC v průběhu diferenciace a z referenčních astrocytů pomocí RNeasy Plus Mini Kit (Qiagen) s QIAshredder (Qiagen) a převedena na cDNA pomocí QuantiTect Reverse Transscription Kit (Qiagen) podle pokynů výrobce. Páry primerů byly otestovány z hlediska specifity, sledovány byly také křivky tání pro vyloučení dimerizace primerů. Na detekčním systému CFX96 Touch Real-Time (Bio-Rad) byla použita následující nastavení: 12 minut při 95 °C pro aktivaci enzymu, poté 15 s při 95 °C pro denaturaci DNA, 40 cyklů po 30 s při 57 °C pro nasednutí primerů a 30 s při 72 °C pro elongaci DNA. Pro normalizaci byly využity geny GAPDH a ATP5F1B.

3.6 Cell surface capture (CSC)

Protokol CSC byl aplikován na 1×10^8 buněk každého vzorku, tedy proliferující HUES7 NSC, NSC indukované k neuronální diferenciaci po dobu 3, 10 a 21 dnů a na 4×10^7 hNT buněk podle dříve publikovaného protokolu[99] s mírnými úpravami[63].

Buňky byly jemně uvolněny škrabkou z kultivačních misek, ošetřeny 1,5 mM jodistanem sodným a následně značeny 3,5 mM biocytin hydrazidem. Poté byly promyty a resuspendovány v 50 mM hydrogenuhličitanu amonném. Solubilizované proteiny byly redukovány, alkylovány a štěpeny trypsinem.

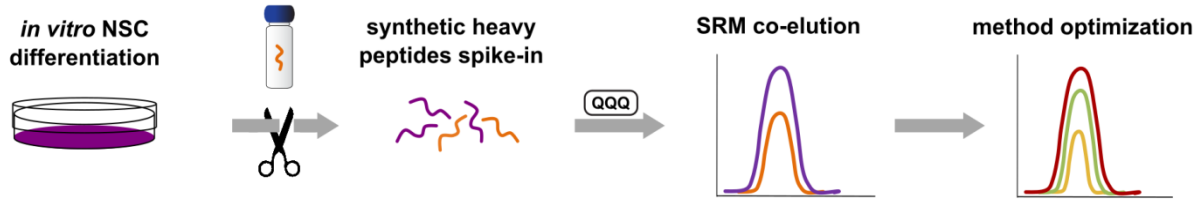
Biotinem značené glykopeptidy byly zachyceny na streptavidinových kuličkách. Kuličky byly resuspendovány v 50 mM hydrogenuhličitanu amonném a pro enzymatické štěpení peptidů z N-glykopeptidů byl použit peptid-N-glykosidáza F (PNGáza F). Vzorky odebraných peptidů byly odsoleny na kolonách C18 Ultra MicroTip.

3.7 Příprava vzorků pro SRM

Vzorky buněk byly zpracovány ve čtyřech biologických replikách pro každou podmínku, paralelně s jejich příslušnými kontrolami (H9 NSC). Proteiny byly po extrakci z buněk redukovány, alkylovány a následně štěpeny LysC a trypsinem. Peptidové směsi byly odsoleny na C18 kolonách a připraveny pro analýzu LC-MS.

3.8 SRM analýza neuronální diferenciace

Pro charakterizaci H9 NSC v průběhu diferenciace jsme vybrali proteinové markery typické pro ESC (NANOG, OCT4 a SOX2), NSC (SOX2, NES, PAX6 a KI67), neurony (DCX, TUBB3, MAP2, VEGF-A a CXCL1, *chemokine C-X-C motif ligand 1*), astrocyty (S100B a GFAP) a oligodendrocyty (GALC, *galactocerebrosidase* a OLIG1, *Oligodendrocyte transcription factor 1*). Proteotypické peptidy vybraných proteinů byly získány z veřejně dostupné databáze SRM Atlas (http://www.srmatlas.org/). Vývoj a validace testů SRM pro měření hladiny proteinů byly provedeny s použitím izotopově značených syntetických peptidů (Thermo Scientific Biopolymers). Ty byly monitorovány pomocí LC-SRM za použití hmotnostního spektrometru 5500 QTrap s trojitým kvadrupólem vybaveného nanoelektrosprejovým iontovým zdrojem (Sciex). Při SRM byly sledovány iontové přechody, tedy páry hodnot m/z prekurzorových iontů (peptidů) a hodnot m/z produktových iontů (peptidových fragmentů) (obr. 7).



Obr. 7. Schéma vývoje metody SRM. Syntetizované peptidy obsahující izotopovou značku byly přidány do trypsinem štěpené peptidové směsi diferencujících NSC. Tyto vzorky byly měřeny pomocí SRM na hmotnostním spektrometru s trojitý kvadrupólem k monitorování koeluce endogenních peptidů (fialový pík) odpovídajících izotopově značeným peptidům (oranžový pík). Vícebarevné píky v grafu optimalizace metody představují detekci různých fragmentových iontů pocházejících z jednoho peptidového prekurzoru (přechody SRM) (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

On-line chromatografické separace peptidů bylo dosaženo pomocí systému Eksigent 425 nanoLC (Eksigent/Sciex) a gradientové eluce (5% - 35% acetonitril s přídavkem 0,1% kyseliny mravenčí) během 30 minut s nastaveným průtokem 350 nl/min. Analýza byla prováděna s Q1 a Q3 v rozlišení 0,7 m/z v polovině maximální šířky píku. Jednotlivé přechody syntetických izotopově značených peptidů byly měřeny nejprve rozpuštěné ve vodě s peptidy iRT (z angl. *Indexed Retention Time*) s časem prodlevy (angl. *dwell time*) 10 ms a celkovým časem cyklu (angl. *cycle time*) ~3,5 s. Syntetické peptidy byly poté přidány do buněčných vzorků pro monitorování společné eluce endogenních (tj. lehkých) peptidů a syntetických (tj. těžkých) peptidů za různých podmínek. Data byla analyzována pomocí programu Skyline a pro kvantifikační experimenty SRM byly s přihlédnutím ke kvalitě získaných dat vybrány peptidy pro deset proteinových markerů (DCX, GALC, GFAP, MAP2, NES, OCT4, OLIG1, S100B, SOX2 a TUBB3), které byly reprezentovány minimálně dvěma proteotypickými peptidy na protein a minimálně čtyřmi přechody na peptid.

V časově definovaných experimentech SRM (angl. *scheduled*), při kterých se zaznamenávají přechody pouze v době experimentálně zjištěné eluce, bylo měřeno celkem 258 přechodů s dobou cyklu 1,7 s, okno retenčního času bylo nastaveno na 4 min. Výsledky SRM byly manuálně kontrolovány v programu Skyline a pro kvantifikaci byly ponechány peptidy detekované s poměrem signálu k šumu více než 3 pro nejintenzivnější přechod. Přechodům pod úrovní šumu byla přiřazena hodnota odpovídající jedné třetině signálu pozadí. Data byla vyhodnocena pomocí programu R s balíčkem MSstats, který kombinuje kvantitativní měření pro peptidy, nábojové stavy a přechody a detekuje proteiny, jejichž množství se mění mezi podmínkami. Množství peptidu a proteinu byly vypočteny z transformovaných (log2) ploch píku jednotlivých přechodů s využitím lineárního modelu se smíšenými efekty.

3.9 SRM analýza povrchových proteinů

Pro proteiny ICAM1, CHL1 (*neural cell adhesion molecule CHL1*) a LAMP1 (z angl. *Lysosome-Associated Membrane Glycoprotein 1*) byly v HUES7 NSC měřeny pomocí SRM dva deglykosylované peptidy, pro protein astrotactin1 jeden dostupný deglykosylovaný peptid. Vývoj a validace testů SRM specifických pro deglykosylované peptidy byly provedeny za použití syntetických izotopově značených peptidů (AQUA peptides, Thermo Fisher Scientific Inc.). Spektra byla získána na hmotnostním spektrometru s trojitým kvadrupólem (6460 Agilent Technologies) vybaveném rozhraním HPLC/ChipCube

(Agilent Technologies). Peptidové směsi s přidanými AQUA peptidy byly separovány pomocí lineárního gradient acetonitrilu (5% - 35%) během 30 minut při průtoku 300 nl/min. Analýza byla provedena s Q1 a Q3 v rozlišení 0,7 m/z s časem prodlevy jednoho přechodu 20 ms a dobou cyklu 2,7 s. Data byla analyzována pomocí programu Skyline a prvních 4–5 přechodů na peptid bylo zachováno pro kvantifikaci SRM.

Retenční čas extrahovaný během vývoje testu byl použit k vytvoření scheduled SRM se 177 přechody a dobou cyklu 1,8 s, okno retenčního času bylo nastaveno na 6 min. Přechody SRM byly manuálně kontrolovány pomocí programu Skyline a pro kvantifikaci byly ponechány pouze peptidy s poměrem signálu k šumu více než 3 a horní 3–4 přechody na peptid. Data byla vyhodnocena pomocí programu R s balíčkem MSstats.

3.10 Shotgun MS analýza povrchových proteinů

Vzorky HUES7 NSC byly analyzovány pomocí nanoLC systému (Eksigent/Sciex) připojenému k hmotnostnímu spektrometru LTQ-Orbitrap XL vybavenému nanoelektrosprejovým iontovým zdrojem (Thermo Fisher Scientific Inc.)[63]. Peptidové směsi byly naneseny z automatického vzorkovače (Eksigent/Sciex) a separovány pomocí lineárního gradientu acetonitrilu (7% -37%) během 60 minut s průtokem 300 nl/min. Ionty byly fragmentovány v LTQ části přístroje v režimu kolizí indukované disociace (CID), zatímco část hmotnostního spektrometru Orbitrap XL byla použita jako citlivý hmotnostní analyzátor. Ionty peptidů v rozmezí 350-1600 m/z byly monitorovány v jednom MS skenu s vysokým rozlišením následovaným pěti MS2 fragmentačními skeny nejintenzivnějších iontů.

Shromážděná spektra včetně běžných proteinových kontaminantů byla prohledána pomocí programu Sorcerer–Sequest s využitím databáze lidských proteinů UniprotKB/Swiss-Prot. Data byla analyzována pomocí programu Trans Proteomic Pipeline a výsledky byly filtrovány na degykosylované peptidy (N[115] deamidace v motivu NxS/T). Transmembránové domény byly predikovány pomocí algoritmu PHOBIUS. Label-free kvantifikace byla provedena na úrovni MS1 pomocí programu Progenesis s balíčkem MSstats.

4 Výsledky

4.1 Monitorování neurální diferenciace pomocí cílené hmotnostní spektrometrie

v publikaci

Targeted mass spectrometry for monitoring of neural differentiation

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METHODS & TECHNIQUES

Targeted mass spectrometry for monitoring of neural differentiation

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ABSTRACT

Human multipotent neural stem cells could effectively be used for the treatment of a variety of neurological disorders. However, a defining signature of neural stem cell lines that would be expandable, non-tumorigenic, and differentiate into desirable neuronal/glial phenotype after *in vivo* grafting is not yet defined. Employing a mass spectrometry approach, based on selected reaction monitoring, we tested a panel of well-described culture conditions, and measured levels of protein markers routinely used to probe neural differentiation, i.e. POU5F1 (OCT4), SOX2, NES, DCX, TUBB3, MAP2, S100B, GFAP, GALC, and OLIG1. Our multiplexed assay enabled us to simultaneously identify the presence of pluripotent, multipotent, and lineage-committed neural cells, thus representing a powerful tool to optimize novel and highly specific propagation and differentiation protocols. The multiplexing capacity of this method permits the addition of other newly identified cell type-specific markers to further increase the specificity and quantitative accuracy in detecting targeted cell populations. Such an expandable assay may gain the advantage over traditional antibody-based assays, and represents a method of choice for quality control of neural stem cell lines intended for clinical use.

KEY WORDS: Neural stem cell, Neural differentiation, Selected reaction monitoring, Mass spectrometry, Cell line characterization, Protein marker

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INTRODUCTION

Neurological disorders affect approximately one-sixth of the human population (United Nations. Nearly 1 in 6 of world's population suffer from neurological disorders – UN report, 2007), and represent a major economic burden for society (United Nations. Nearly 1 in 6 of world's population suffer from neurological disorders – UN report, 2007; Wittchen et al., 2011; World Health Organization. Neurological disorders: public health challenges, 2006). Since the figures are expected to grow (World Health Organization. Neurological disorders: public health challenges, 2006), it is of utmost importance to develop an effective therapy, as currently this is mostly limited to symptomatic treatment, physiotherapy, and occasional surgical interventions. The adult central nervous system (CNS) was long considered a relatively static tissue with very limited regenerative capacity. Nevertheless, ground-breaking discoveries throughout the past two decades demonstrated that in humans, new neurons were produced continuously from neural stem cells (NSCs) residing mainly in the subventricular zone, in the dentate gyrus of the hippocampus (Doetsch et al., 1999; Eriksson et al., 1998; Johansson et al., 1999), and possibly in the striatum (Ernst et al., 2014). Human NSCs can be derived from the fetal CNS, embryonic stem cells (ESCs), or induced pluripotent stem cells (iPSCs), and such *in vitro*-propagated cells survive, divide, migrate, and differentiate into neurons and glial cells in host CNS tissues upon transplantation (Carpenter et al., 1999; Flax et al., 1998; Kobayashi et al., 2012; Svendsen et al., 1997; Vescovi et al., 1999; Yuan et al., 2013; Zhang et al., 2001).

In vitro-propagated NSCs cultured in monolayer require fibroblast growth factor-2 (FGF-2) and/or epidermal growth factor (EGF) to survive, retain multipotentiality, and neurogenic efficiency (Carpenter et al., 1999; Conti and Cattaneo, 2010; Flax et al., 1998; Vescovi et al., 1999). Simple withdrawal of the mitogens leads to a spontaneous differentiation mainly into neurons, then astrocytes, and oligodendrocytes (Cattaneo and McKay, 1991; Vescovi et al., 1999; Zhang et al., 2001). Differentiated cells die in the absence of FGF-2 (Vescovi et al., 1999), which can be prevented by using either low levels of FGF-2 (Vescovi et al., 1999) or supplements such as N-2 or serum (Carpenter et al., 1999; Flax et al., 1998), trophic factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor or signalling molecules such as dibutyryl cyclic AMP (Lee et al., 2007; Yuan et al., 2011). Other protocols were developed to direct the NSC differentiation towards particular neural cell types, such as using fetal bovine serum (FBS) together with the N-2 supplement for astrocytes (Meyer et al., 2014). NSCs can also be 'primed' or 'pre-differentiated' to enrich for cells of particular interest (Yuan et al., 2011), or genetically modified to overexpress relevant proteins (Klein et al., 2005), and this self-production and/or secretion of protein(s) may significantly affect the uniformity of such cell lines.

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NSCs can be derived from multiple sources, and properties of such NSC lines differ (Conti and Cattaneo, 2010). Many protocols generate a rather heterogeneous population containing NSCs, committed neuronal and glial cells, or neural crest cells. In the case of ESC- or iPSC-derived NSCs, residual undifferentiated pluripotent stem cells can also be present in cultures, which may cause tumour formation after *in vivo* transplantation (Yabut and Pleasure, 2016). Thus, both differentiation potential and purity of human NSC lines should be periodically screened during the production period, and only a population of NSCs that fulfils the release criteria used for *in vivo* grafting assays.

To develop a potent, specific, and predictable screening assay that defines the NSCs clones of high purity, several criteria need to be met, including the ability to (i) define the NSCs population by the presence of specific markers, (ii) identify the presence of pluripotent stem cells or other cell type contaminants, including the mesoderm and endoderm derivatives, and (iii) offer a quick turnaround from data analysis to interpretation.

Morphology of live cells in culture is regularly checked as a part of good laboratory practice. Next-generation (deep) RNA sequencing offers the potential for a detailed characterization of human NSC lines and for the discovery of novel NSC markers (Bohaciakova et al., 2019). Deep RNA sequencing, however, is currently not fast enough to serve as a screening method, and protein effector levels can be predicted from the RNA levels only with limited accuracy. Although traditional antibody-based screenings such as immunofluorescence (IF) imaging, western blotting, or microarrays are well established for the detection of proteins, their throughput potential is relatively low. Immunoassays such as ELISA or flow cytometry may increase the throughput, but their multiplexing capacity is limited (Kupcova Skalnikova et al., 2017). Mass cytometry, flow cytometry augmented by mass spectrometry (MS)-based detection improves multiplexing potential. Imaging mass cytometry, a technique combining IF and mass cytometry (Bodenmiller, 2016), allows for simultaneous and spatially-resolved quantification, but cannot ensure rapid read-out and analysis.

The application of quantitative proteomics provided essential insights into NSC biology, generating a number of differential protein maps and partial functional networks (Shoemaker and Kornblum, 2016; Zizkova et al., 2015). MS-based quantifications following enrichment strategies for capturing candidate markers of NSCs were performed (Melo-Braga et al., 2014; Song et al., 2019; Tyleckova et al., 2016) using a conventional shotgun approach, where a subset of peptides was automatically and in part stochastically measured in the process of data-dependent precursor selection (Aebersold and Mann, 2003). Recently, we applied the data-independent acquisition MS method that combined global feature detection with targeted data extraction to simultaneously quantify thousands of proteins in the course of NSC differentiation (Červenka et al., 2021). This altogether helped to improve our understanding of the NSC differentiation and to identify potential protein markers of distinct steps in this process. However, such studies are not suitable for routine cell line characterization due to time requirements for data processing.

We aimed to develop an assay that would allow fast, efficient, and accurate monitoring of human NSC cultures using a targeted MS approach based on selected reaction monitoring (SRM). The essence of the SRM is the generation of specific, quantitative MS assays for each protein of interest and their subsequent application to multiple samples (Lange et al., 2008). To achieve this, several independent proteotypic (detectable and unique) peptides of the same protein are targeted, substantially increasing the confidence in the specific detection. The endogenous peptides are measured together with isotopically labelled reference peptides, and their quality can be verified by a fragment ion spectrum. Multiple data points are integrated to quantify proteins of interest, increasing the method statistical power and the precision of determined abundance changes. All this offers higher data reliability compared to the antibody-based methods routinely used for protein quantification. Samples can be processed in a single 30-min multiplexed MS method which makes it possible to collect and analyse the data about a cellular state in a matter of hours without the computational overhead (Soste et al., 2014).

Here we present a novel SRM assay to measure qualitatively and quantitatively the levels of protein markers broadly used to probe neural differentiation, i.e. POU domain, class 5, transcription factor 1 (POU5F1; also known as octamer-binding transcription factor 4, OCT4), transcription factor SOX-2 (SOX2), nestin (NES), doublecortin (DCX), tubulin beta-3 chain (TUBB3), microtubule-associated protein 2 (MAP2), protein S100-B (S100B), glial fibrillary acidic protein (GFAP), galactocerebrosidase (GALC), and oligodendrocyte transcription factor 1 (OLIG1). Such assay can be used to monitor the purity and the differentiation potential of human NSCs, and to identify their optimal culture conditions.

RESULTS

Markers selection and SRM method development

We aimed to target a set of protein markers routinely used in NSC differentiation studies (Table S1), including ESC markers (homeobox protein NANOG, NANOG; OCT4), NSC markers (SOX2; NES; paired box protein Pax-6, PAX6; proliferation marker protein Ki-67, MKI67), neuronal markers (DCX, TUBB3, MAP2), astrocyte markers (GFAP, S100B), and oligodendrocyte markers (GALC, OLIG1). We also intended to test the ability to detect low-abundant proteins previously found in our differentiation experiments, namely vascular endothelial growth factor A (VEGF-A) (Červenka et al., 2021) and growth-regulated alpha protein (CXCL1) (unpublished work, Institute of Animal Physiology and Genetics of The Czech Academy of Sciences).

The level of endogenous peptides is typically stoichiometric to the level of proteins (quantotypic). We developed SRM assays using heavy-labelled synthetic reference peptides (Table S2) that do not recapitulate the complexity of post-translational or translational modifications. Incomplete digestion during sample processing may also impact the quantotypic properties, so we performed preliminary measurements of NSCs differentiated with BDNF and GDNF for 21 days. This allowed us to spot discrepancies, exclude outlier peptides (if present), and ensure accurate quantification of protein levels.

For quantitative measurements, we had selected proteins successfully detected by SRM in our conditions (Fig. 1), and evaluated their capability to provide a read-out for NSCs and their differentiated counterparts by immunocytochemistry (Fig. 2; Table S3), and by gene expression analysis (Fig. 3; Table S4). Then, we assembled optimal coordinates of specific assays for ten markers (OCT4, SOX2, NES, DCX, TUBB3, MAP2, GFAP, S100B, GALC, OLIG1) into a multiplexed SRM method (Table S5). Proteins were represented by two to eight proteotypic peptides with good quantotypic properties, accurately representing the abundance level, and their four to ten most suitable transitions.

BDNF and GDNF differentiation defined by immunocytochemistry and gene expression analysis

NSCs generated from the NIH approved human ESCs line H9 were cultured in the NSC proliferation medium with EGF and FGF-2.

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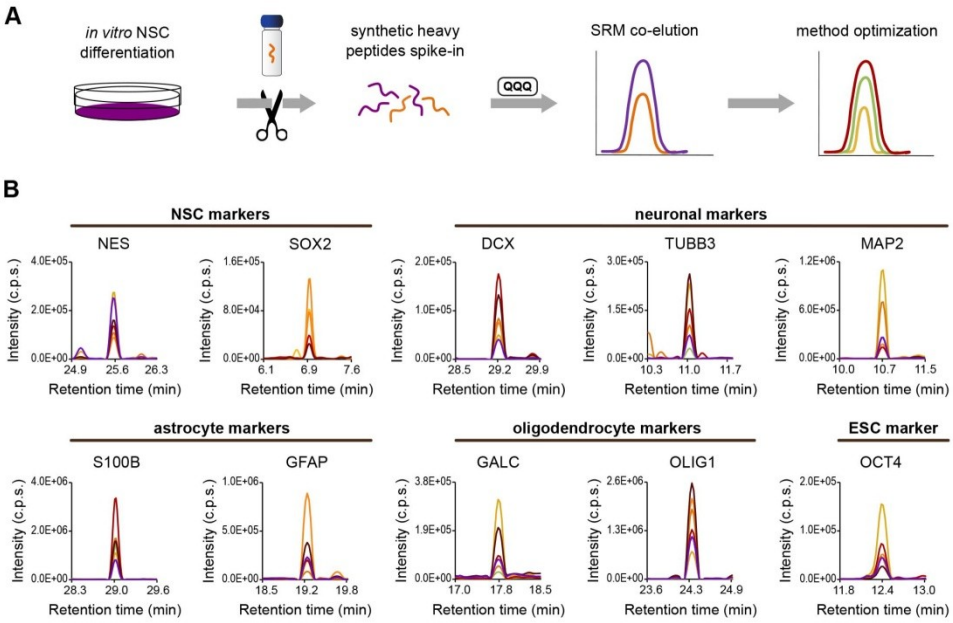

Fig. 1. Design of multiplexed SRM method. (A) Synthesized peptides (orange string in the vial) containing a heavy-isotope label were spiked into peptide mixtures extracted from differentiating NSCs after trypsin cleavage (purple strings). These samples were measured by SRM on a triple quadrupole to monitor the chromatographic co-elution of endogenous peptides (purple peak) and spiked-in heavy surrogates (orange peak), and a match in relative intensities of fragment ions. Multiple coloured traces in the method optimization graph represent the detection of different fragment ions from common peptide precursor (SRM transitions). (B) Optimal coordinates were assembled into a multiplexed method, and representative heavy peptides of protein markers are displayed.

The cells were directed into neurons using the differentiation medium without EGF and FGF-2, and supplemented by BDNF and GDNF (BG) to support cell survival for 7, 14, 21, and 28 days (Fig. 2A). To evaluate the cellular identity of proliferating NSCs and differentiating cells at protein and transcript levels, we applied antibody-based IF imaging and quantitative reverse transcription polymerase chain reaction (RT-qPCR).

All protein markers analysed, excluding OCT4 and GFAP, were detected in NSCs by IF imaging, fibrillar localization of NES, DCX, TUBB3, MAP2, and S100B was mainly apparent in differentiating cells, and only sporadic positivity for GALC and OLIG1 was detected in the later stages (Fig. 2B). Once the BG differentiation had been triggered, the mRNA level of neuronal (DCX, TUBB3, and MAP2) and NSC (NES, SOX2) markers was strongly induced (Fig. 3A). In the second week, the expression of neuronal markers had further increased, and remained stably high, while the expression of NSC markers had gradually decreased (Fig. 3A). Glial markers had dropped in the first week which was followed by steeply rising levels of the astrocyte marker S100B but steady levels of oligodendrocyte markers (GALC, OLIG1) (Fig. 3A).

The clustering of expression profiles (Fig. 3B) showed a separation of NSC (NES, SOX2) and neuronal (DCX, TUBB3, MAP2) markers from glial lineage markers (S100B, OLIG1, GALC). As we expected, our IF and RT-qPCR data showed an induced expression of neuronal markers and a reduced expression of glial markers at the early stages of neuronal differentiation, which was followed by a reduced expression of NSC markers in the later stages.

BDNF and GDNF differentiation defined by SRM

The BG differentiation peptide samples were subjected to simultaneous quantitative measurement by SRM (Table S6). Only proteins detected with ≥2 peptides in either BG differentiated cells or control cells (NSCs) were assigned as quantifiable. This included neuronal and NSC markers (DCX, TUBB3, MAP2, NES, SOX2), and the astrocyte marker S100B (Fig. 4A). If only one peptide of a protein had been detected, this marker was assigned as detectable in a particular condition (GFAP, GALC, OLIG1) (Table S6). In agreement with IF imaging results, OCT4 was not detected by SRM in BG differentiating NSCs (Table S6).

DCX was quantifiable only in differentiating BG cells and not in proliferating NSCs, reaching its maximum level after 3 weeks of differentiation with the highest abundance change recorded in our study. MAP2 and TUBB3 were gradually rising from day 7 and 14, respectively. NES and SOX2 were decreasing from day 7 and 14, respectively. Only one of two analysed SOX2 peptides remained detectable after 4 weeks of differentiation. SRM quantification results show that the method enables monitoring of NSC differentiation (Fig. 4A). All neuronal markers are increased in differentiating BG cells, and all NSC markers are decreased in these cells.

A significant positive correlation over the differentiation time-course was observed for the neuronal markers TUBB3 and MAP2, DCX and MAP2, but also for the glial marker S100B with TUBB3 (Fig. 4B). Despite differences in the S100B peptides

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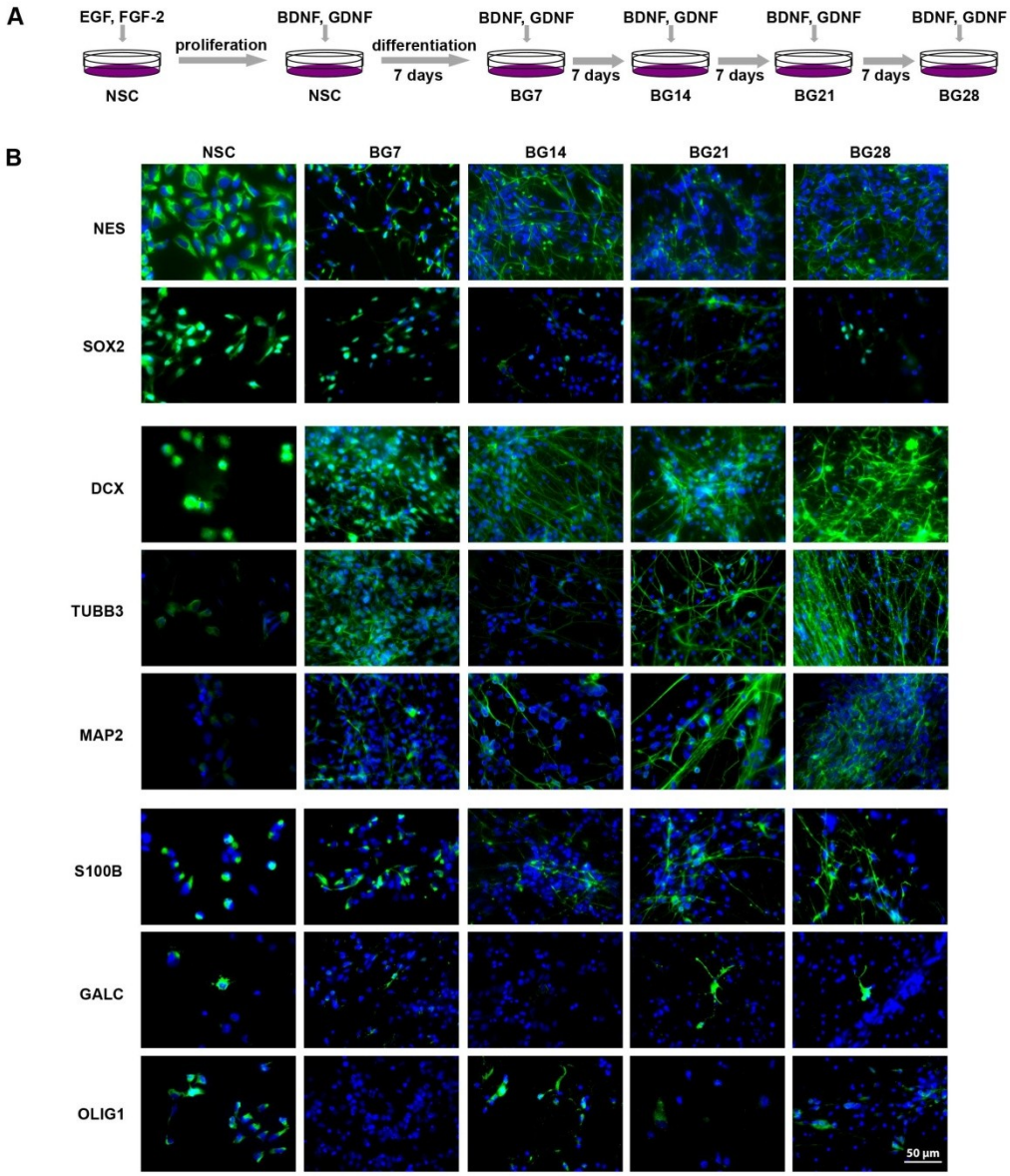

Fig. 2. NSC differentiation analysed by immunocytochemistry. (A) Scheme of NSC differentiation into neurons by the exchange of EGF and FGF-2 for BDNF and GDNF (BG) for 7, 14, 21, and 28 days. (B) Representative IF images of BG differentiation show protein markers in green; cell nuclei counterstained by DAPI in blue. Scale bar: 50 µm. Images of negative controls (no primary antibody) are shown in Table S3 with the table of used antibodies.

performance (Fig. 4A), the changes at the protein level reliably reflected the changes at the mRNA level (Fig. 4C). The S100B protein level decreased in the first week, and returned to its original level in the later stages of BG differentiation (Fig. 4A). The levels of DCX and MAP2 measured by SRM also positively correlated with mRNA levels measured by RT-qPCR (Fig. 4C;

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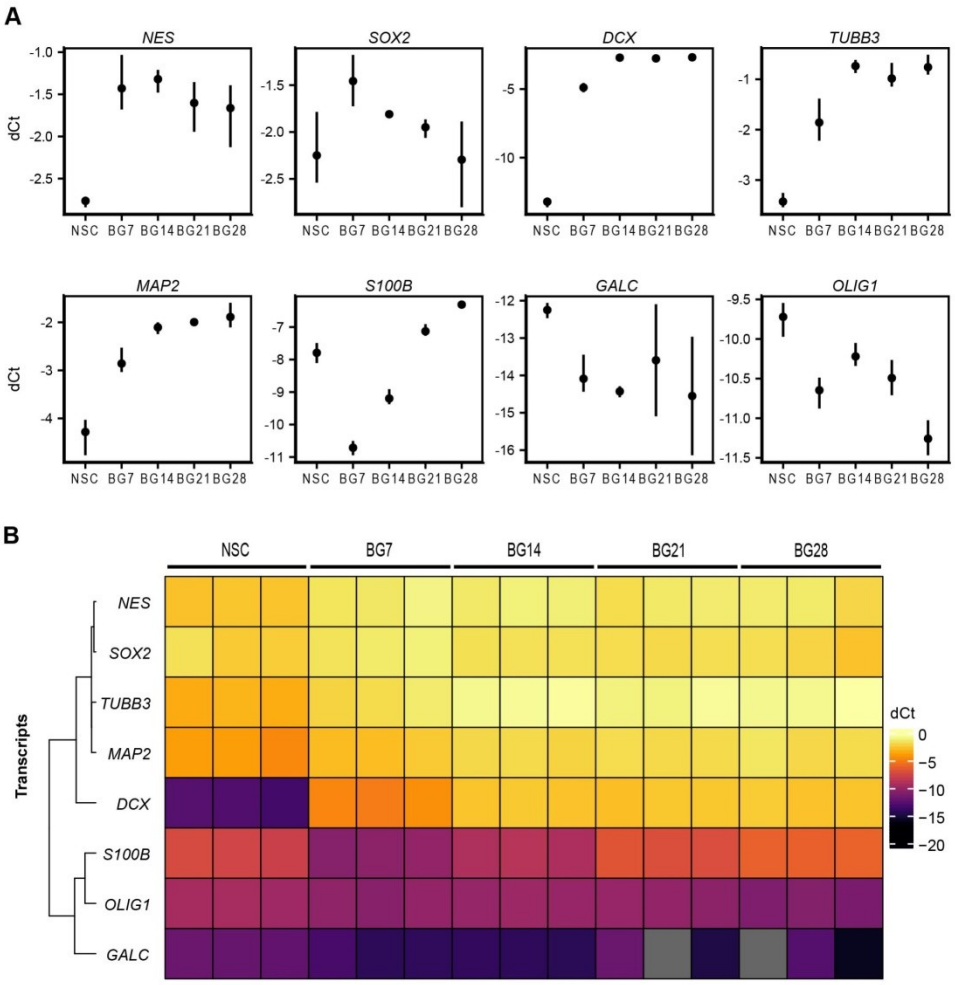

Fig. 3. Analysis of gene expression during NSC differentiation. (A) mRNA levels analysed by RT-qPCR. Individual transcripts were normalized to two housekeeping mRNA controls (*GAPDH* and *ATP5F1B*). Data from three independent experiments are displayed as mean (point) dCt values ±95% confidence intervals (vertical lines). (B) A heatmap of normalized dCt values from A shows similar co-expression profiles of neural and glial markers over the course of *in vitro* differentiation. Primers are listed in Table S4.

Table S7). The significant negative correlation of SOX2 versus DCX (Fig. 4B) confirms the switch from NSCs to differentiating neuronal states.

Our data indicate that major changes occur in the first week of the BG differentiation (Figs 3 and 4), so we zoomed in, and analysed differentiating NSCs daily for the first 8 days. We found that DCX and TUBB3/MAP2 increased from day 2 and 3, respectively, NES and SOX2 decreased from day 4, and S100B decreased until day 8 (Fig. 5). The expression of neuronal and NSC markers, and the astrocyte marker S100B is regulated at the very early stages of *in vitro* differentiation.

SRM monitoring of differentiating NSCs, ESCs, and astrocytes

Next, we tested a panel of additional culture conditions. NSCs were directed into neurons using the differentiation medium without EGF and FGF-2 supplemented with different combinations of BDNF and GDNF, and into astrocytes using FBS (Fig. 6A). Our recent data revealed that these neurotrophic factors affected the later stages of differentiation (Červenka et al., 2021), so we employed our SRM assay to depict this effect after 4 weeks of differentiation. As a reference, pluripotent ESCs and mature astrocytes were processed for MS analysis (Fig. 6A).

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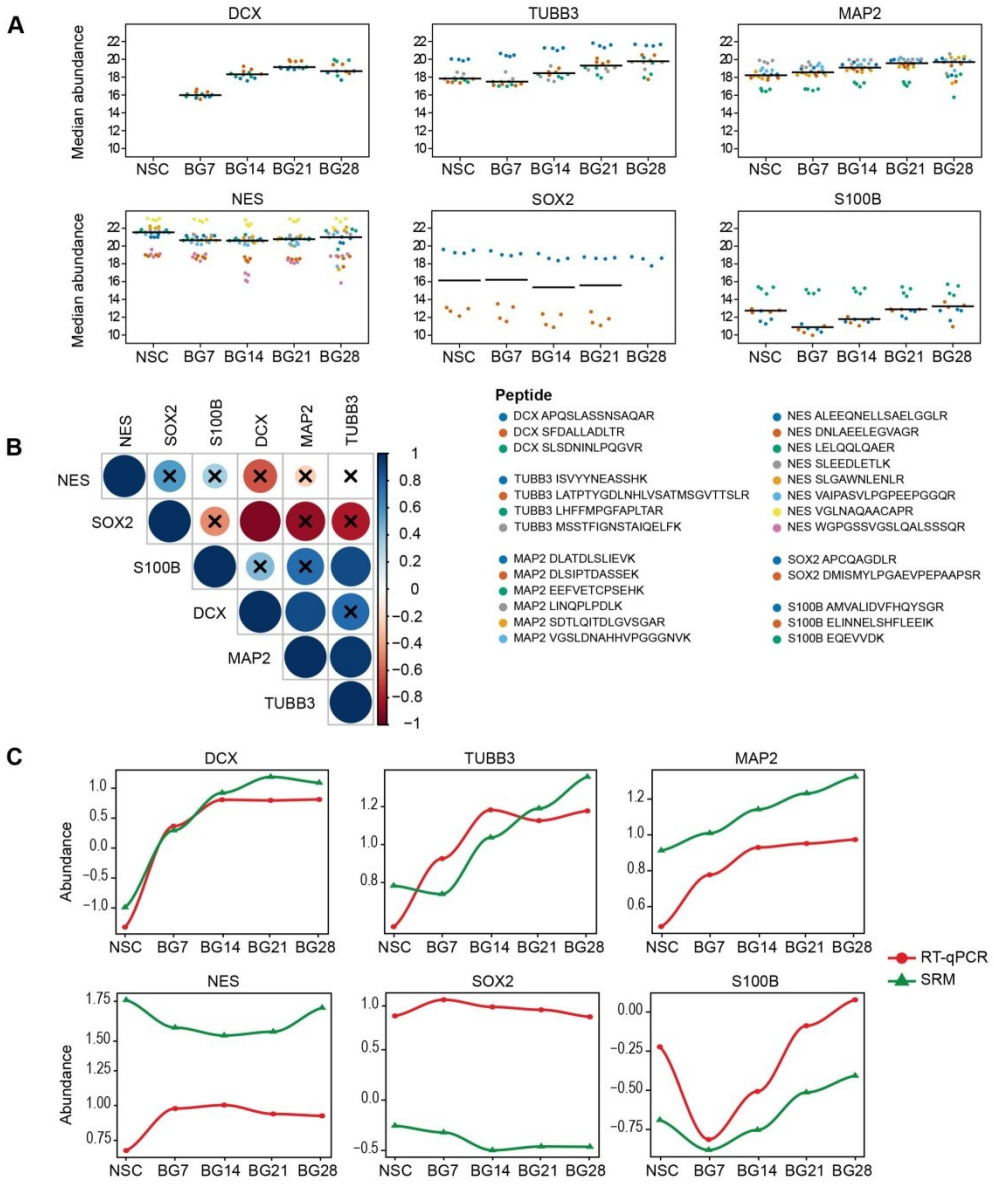

Fig. 4. Simultaneous quantification of 28-day BG differentiation. (A) Median abundances (black lines) of each protein marker in a given time interval of BG differentiation (7, 14, 21, 28 days). Dots of the same colour represent peptide abundances in four biological replicates. This is defined as the median of log2-transformed peak area of all transitions of the same peptide. Quantification results can be found in Table S6. (B) A correlogram is depicting pair-wise Pearson correlations of individual protein markers over the differentiation time-course. Colour and dot sizes indicate correlation strength, correlations without cross are statistically significant (P<0.05). (C) Plots are depicting a correlation of the transcripts levels (RT-qPCR, data from Fig. 3A) and the proteins levels (SRM, data from Fig. 4A) over time. The dCT values for mRNA levels and the log2-transformed values (abundances) for proteins were scaled and centred to mean 0 and standard deviation 1 across all measured targets to allow display in the same graph. A table of corresponding Pearson correlation coefficients is provided as Table S7.

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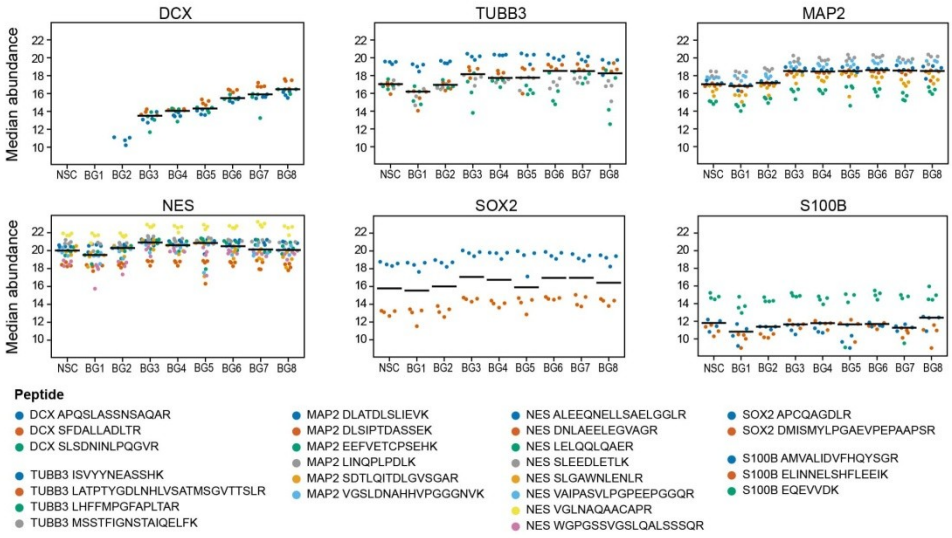

Fig. 5. SRM quantification of 8-day BG differentiation. Median abundances (black lines) of each protein marker in a given time interval of BG differentiation (1–8 days). Dots of the same colour represent peptide abundances in four biological replicates. This is defined as the median of log2-transformed peak area of all transitions of the same peptide.

SOX2, DCX, and MAP2 were quantifiable in the course of neuronal differentiation induced with BDNF and/or GDNF (BG28, B28, G28), in the spontaneously differentiated NSCs (S28), and in the astroglial differentiation induced by FBS (Astro1) (Fig. 6B; Table S6). NES and TUBB3 were detected by SRM at a quantifiable level in all conditions (Fig. 6B), regardless of their expected specificity. OCT4 was quantifiable in the reference ESCs (ESC1, ESC2) and GFAP in the reference astrocytes (Astro2), exclusively (Fig. 6A; Table S6). In agreement with our SRM data (Fig. 6B), the expression of OCT4 pluripotent ESC marker was previously confirmed in the ESC1 and ESC2 cell lines (The International Stem Cell Initiative*, 2007). S100B astrocyte marker was recognized as a suitable protein for quantification in both astrocyte conditions, and in NSCs induced to neuronal differentiation (Fig. 6B).

Our neural cell cultures do not contain detectable amounts of terminally differentiated oligodendrocytes. GALC levels could be quantified by SRM in the pluripotent ESCs and in the astrocyte differentiating NSCs (Table S6), highlighting the validity of this protein as a target for stem cell studies. OLIG1 was identified in differentiating NSCs only as detectable, without possible quantification. This marker was retained in the assay for its prospective use in oligodendrocyte differentiation studies where OLIG1 levels are expected to rise and for its correlation with GALC levels. Protein abundance changes prove the validity of all protein markers, except OLIG1, for their simultaneous quantification by SRM (Table S6).

The SRM data showed that neuronal markers, and the astrocyte marker S100B were strongly induced, while NSC markers were mostly reduced in all differentiation conditions (Fig. 6B). A weak signal of the astrocyte marker GFAP was detected only in the BG-induced NSCs for one of its unique peptides (Table S6). Different levels of astrocyte markers were identified in the Astro1 cells derived from NSCs and in the Astro2 mature astrocytes (Fig. 7). S100B increased in abundance in the astrocyte differentiating NSCs, but not in the mature astrocytes (Fig. 7A,B). All four peptides of the GFAP marker were detected specifically in the mature astrocytes, but not in the astrocyte differentiating NSCs (Fig. 7A). In mature astrocytes, antibody-based imaging confirmed strongly positive cells for GFAP (Fig. 7C), which was negative in all differentiation conditions of H9-derived NSCs (data not shown). BG cells were positive for S100B (Fig. 2B) without expected morphological changes, compared to mature astrocytes (Fig. 7C). Importantly, proteins marked in our study as quantifiable in the pluripotent ESC1 and ESC2 cells (OCT4, GALC) were also observed in the Astro1 cells exposed to FBS (less defined culture conditions) (Table S6). Based on SRM, we demonstrate that all the differentiation conditions we considered have pleiotropic effects, and simple removal of EGF and FGF-2 is sufficient for triggering neuronal phenotype changes. The astrocyte differentiating H9-derived NSCs manifest rather neuronal than astroglial phenotype.

DISCUSSION

The animal *in vivo* grafting experiments with human NSCs derived from fetal tissue, ESCs, or iPSCs have accumulated convincing and valuable data to support cell-replacement therapies in neurological disorders and CNS injuries (Cizkova et al., 2007; Hefferan et al., 2012; Jensen et al., 2013; Kelly et al., 2004; Lu et al., 2012, 2014; Svendsen et al., 1997; van Gorp et al., 2013; Yuan et al., 2013). Fetal tissues come with inherent ethical and logistical issues (Barker and de Beaufort, 2013), and it is evident that the source of such a tissue is limited. However, fetal cerebral tissue grafting experiments into human patients with neurodegenerative diseases provided us with invaluable information about feasibility, safety, and experimental procedures. It thus paved the way for the use of proliferating NSC lines generated from a single donor (fetal tissue, embryo, or skin biopsy-reprogrammed cells) that may represent the

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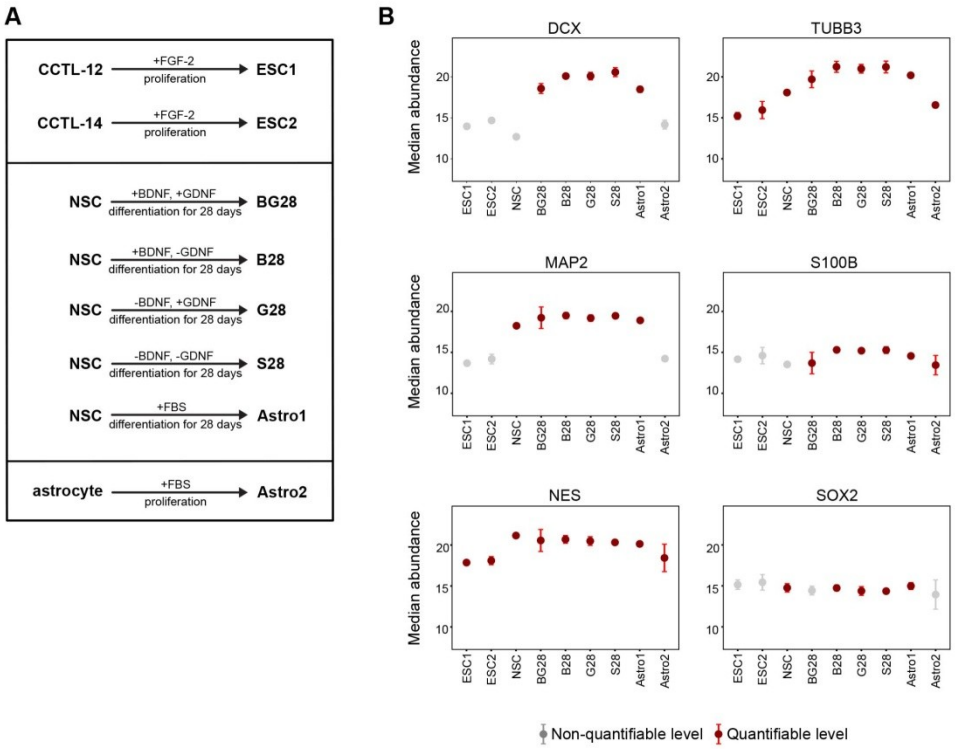

Fig. 6. SRM monitoring of NSC differentiation. (A) A panel of additional conditions for validation involved two reference ESC lines (ESC1, ESC2), NSCs differentiated with both BDNF and GDNF (BG28), BDNF only (B28), GDNF only (G28), spontaneously differentiated NSCs (S28), astrocyte differentiating NSCs (Astro1), and reference mature astrocytes (Astro2). (B) Condition plots from quadruplicate cultures were generated using MSstats; graphs show median signal and 95% confidence intervals. Red colour means that the protein was quantifiable in this condition (≥2 peptides per protein were detected in ≥3 biological replicates), grey colour means that protein abundances were below quantification levels in particular conditions.

cells of choice. While the development of new grafting and immunosuppression strategies is ongoing (Bjarkam et al., 2010; Boulis, 2010; Cunningham, 1998; Marsala, 2014; Usvald et al., 2010), it is essential to establish in parallel a reliable and reasonably fast screening protocol that would assess the potential of the selected NSCs as well as their safety.

To address shortcomings of antibody-based screens, targeted MS analysis by SRM can be used not only to accurately verify protein abundance changes emerging from global transcriptome and proteome profiling (Červenka et al., 2021; Donega et al., 2019; Tyleckova et al., 2016; Yocum et al., 2008), but rather to identify markers that would provide a reliable read-out for the differentiation potential of NSCs. We had developed a quantitative high-throughput assay for neuroscience studies, and evaluated its capability to monitor neurogenic potential and maturity of lineage-directed populations of NSCs. A variety of expected responses were detected by SRM, including increased neuronal markers from very early stages of *in vitro* differentiation and decreased NSC multipotency markers in later stages. Persisting expression of NES and SOX2 in differentiated cells might indicate that multipotent NSCs are still present in this population, providing a potential source for ongoing proliferation and differentiation upon transplantation into the host CNS. Alternatively, it may suggest persistent NES and SOX2 expression in non-neuronal populations, e.g. differentiating glia. Either way, it would make NES and SOX2 ideal negative selection markers for pure neuronal populations.

Our data show that DCX, TUBB3, and MAP2 represent more neuro-specific markers compared to NES and SOX2. For this reason, no single marker should be used as definitive proof of a particular cell type. Instead, a quantitative evaluation of several markers in a combinatory assay should be used to identify a protein profile (cell signature) of a selected cell population. Combinatory quantitative assays targeting protein markers may indeed represent a powerful method that would report on the multidifferentiation potential of NSCs, both *in vitro* and *in vivo* (Nagato et al., 2005). Dunkley et al. (2015) introduced a human pluripotent stem cell-derived cellular model of neuronal development. The SRM-based protein profiling applied in this study enabled the identification of time-dependent patterns conserved across multiple cell lines. However, care should be taken to include only reliable and independently verified markers, to avoid measuring uninformative markers.

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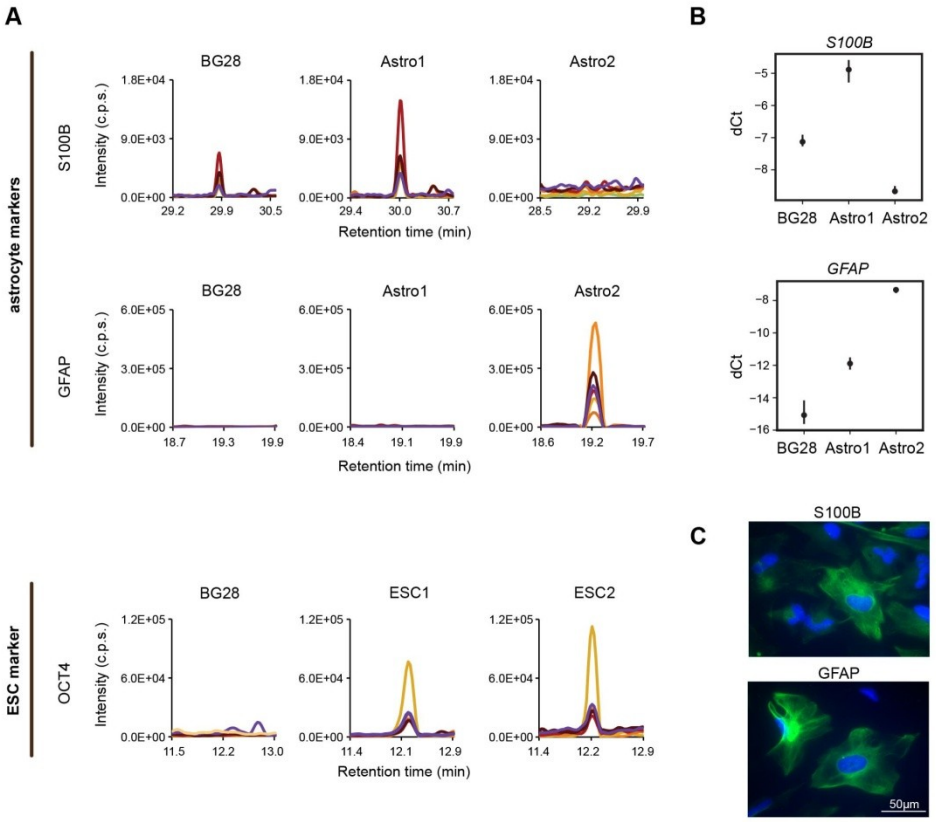

Fig. 7. Astrocyte and ESC markers. (A) S100B and GFAP astrocyte markers in NSCs directed into astrocytes (Astro1) and in mature astrocytes (Astro2), OCT4 marker of ESCs in reference ESC lines (ESC1, ESC2). Representative endogenous peptides are displayed as a chromatographic trace of peptide elution and detection by SRM. Coloured traces represent the detection of different SRM transitions. (B) *S100B* and *GFAP* mRNAs analysed by RT-qPCR. Individual transcripts were normalized to two housekeeping controls (*GAPDH* and *ATP5F1B*) from three independent experiments. Mean ±95% confidence intervals are shown as black points and vertical lines. (C) Representative IF images of mature astrocytes (Astro2) show GFAP and S100B protein markers in green, cell nuclei counterstained by DAPI in blue. Scale bar: 50 µm. Images of negative controls (no primary antibody) are shown in Table S3 with the table of used antibodies.

Ideally, reference pure cell populations would be used as controls for individual markers, but the post-mitotic nature of terminally differentiated neurons and oligodendrocytes makes this impossible for human cells. Immortalized or progenitor human cell lines still depend on *in vitro* differentiation and/or suffer from biased protein patterns (Conti and Cattaneo, 2010; Melo-Braga et al., 2015). FBS had been used in our study to differentiate NSCs into astrocytes, and the OCT4 marker became detectable, indicating the potential of H9-derived NSCs to dedifferentiate and manifest pluripotent traits. The GALC marker did not reach the limit of quantification in the course of BG differentiation, but appeared at a quantifiable level in pluripotent ESCs and in the FBS-induced NSCs.

S100B levels varied considerably in H9-derived NSCs exposed to various stimuli. Although S100B is a broadly accepted marker of astroglial cells, its dynamic expression was reported in NSCs in developing rat brain (Patro et al., 2015), and in human NSC lines (Lam et al., 2019). S100B expression in rat neural progenitor cells correlated with their proliferative potential. When the progenitor cells had stopped dividing, S100B was downregulated, and its expression was restored in mature astrocytes, together with an astrocyte marker GFAP (Patro et al., 2015). GFAP was readily detected in our mature astrocytes, and was at detection, but not quantification limit in BG neuronal differentiation cells, demonstrating the presence of sporadic astrocytes in this population. As we reported recently, NSCs derived from human ESCs showed no detectable GFAP signal during 3–6 weeks of the FBS-induced *in vitro* differentiation. However, 2–6 months after *in vivo* grafting into immunosuppressed rats and minipigs, a high number of GFAP positive human astrocytes is clearly detectable (Bohaciakova et al., 2019). These findings resemble *in vivo* embryonal development of the human cerebral cortex, where no expression of GFAP was detectable at week 11, whereas S100B was expressed (Vinci et al.,

2016). Our results suggest that classical protocols are not optimal for *in vitro* differentiation of mature astrocytes from the ESC-derived NSCs.

Here we show that the SRM-based quantification of suitable proteins/peptides is a powerful tool to report on the presence of pluripotent, multipotent, and committed neuronal and glial cells. SRM allows fast and reproducible detection of a predefined set of proteins, spanning a broad range of abundances (Lange et al., 2008). Some of the potential specific markers were not accessible by SRM due to the lack of tryptic peptides specific for a single protein with respect to the human proteome (NANOG). For other targets, specific SRM assays could be developed using synthetic heavy-labelled peptides, but were below the limit of detection in our cultures (PAX6, MKI67, VEGF-A, and CXCL1). These proteins can be substituted by other markers, such as minichromosome maintenance complex components as additional markers of proliferation, or other relevant proteins expected from the literature to report on neural (stem) cell populations and their derivatives (Zizkova et al., 2015).

The quantitative SRM assay presented here can be applied to an unlimited number of human NSC lines at high throughput and reproducibility, and using ~300,000 cells to be able to perform this high-accuracy quantitative measurement repeatedly. Since the *in vitro* differentiation takes long periods of time, we suggest running the assay before commencing large-scale experiments to ensure high reproducibility. On top of that, relatively low cell numbers required for a successful SRM measurement brings the possibility of using identical samples for multiple high-throughput screens, including bulk or single-cell deep RNA sequencing, thus offering unique gene/protein expression cross-validation.

We propose the application of the developed neural cell SRM assay as quality control for optimizing culture conditions during NSCs propagation and differentiation. The multiplexing capacity enables to include broad spectra of targets (~150 proteins) that could be selected from relevant molecular pathways (e.g. cell cycle, apoptosis, stress response, etc.) and measured together with the current panel of markers within the 30-min MS method (Soste et al., 2014). However, novel candidate markers need to be screened for their biological relevance and MS detectability with respect to the number of targeted peptides and their suitability for quantification. Fluorescence activated cell sorting (FACS) analysis of proliferating NSCs could increase the throughput in the SRM assays validation step, which could be further combined with a sorting strategy coupled to SRM. This would make it possible to distinguish between maturity and purity of neural populations generated from NSCs, adding another level of information.

Conclusions

In summary, we developed a novel SRM-based assay that could be easily employed to assess the neurogenic/gliogenic potential of NSCs during the propagation phase. The assay can be further exploited in *in vitro* experiments which could lead to improved or even novel differentiation protocols. The sensitivity and speed could eventually allow for testing of banked NSCs to test their differentiation potential upon long-term storage. Moreover, the SRM assay can be simply adapted to the analysis of additional cell types and experimental approaches.

MATERIALS AND METHODS

Neural stem cells differentiation

Unless otherwise stated, cell culture reagents were obtained from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were maintained at 37°C in 5% CO_2 in a humidified atmosphere.

Gibco Human Neural Stem Cells (H9-derived) generated from the NIH approved human ESCs (WA09; 46, XX) had been obtained from Life Technologies (catalogue number 510088, lot number 1402001, Thermo Fisher Scientific Inc.) and cultured as described previously (Červenka et al., 2021) with modifications. Briefly, the H9-derived NSCs (condition NSC) were grown on 20 µg/ml poly-L-ornithine and 5 µg/ml laminin-coated plates (both from Sigma-Aldrich, St. Louis, MO, USA) in the NSC proliferation medium containing KnockOut Dulbecco's modified Eagle's medium (DMEM)/F-12, 2 mM GlutaMAX, 1% penicillin-streptomycin, 2% StemPro Neural Supplement, 20 ng/ml human recombinant FGF-2, and 20 ng/ml human recombinant EGF. The NSC proliferation medium was changed every other day, and cells were passaged every 5–7 days using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA). Once the NSC culture had been established, low-passage cells were directed toward a specific lineage using an appropriate differentiation medium.

For directed differentiation into neurons, the NSC proliferation medium was switched to neuronal differentiation medium by exchanging FGF-2 and EGF for human recombinant BDNF and human recombinant GDNF (10 ng/ml of each; both from PeproTech, Rocky Hill, NJ, USA). Half of the differentiation medium was changed every other day, directing the NSC differentiation into neurons upon the treatment with BDNF and GDNF for 7, 14, 21, and 28 days (conditions BG7, BG14, BG21, BG28).

In the zooming experiment, NSCs were supplemented with BDNF and GDNF for 1–8 days (conditions BG1-8).

To evaluate the effect of BDNF and GDNF, these factors were applied exclusively for 28 days (conditions B28, G28). For spontaneous neuronal differentiation, neither BDNF nor GDNF was used, and NSCs differentiated by removing FGF-2 and EGF (condition S28).

For differentiation into astrocytes, NSCs were grown on Geltrex-coated plates, and the NSC proliferation medium was switched to astrocyte differentiation medium containing KnockOut DMEM/F-12, 1% N-2 Supplement, 2 mM GlutaMAX, 1% FBS, 1% penicillin-streptomycin. The astrocyte differentiation medium was changed every 3–4 days, directing the NSC differentiation into astrocytes for 28 days (condition Astro1).

Reference cell populations

Gibco Human Astrocytes generated from brain progenitor-derived astrocytes were obtained from Life Technologies (part number K1884, lot number 1640797, Thermo Fisher Scientific Inc.). Astrocytes (condition Astro2) were cultured according to the manufacturer's instructions on Geltrex-coated plates in the astrocyte proliferation medium containing Gibco Astrocyte Medium, 1% N-2 Supplement, 1% penicillin-streptomycin, and 10% FBS. The astrocyte proliferation medium was changed every other day, and cells were passaged every 3–4 days using 0.05% trypsin/EDTA.

Human ESC lines CCTL-12 [46, XX, del(18); condition ESC1] and CCTL-14 (46, XX; condition ESC2) (The International Stem Cell Initiative*, 2007) were grown on gelatin-coated plates in the presence of mitotically inactivated primary mouse embryonic fibroblasts (derived from 12.5-day-old mouse embryos, strain CF1; density 24,000 cells/cm^2). DMEM/F-12 was supplemented with 15% knockout serum replacement, 2 mM L-Glutamine, 1× minimum essential medium non-essential amino acids, 0.5% penicillin-streptomycin, 100 µmol/lβ-2 mercaptoethanol (Sigma-Aldrich), and 4 ng/ml FGF-2 (PeproTech). The embryonic culture medium was changed every day, and cells were manually passaged every 5–7 days. For sample preparation, ESC colonies were manually detached from the cell culture dish to avoid contamination with mouse embryonic fibroblasts.

Immunocytochemistry

Selected markers were monitored in BG-differentiating NSCs and in astrocyte conditions by IF imaging. Cells were seeded on Nunc Lab-Tek chambered slides. Cells cultured as described above were washed with pre-heated phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 15 min, and washed three times with PBS. The cells were then permeabilized and blocked with 0.1% Triton X-100, 5% goat serum, and 1% bovine serum albumin in PBS for 45 min. For the cell surface marker GALC, Triton X-100 was omitted. Antibodies (Table S3) were diluted in 5% goat serum in PBS and incubated with the cells overnight at 4°C. After

three washing steps with PBS, antibodies were detected using fluorescently-labelled secondary antibodies (goat anti-mouse or goat anti-rabbit; Alexa Fluor 488; both from Thermo Fisher Scientific Inc.) diluted to 1:500 in 5% goat serum in PBS for 60 min in the dark. After three washing steps with PBS, DNA was stained with DAPI. In the negative controls, primary antibodies were omitted (Table S3). Fluorescent images were captured using an inverted fluorescent microscope in 16-bit depth (DMI6000 B; Leica Microsystems, Wetzlar, Germany) and assembled in ImageJ software (v1.49k, National Institutes of Health, Bethesda, MA, USA) (Schneider et al., 2012).

Quantitative reverse transcription PCR

Gene expression analyses of selected markers were performed as described previously (Červenka et al., 2021). Briefly, total RNA was isolated from BG-differentiating NSCs and astrocyte conditions by RNeasy Plus Mini Kit (Qiagen) with QIAshredder (Qiagen), and converted into cDNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. The reaction mix for one quantitative PCR contained 5× HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne), 125 nM of each primer (Table S4), 25 ng of cDNA template, and PCR water. Following settings were used on CFX96 Touch Real-Time detection system (Bio-Rad): 12 min at 95°C for enzyme activation, then 15 s at 95°C for denaturation with 40 cycles of 30 s at 57°C for annealing, and 30 s at 72°C for an extension. Cycle threshold (Ct) values were normalized to the average of two housekeeping genes Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ATP synthase subunit beta, mitochondrial (*ATP5F1B*).

Sample preparation for MS analysis

Cell samples in four bioreplicates for each condition were washed with PBS and resuspended in a buffer containing 8 M urea (Sigma-Aldrich), 50 mM ammonium bicarbonate (NH_4HCO_3, Sigma-Aldrich), and 5 mM EDTA (Carl Roth GmbH, Karlsruhe, Germany). The cells were disrupted by vortexing (ten consecutive rounds of 1.2 min) and by sonicating on ice (15 min). The samples were centrifuged at 20,000 ***g*** for 15 min (4°C) to remove any remaining debris, and protein concentrations were determined (Pierce 660 nm Protein Assay, Thermo Fisher Scientific Inc.). The protein extracts were then supplemented with ProteaseMAX Surfactant (Promega, Madison, WI, USA) to a final concentration of 0.1%. After vortexing and sonicating as described above, proteins were reduced with 10 mM tris(2-carboxyethyl)phosphine for 30 min at 32°C and alkylated with 40 mM iodoacetamide for 45 min at 25°C, in the dark. Samples were diluted with freshly prepared 0.1 M NH_4HCO_3 and 0.01% ProteaseMAX to a final concentration of 1 M urea, and incubated at 37°C with sequencing-grade Lysyl Endopeptidase (Wako Chemicals GmbH, Neuss, Germany) and sequencing-grade porcine trypsin (Promega) proteases for 4 h and 14 h, respectively, both in an enzyme/substrate ratio of 1/100 (w/w). The digestion was stopped by acidification with formic acid (FA) to a final pH <3. The peptide mixtures were loaded onto C18 spin columns (The Nest Group Inc., Southborough, MA, USA) to desalt according to the manufacturer's instructions. Peptides were eluted with 80% acetonitrile. Peptide samples were desiccated on a vacuum centrifuge and re-solubilized in 0.1% FA for LC-MS analysis. Samples were processed in parallel with their respective controls (NSCs).

SRM assays development

Proteotypic peptides matching 14 frequently used protein markers of the NSC differentiation (Table S1) were retrieved from publicly available resources of targeted proteomics assays (SRM Atlas, http://www.srmatlas.org/). Development and validation of SRM assays to measure protein abundances were performed as previously described (Soste et al., 2014) using heavy-labelled unpurified synthetic peptides (Thermo Scientific Biopolymers, Thermo Fisher Scientific Inc.). These were mixed and monitored by LC-SRM using a 5500 QTrap triple-quadrupole/ion-trap mass spectrometer (Sciex, Framingham, MA, USA) equipped with a nano-electrospray ion source (Sciex). On-line chromatographic separation of the peptides was achieved with an Eksigent 425 nanoLC system (Eksigent/Sciex) equipped with a 20-cm fused-silica column with a 75-µm inner diameter (New Objective, Woburn, MA, USA), packed in-house with ProntoSIL C18 AQ 3 µm beads (Bischoff Analysentechnik GmbH, Leonberg, Germany). The peptide mixtures were loaded and separated with a linear gradient from 5% to 35% acetonitrile over 30 min at a flow rate set to 350 nl/min. The instrument was operated as described in Soste et al. (2014). SRM analysis was conducted with Q1 and Q3 operated at unit resolution (0.7 m/z half-maximum peak width) with a dwell time of 10 ms and a cycle time ∼3.5 s. For each peptide, doubly and triply charged precursor ions, and the 20 most probable singly or doubly charged fragment ions from the b- and y-ion series were selected using Skyline (v3.1.1.7490, release date 20 May 2015, MacCoss Lab Software, University of Washington, Seattle, WA, USA) (MacLean et al., 2010) and measured by SRM. The indexed retention time peptides (Biognosys AG, Zürich, Switzerland) were annotated and used to schedule the acquisition of selected SRM traces within retention-time (RT) windows. Synthesized peptides containing a heavy-isotope label were then spiked into cell samples, and corresponding heavy and light transitions were targeted to monitor the co-elution of endogenous (i.e. light) peptides and the spiked-in (heavy) surrogates in different conditions. The raw data can be accessed at http://www.peptideatlas.org/PASS/PASS00872. Data were analysed with Skyline, and ten validated markers represented by ≥2 proteotypic peptides per protein and the ≥4 most suitable transitions (precursor and fragment ion pairs) per peptide were experimentally selected for quantification experiments.

Protein quantification

To track the system performance, commercial predigested Beta-Galactosidase (Sciex) was diluted with indexed retention time peptides (Biognosys AG) and Glu-1-Fibrinopeptide B (Sciex) to a working solution of 20 fmol/µl, and monitored prior to analysis. In time-scheduled SRM experiments, protein markers were targeted in all bioreplicates of each condition (total 258 transitions, 4-min RT window, 1.7-s cycle time, 1 µg peptides, 30-min gradient) (Table S5). Corresponding conditions and controls (NSCs) were analysed with the same RT window using the instrument settings described above. The raw data can be accessed at http://www.peptideatlas.org/PASS/PASS00873. SRM peaks were manually inspected using Skyline by checking for co-elution, peak shape similarity, a match in relative intensities of fragment ions and retention times compared to the assay development phase. Only SRM peaks detected with a signal-to-noise ratio of >3 for at least the top transition were considered for quantification. Raw SRM data (peak areas) were exported from Skyline and, for transitions below the transition specific background level, the peak areas were assigned to one-third of the background level. Protein significance analysis was performed using an open-source statistical environment R (R Core Team, 2020) (version 4.0.2) with package MSstats (version 3.20.1), which combines the quantitative measurements for peptides, charge states, and transitions, and detects proteins that change in abundance between conditions while controlling the false discovery rate (Chang et al., 2012; Choi et al., 2014). The peptide and protein abundances were calculated from log2-transformed peak areas of individual transitions. A linear mixed-effects model was used for the relative quantification of a given condition with respect to its control (NSCs). Significant abundance changes were reported as log2 fold-changes with standard error, T value, degrees of freedom, and *P*-value adjusted for multiple comparisons (Table S6). A false discovery rate-adjusted *P*-value cut-off of 0.05 was used. The R environment (R Core Team, 2020) was used to generate a variety of different plots.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.S., H.K., P.V.; Methodology: R.S., M.K., P.V.; Formal analysis: R.S., P.V.; Investigation: R.S., M.K., J.C., D.B., K.V.K., T.N., K.B., A.S.; Resources:

D.B., J.M., P.V.; Data curation: R.S., P.V.; Writing - original draft: R.S., M.K., J.C., M.H.P., M.M., J.M., H.K., P.V.; Writing - review & editing: R.S., M.K., J.C., M.H.P., D.B., K.V.K., A.S., M.M., P.V.; Visualization: R.S., M.K., J.C., A.S., P.V.; Supervision: R.S., M.M., J.M., H.K., P.V.; Project administration: R.S., P.V.; Funding acquisition: M.H.P., D.B., J.M., P.V.

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Data availability

The datasets generated and analysed during the current study are available in the Peptide Atlas repository (http://www.peptideatlas.org/PASS/PASS00872, http://www.peptideatlas.org/PASS/PASS00873). The datasets supporting the conclusions of this article are included within the article and its additional file.

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4.2 Identifikace klíčových proteinů neuronální diferenciace pomocí analýzy povrchového N-glykoproteomu

v publikaci

Surface N-glycoproteome patterns reveal key proteins of neuronal differentiation

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Surface N-glycoproteome patterns reveal key proteins of neuronal differentiation

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Cell-replacement therapies

ABSTRACT

Pluripotent stem cell-derived committed neural precursors are an important source of cells to treat neurodegenerative diseases including spinal cord injury. There remains an urgency to identify markers for monitoring of neural progenitor specificity, estimation of neural fate and follow-up correlation with therapeutic effect in preclinical studies using animal disease models. Cell surface capture technology was used to uncover the cell surface exposed N-glycoproteome of neural precursor cells upon neuronal differentiation as well as post-mitotic mature hNT neurons. The data presented depict an extensive study of surfaceome during neuronal differentiation, confirming glycosylation at a particular predicted site of many of the identified proteins. Quantitative changes detected in cell surface protein levels reveal a set of proteins that highlight the complexity of the neuronal differentiation process. Several of these proteins including the cell adhesion molecules ICAM1, CHL1, and astrotactin1 as well as LAMP1 were validated by SRM. Combination of immunofluorescence staining of ICAM1 and flow cytometry indicated a possible direction for future scrutiny of such proteins as targets for enrichment of the neuronal subpopulation from mixed cultures after differentiation of neural precursor cells. These surface proteins hold an important key for development of safe strategies in cell-replacement therapies of neuronal disorders.

Biological significance: Neural stem and/or precursor cells have a great potential for cell-replacement therapies of neuronal diseases. Availability of well characterised and expandable neural cell lineage specific populations is critical for addressing such a challenge. In our study we identified and relatively quantified several hundred surface N-glycoproteins in the course of neuronal differentiation. We further confirmed the abundant changes for several cell adhesion proteins by SRM and outlined a strategy for utilisation of such N-glycoproteins in antibody based cell sorting. The comprehensive dataset presented here demonstrates the molecular background of neuronal differentiation highly useful for development of new plasma membrane markers to identify and select neuronal subpopulation from mixed neural cell cultures.

1. Introduction

With advances in stem cell research and related technologies, there is a growing interest in using embryonic stem cell (ESC) — or induced pluripotent stem cell derived committed neural precursor cells (NPCs) in the treatment of severe neurodegenerative indications including spinal trauma. Animal model studies and clinical data from human spinal trauma trial clearly demonstrate the treatment potential of NPCs and warrant their further research [1–5].

To address the issue of availability of well characterised and expandable neural cell lineage-specific populations, it is indispensable to understand the spatio-temporal profile of cellular protein expression patterns during neural differentiation. Furthermore, there is an urgent need to identify the proteins specific for the particular neural cell subtypes. Such proteins may facilitate isolation of neural cell subpopulations as well as the monitoring of their fate after engraftment for follow-up correlation with therapeutic effect in preclinical cell transplantation using animal disease models [6].

Abbreviations: ASTN1, astrotactin 1; CHL1, neural cell adhesion molecule L1-like protein; CSC, cell surface capture; DCX, doublecortin; ESC, embryonic stem cell; EPHB1, ephrin type-B receptor 1; ICAM1, intercellular adhesion molecule 1; LAMP1, lysosome-associated membrane glycoprotein 1; NPC, neural precursor cell; NSC, neural stem cell; POL, poly-L-ornithine/laminin; THS7A, thrombospondin type-1 domain-containing protein 7A; TUJ1, tubulin beta-3 chain.

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Among cellular proteins of stem cells, several cell surface proteins are known to play an important role in neuropoiesis and these are referred to as "stemness markers" [7]. They include prominin-1 (CD133), stage specific embryonic antigen-1 (SSEA-1; CD15) derived from stem cells of teratocarcinomas or structurally related forebrain-surface-embryonic antigen (FORSE-1). Other proteins, like polysialylated neural cell adhesion molecule (PSA-NCAM; CD56) and CD24, can distinguish differentiated neuronal precursors, whilst expression of A2B5 cell surface ganglioside epitope is typical for glial precursor cells [8,9], or CD44 and PDGFR (CD140a) [10], which characterise astrocyte and oligodendrocyte precursors respectively. Advances in proteomic approaches and technologies can improve identification of cell surface proteins and understanding of their contribution to the molecular mechanisms of neural differentiation. One approach is to systematically study the involvement of already known cell surface proteins like CD molecules. The key advantage of this approach is the availability of the antibodies, whilst the disadvantage is that only limited subset of cell surface proteins can be monitored. To disclose new molecules and obtain more comprehensive results, proteome-wide mass spectrometry studies may be performed in addition to CD molecule targeted analysis. Cell Surface Capture (CSC) technology enables the selective enrichment of cell surface-exposed plasma membrane N-glycoproteins through chemical labelling on live cells, which represents a very effective approach for extracting and identifying such proteins [11]. However, studies on the characterisation of cell surface proteins during neural differentiation of pluripotent cells are to date rare and include an extensive quantitative comparative study of membrane proteome between human ESCs and NSCs [12] and surfaceome profiling of regulators of NSC functionality [13].

In the present study, we determined the composition and relatively quantified the cell surface N-glycoproteome of the NPCs at onset and upon neuronal differentiation, as well as in mature hNT neurons using CSC technology combined with high resolution LC–MS/MS. The changes in cell surface protein abundance levels of ICAM1, CHL1, LAMP1, and astrotactin1 were confirmed by SRM. The immunofluorescence staining of ICAM1 combined with flow cytometry analysis demonstrated a possible way to access the neuronal subpopulation(s) for development of strategies for cell-replacement therapies of neurodegenerative diseases.

2. Materials and methods

2.1. Cell culture

Human NPCs were derived from ESCs HUES-7 as described previously [10,14]. This part of the study was performed at the University of California, San Diego (Institutional Review Boards Protocol Number 101323). Unless otherwise stated, cell culture reagents were obtained from Life Technologies (Thermo Fisher Scientific Inc., Waltham, MA). Briefly, HUES-7 cells were cultured on a mouse embryonic fibroblast feeder layer in medium containing Knockout-DMEM, 10% plasmanate, 10% Knockout Serum Replacement, non-essential amino acids, 20 mM GlutaMAX, 1% penicillin/streptomycin and 10 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN). Neural induction was triggered by generation of embryonic bodies from which NSCs were obtained after depletion of embryonic bodies-rosette negative ectoderm epithelial-like cells and other contaminating cells. NSCs were cultured on 20 μg/ml poly-L-ornithine and 5 μg/ml laminin (both from Sigma-Aldrich, St. Louis, MO) (POL) coated plates in NSC growth medium containing DMEM/F12 GlutaMAX with 0.5% N_2 and 0.5% B27 serum supplements, 1% penicillin/streptomycin and 20 ng/ml bFGF.

The $CD184^+/CD271^-/CD44^-/CD24^+$ subpopulation of NSCs, assigned as NPCs, was sorted out using FACS ARIAII (Becton Dickinson, San Jose, CA) and collected in NSC growth medium. The NPCs were seeded on POL-coated plates in the NSC growth medium and maintained at 37 °C in 5% CO_2 in humidified atmosphere. The medium was changed every other day and cells were passaged every 3–4 days using 0.05% trypsin/EDTA. For neuronal differentiation, the growth medium was switched to differentiation medium by exchange of bFGF for brain derived neurotrophic factor (BDNF) and glial cell line derived neurotrophic factor (GDNF) (20 ng/ml of each; both from Peprotech, Rocky Hill, NJ) when the cells reached ~70% confluency. The half of the medium was then changed every other day.

The postmitotic hNT neurons derived from human teratocarcinoma (Layton Bioscience, Atherton, CA) were plated on POL-coated dishes and cultured at 37 °C in 5% CO_2 humidified atmosphere in DMEM/F12 GlutaMAX media with 10% FBS and 1% penicillin/streptomycin. After 5 days, hNT cells were harvested for the follow-up analyses without passaging.

2.2. Cell surface capture technology

The CSC was applied to two biological replicates of 1×10^8 cells of each sample, including proliferating NPCs, NPCs induced to neuronal differentiation for 3, 10 and 21 days (3dNPC, 10dNPC and 21dNPC) as well as 4×10^7 hNT cells according to the previously published protocol [11] with slight modifications. Two biological replicates of each sample were pooled from four cell samples cultured separately in order to reach final total number (1×10^8 cells, resp. 4×10^7) of the cells required for CSC. Then, each pooled biological replicate of each sample was labelled and analysed separately using MS. We considered this approach due to: i) demand for high amount of the cells for CSC and follow-up LC–MS/MS analysis, ii) time course of the study, iii) application of the Progenesis software for normalisation of LC–MS/MS data to reduce technical variations, and iv) availability of the MSstat software package suitable for experiments with such complex design comparing more than two experimental conditions and time changes using linear mixed-effects models.

The cells were washed with ice-cold PBS, scraped and treated with 1.5 mM sodium meta-periodate (Sigma-Aldrich) in labelling buffer (0.1% foetal calf serum in PBS) for 15 min at 4 °C with gentle agitation in the dark. This was followed by labelling with 3.5 mM biocytin hydrazide (Biotium, Hayward, CA) in labelling buffer for 1 h at 4 °C with gentle agitation. The cells were then washed with PBS, resuspended in 50 mM ammonium bicarbonate (Sigma-Aldrich), and homogenised by sonication in Vial Tweeter (70 s, 100% amplitude, 0.8 cycle time). The nuclei were removed by centrifugation at 2500 *g* for 10 min. The Rapigest detergent (Waters, Milford, MA) was then added to a final concentration of 0.1% (*v*/*v*) and solubilised proteins were reduced and alkylated using 5 mM tris(2-carboxyethyl) phosphine and 10 mM iodoacetamide (Thermo Fisher Scientific Inc), respectively.

The protein concentration in each sample was determined by micro-BCA protein assay (Thermo Fisher Scientific Inc.). Sequence grade trypsin (Promega, Madison, WI) was then added (1:50 protease/protein ratio) and the samples were incubated overnight at 37 °C under agitation which was followed by trypsin inactivation at 96 °C for 12 min. The biotin-tagged glycopeptides were captured on streptavidin beads (Thermo Fisher Scientific Inc.) during 1 h incubation and then thoroughly washed to remove non-bound peptides. The beads were resuspended in 50 mM ammonium bicarbonate and 500 units of the peptide-N-glycosidase F (PNGaseF; New England Biolabs, Ipswich) were used for enzymatic cleavage of peptides from N-glycopeptides (overnight agitation at 37 °C). Samples of collected peptides were acidified with formic acid to a final pH <3 and loaded onto C18 Ultra MicroTip columns (The Nest Group Inc., Southborough, MA) to desalt according to the manufacturer's instructions. Peptide mixtures were evaporated on a vacuum centrifuge to dryness and subsequently solubilised for LC–MS analysis in 0.1% formic acid in 2% acetonitrile (Thermo Fisher Scientific Inc.).

2.3. Mass spectrometry analysis

Each biological replicate was analysed by LC–MS/MS using a nano-LC system (Eksigent/ABSciex, Framingham, MA) with fused-silica

column (10-cm × 75-μm inner diameter) packed in-house with Magic C18 AQ 3-μm beads (Michrom Bioresources, Auburn, CA) and connected to a LTQ-Orbitrap XL mass spectrometer equipped with nanoelectrospray ion source (Thermo Fisher Scientific Inc.). The peptide mixtures (~500 ng) were loaded from an auto-sampler (Eksigent/ABSciex, Framingham, MA), cooled to 4 °C and separated using linear gradient from 7% to 37% acetonitrile in 60 min with the flow rate set to 300 nl/min. The ions were fragmented in the LTQ part of the instrument in collision induced dissociation (CID) mode, whilst Orbitrap XL part of mass spectrometer was utilised as a sensitive mass analyser. The peptide ion mass to charge range of 350–1600 was monitored in one high resolution (60,000) MS1 scan followed by five MS2 fragmentation scans on the most intense ions in CID mode with a collision energy of 32eV After each MS2 scan, precursor ion masses were temporarily excluded from fragmentation for 30 s. Ions with a charge state of 1 were excluded from MS2 fragmentation.

2.4. Protein identification, quantification and classification of their functionalities

Raw data was converted to mzXML data format using ReAdW software (v4.3.1) [15] and the collected spectra including common protein contaminants were searched against the human UniprotKB/Swiss-Prot protein database (v57.15) using Sorcerer–Sequest (40,521 entries in total; Sage-N Research, Milpitas, CA; [16]). The search parameters included: trypsin as the digesting protease, the tolerance of peptides with one tryptic termini and one missed cleavage site, carbamidomethylation of cysteines (+57.0214 Da) as a fixed modification of asparagines (+0.984 Da) and oxidation of methionine (+15.99492 Da) as variable modifications. The monoisotopic peptide mass tolerances were set to 0.04 Da. Each sample was searched separately and identifications were filtered with FDR <1%, calculated on the basis of a target-decoy approach [17].

A Trans Proteomic Pipeline was used for the statistical analysis of MS/MS data (v4.4; [18]). Search results of protein identification were filtered for the peptides containing N[115] deamidation inside the N-glycosylation NxS/T motif. The alphabetically first UniProtKB-Swiss-Prot identifier was used for peptide identifications leading to indistinguishable protein group identification. The proteins identified on the basis of only one independent spectrum of a peptide in a single experiment were discarded. Protein transmembrane domains were predicted using the PHOBIUS algorithm (v1.01) [19].

Deglycosylated peptide spectrum identifications over all cell types/conditions were reported for each protein and provided an estimation of overall glycoprotein abundance. Progenesis software (v2.0, Nonlinear Dynamics, Waters, Milford, MA) [20] was used to visualise, align, and normalise label-free LC–MS/MS data, generating complete data sets with no missing values (http://www.nonlinear.com). MS1 intensities of the individual deglycosylated peptides were extracted and protein quantification was then performed using the MSstats software package [21] utilising a linear mixed-effects model. The differentiated 21dNPCs as well as proliferating NPCs were also purposely pair-compared with the post-mitotic hNT neurons to observe commonalities at the protein level that would reflect differentiation status independent on genetic background of the cells. The results were summarised as protein fold changes with standard errors, T values, degrees of freedom, and P-values adjusted for multiple comparisons, where the changes reaching $P \le 0.05$ were considered as significant.

The PANTHER (Protein ANalysis THrough Evolutionary Relationships, version 9.0) database was used to assign functionalities of identified proteins according to Gene Ontology (http://www.pantherdb.org). [22]. To identify the main protein classes with the impact on NSC differentiation status, UniProt accession numbers of target proteins regulated in the course of differentiation were submitted to the PANTHER database. PANTHER Protein Class was used to categorise proteins according to protein families (groups of evolutionarily related proteins with the same function).

2.5. Selected reaction monitoring

The selection of proteins for further validation using SRM was based on the following criteria: i) significantly ($P \le 0.05$) altered abundance in differentiated 21dNPCs in comparison to NPCs; ii) at least two fully tryptic deglycosylated peptides for SRM; and iii) presence of at least one transmembrane domain predicted by Phobius software and independently confirmed at the protein level using annotated UniProtKB/Swiss-Prot database (http://www.uniprot.org/). Among several candidates, ICAM1 (CD54) showed the highest up-regulation in proliferating NPCs whilst CHL1 and LAMP1 (CD107a) were increased in differentiated 21dNPCs. In addition, brain protein astrotactin1, with only one N-deglycosylated peptide found in our study, was also considered for validation and the corresponding peptide was targeted in SRM. For each of the other proteins (ICAM1, CHL1 and LAMP1) two deglycosylated peptides were measured using SRM.

Development and validation of deglycosylated peptide-specific SRM assays was performed as previously described [23] using synthetically heavy-labelled peptides (AQUA peptides, Thermo Fisher Scientific Inc.) with aspartic acid replacing the asparagines corresponding to the mass modification introduced by PNGaseF in our experimental set up. To extract the SRM coordinates, doubly or triply charged precursor ions and doubly or singly charged fragment ions of the b- and y-ion series of the peptides were monitored. Spectra were acquired on a triple quadrupole mass spectrometer (6460 Agilent Technologies, Santa Clara, CA) equipped with HPLC/ChipCube interface (Agilent Technologies) and operating in positive ion SRM mode. The peptide mixtures with spiked AQUA peptides were injected from a micro Well-plate sample (Agilent Technologies) cooled to 6 °C and separated on HPLC-chip (5 μm Zorbax C18, enrichment column 160 nl, analytical column 75 μm × 15 cm; Agilent Technologies) with a linear gradient from 5 to 35% acetonitrile over 30 min at a flow rate of 300 nl/min. SRM analysis of AQUA heavy-labelled peptides including the endogenous (light) version of iRT peptides (Biognosys AG, Schlieren, Switzerland) was conducted with Q1 and Q3 operated at unit resolution (0.7 m/z half-maximum peak width) with a dwell time >20 ms and a cycle time <2.7 s (178 transitions). Data was analysed with Skyline, and the top 4–5 transitions per peptide were retained in the final SRM method.

Retention time (RT) extracted during the assay refinement was used to schedule SRM acquisition. LC–SRM method (177 transitions, 6-min RT window, 1.8-s cycle time) was used and the samples in two biological (independent cultures) and two technical (MS runs) replicates of each sample, including proliferating NPCs, NPCs induced to neuronal differentiation (3dNPC, 10dNPC and 21dNPC) as well as hNT cells, were analysed using the instrument settings described above. SRM peaks were manually inspected using Skyline by checking for co-elution, peak shape similarity, a match in relative intensities of fragment ions and RTs compared to the assay development step. Only SRM peaks with a signal-to-noise ratio >3 were considered and the top 3–4 transitions per peptide with no obvious interference were retained for quantification.

Peak areas were exported from Skyline and further analysed using the MSstats [24]. The log-transformed values were normalised based on signals of all peptides across MS runs and linear mixed-effects model was then applied for protein significance analysis. Multiple SRM transitions per peptide and multiple peptides per protein (except for astrotactin-1 where only one peptide was measured) were analysed. Relative quantification of the samples of differentiated NPCs was performed with respect to proliferating NPCs and significant changes were reported as $\log_2$ fold changes with standard errors, T values, degree of freedom, and P values adjusted for multiple comparisons to control the FDR in the list of differentially abundant proteins. The fold-

change cut-off was calculated on the basis of the number of biological replicates, peptides per protein, and transitions per peptide while retaining a statistical power >0.5 ($P \leq 0.05$) [24].

2.6. Flow cytometry

Approximately 2×10^5 proliferating NPCs and 21dNPCs were used to perform flow cytometry analysis using cell surface immunostaining. The cells were washed with PBS containing 0.1% BSA and blocked with 0.5% BSA in PBS for 15 min at 4 °C. For single-colour staining, primary antibody CD54 (ICAM1) R-Phycoerythrin conjugated (monoclonal mouse antibody, IgG1, clone 84H10; Beckman Coulter Czech Republic s.r.o., Prague, Czech Republic), was incubated with cells to allow binding for 30 min at 4 °C. After two wash steps with PBS, the cells were incubated with propidium iodide to stain for exclusion of dead cells and analysed with a FACS ARIAII cytometer (Becton Dickinson, San Jose, CA). At least 40,000 cells were acquired for the analysis. The NPC or 21dNPC populations were gated from all measured events by their forward and side light scatter characteristics and viable single cells were sequestered from dead cells by the second gate. Finally, positively stained cells were gated according to the mouse IgG1 R-Phycoerythrin ICAM1 isotype control (Miltenyi Biotec — BIOCHEM spol. s.r.o., Trencin, Slovakia). The gating strategy is shown in Supplementary Fig. 1. The experiment was repeated twice for conformity of results.

2.7. Immunocytochemistry

Immunocytochemistry aimed at monitoring neuronal differentiation was carried out on NPCs, 3dNPCs, 10dNPCs, 20dNPCs, and 30dNPCs using known markers of neuronal induction. These markers included tubulin beta-3 chain (TUJ1), neuronal migration protein doublecortin (DCX) and microtubule-associated protein (MAP2). In addition, antigen Ki-67, expressed predominantly by proliferating NPCs was monitored.

3. Results

3.1. Immunofluorescence imaging of NPCs in the course of neuronal differentiation

The human NPCs ($CD184^+/CD271^-/CD44^-/CD24^+$) were seeded on POL plates in NSC growth medium containing bFGF to maintain undifferentiated NSC-like morphology (middle-sized nuclei, adherent monolayer culture) with high expression of Sox2 and nestin, whose expression is largely related to the Ki-67 marker of proliferating cells [10]. After switching to differentiation medium, the cells differentiated into neurons (smaller nuclear content, fibrillar structures, adherent 3D culture) confirmed by DCX and MAP2 expression, 3 days after triggering of differentiation (Supplementary Fig. 2), indicating the appearance of neurons with varying degree of maturity. The expression of DCX, MAP2, as well as TUJ1 was evident 10 days after differentiation with the majority of cells committed to the neuronal fate despite the presence of Ki-67 positive cells until this time interval (Supplementary Fig. 2). The quiescent cells differentiated for 20 and 30 days, with noticeably weaker expression of nuclear proliferation marker Ki-67 manifested as differentiated mature neurons which strongly expressed neuronal markers MAP2 and TUJ1 (Supplementary Fig. 2). This characterisation confirmed the usability of this model and justified time points for studying the neuronal differentiation and temporal protein patterns that drive this process.

3.2. Identification of cell surface N-linked glycoproteins in NPCs and upon neuronal differentiation

We utilised CSC technology in combination with high accuracy LC–MS/MS to determine the composition of the cell surface N-glycoproteome of the NPCs upon their neuronal differentiation, as well as in post-mitotic mature hNT neurons (Fig. 1). The raw data can be accessed at http://www.peptideatlas.org/PASS/PASS00649. The list of total N-glycoproteins identified in the present study along with all reported peptides with a chemical deamidation at the consensus amino acid sequence motif for N-glycosylation (NxS/T) is provided in Supplementary Table 1. In total, 522 N-glycoproteins were considered confidently identified with FDR <1%. The majority of cell surface proteins were identified using up to 3 N-deglycosylated peptides and different numbers of transmembrane domains (Table 1A). Proteins without a predicted transmembrane domain were mainly annotated as extracellular matrix proteins, secreted or intracellular membrane proteins, or proteins with GPI anchor.

The CSC technology revealed a high number of proteins that were categorised into protein classes by the PANTHER database (Table 1B). Complete information on the distribution of different functional protein classes identified in the different development stages is visualised in Supplementary Table 2. Proliferating NPCs appeared to have slightly

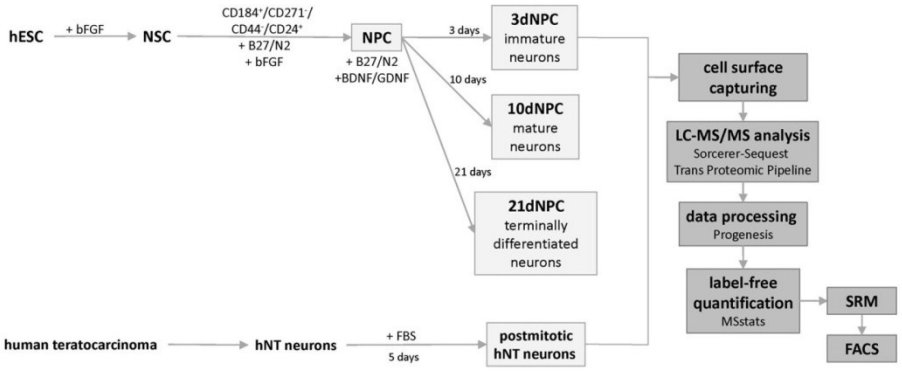

Fig. 1. Experimental workflow. Neural stem cells (NSC) were derived from human embryonic stem cells (hESC) and sorted $CD184^+/CD271^-/CD44^-/CD24^+$ neural precursor cells (NPC) were used to induce neuronal differentiation for 3, 10 or 21 days (Supplementary Fig. 1). Using cell surface capture (CSC) technology coupled to LC–MS/MS, cell surface proteins of NPCs upon their neuronal differentiation and hNT neurons were identified and characterised (Table 1, Supplementary Table 2) and their list along with confidence scores is presented in Supplementary Table 1. The proteins were relatively quantified and statistically evaluated to select the abundance changes (Fig. 2, Supplementary Tables 3 and 4). Several abundance-modulated N-glycoproteins were subjected to validation by SRM (Fig. 3, Supplementary Tables 5-7) and flow cytometry (Fig. 4).

Table 1
Distribution of N-glycosylation sites (A), transmembrane domains (A) and functional classification of the proteins identified (B).

Tab 1A

N-glycosites	**0**	**1**	**2**	**3**	>**3**
	0	268 (50.7%)	149 (28.2%)	56 (10.6%)	55 (10.6%)
Phobius transmembrane domain	**0** 164 (31.0%)	**1** 221 (41.8%)	**2** 25 (4.7%)	**7** 19 (3.6%)	>**10** 46 (8.7%)

Tab 1B

Panther protein class	Hits	% NPC	% 21dNPC	% hNT
Receptor	143	19.6	19.1	18.3
Hydrolase	78	9.4	9.8	11.2
Transporter	74	10.1	10.5	12.4
Cell adhesion molecule	74	11.6	9.5	9.0
Signalling molecule	60	8.4	8.3	5.7

higher representation of cell adhesion proteins and receptors, whilst hydrolase and transporters were slightly enriched in the differentiated 21dNPCs and hNT cells (Table 1B). The cell adhesion molecules have been shown to play a vital role in NSCs and NPCs development through transmission of the signals from stem cell niche and several of cell adhesion molecules have been studied for potential to promote functional recovery of neural disorder or injury [25]. Neuronal membrane transport proteins on differentiated cells function to carry neurotransmitters across these membranes and to direct their further transport to specific intracellular locations. There are many highly diverse neurotransmitter transporters with selective uptake systems associated specifically with particular neurotransmitter [26].

3.3. The abundance changes of identified N-glycoproteins in NPCs upon neuronal differentiation

Progenesis was used to align and normalise the acquired LC–MS/MS data. MS1 intensities of the individual peptides were then extracted (Supplementary Table 3) and quantified by MSstats (Supplementary Table 4). Differentiated cells (3dNPC, 10dNPC, 21dNPC) were compared to the control sample (NPC), and post-mitotic neurons (hNT) were compared to NPC or 21dNPC in order to link their differentiation status. In comparison with NPCs, abundances of 1, 29, and 55 proteins were significantly different with $P \leq 0.05$ in 3dNPCs, 10dNPCs, 21dNPC, respectively, indicating increasing number of alterations as differentiation proceeded (Supplementary Table 4). In addition, 164 and 187 proteins were differentially abundant in hNT neurons when compared to differentiated 21dNPCs and NPCs, respectively, ($P \leq 0.05$, Supplementary Table 4) which may reflect the distinct differentiation state of 21dNPCs and hNT neurons as well as their genetic origin.

Five proteins showed abundance changes in the whole time course of neuronal differentiation (Fig. 2, Supplementary Table 4). Among them, thrombospondin type-1 domain-containing protein 7A (THS7A; 9UPZ6) was found to be significantly higher on day 3 and in all the following time intervals of neuronal differentiation whilst teneurin-3 (Q9P273), contactin-associated protein 1(P78357), and interleukin-13 receptor subunit alpha-2 (Q14627) decreased from day 3 of differentiation. Three proteins including sodium channel protein type 2 subunit alpha (Q99250), neural cell adhesion molecule CHL1 (CHL1; O00533) and voltage-dependent calcium channel subunit alpha-2delta (; P54289) were up-regulated with the high fold change from day 10 to 21 of neuronal differentiation. On the contrary four proteins such as transmembrane protein 194A (O14524), junctional adhesion molecule C (Q9BX67), receptor-type tyrosine-protein phosphatase zeta (P23471), or omega (Q16827)) were down-regulated from day 10 to 21 of neuronal differentiation (Fig. 2, Supplementary Table 4). Additionally, there were 17 proteins regulated only on day 10 of NPC differentiation such as fibronectin type III domain-containing protein 5 (Q8NAU1) and crumbs homologue 2 (Q5IJ48) (Fig. 2, Supplementary Table 4).

The highest number of regulated proteins compared to other time points was evident on day 21 of neuronal differentiation. Eight proteins increased in differentiated cells and reached significant alterations with fold change higher than 10, including tomoregulin-2 (TEFF2; Q9UIK5) and ephrin type-B receptor 1(EPHB1; P54762) (Fig. 2, Supplementary Table 4). One example of hydrolase family was represented by leucyl-cystinyl aminopeptidase (Q9UIQ6). Among 22 proteins decreased on day 21 of differentiation, dominant alterations were observed for intercellular adhesion molecule 1 (ICAM1; P05362), which was together with

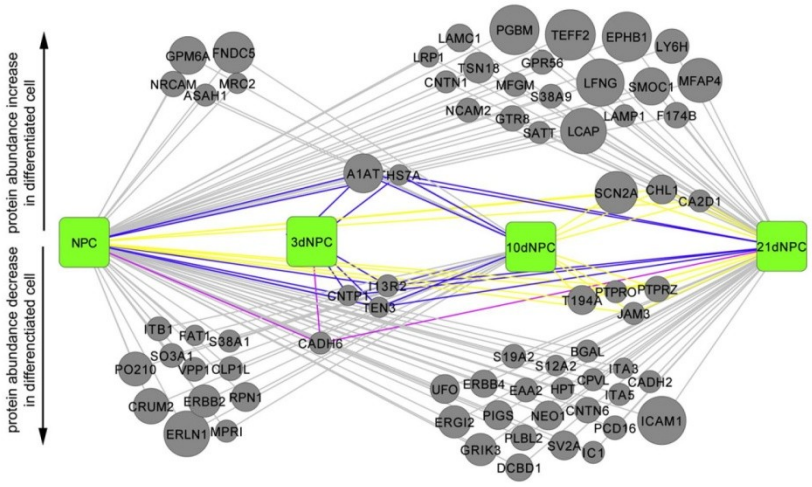

Fig. 2. Visualisation of the abundance alterations of identified N-glycoproteins in the course of NPC neuronal differentiation. Square green nodes represent cells and round nodes of grey colour and different size depict proteins differentially abundant between the cell types. The size of nodes is proportional to fold-change. The colours of edges depict overlaps in comparisons.

protocadherin-16 (PCD16; Q96JQ0), integrin alpha-5 (P08648) and cadherin-2 (P19022), representative of cell adhesion molecules significantly down-regulated in 21dNPCs (Fig. 2, Supplementary Table 4).

Comparison between 21dNPCs and hNT indicated several co-regulated glycoproteins. Among them, ICAM1 and transmembrane protein 194A appeared to be commonly down-regulated whilst neural cell adhesion molecule 2 (O15394), and lysosome-associated membrane glycoprotein 1 (LAMP1; P11279) were similarly up-regulated in both 21dNPCs and hNT differentiated cells (Supplementary Table 4), hence they may represent common targets of neuronal differentiation. Taking into account the significant P values of the observed changes, ICAM1 and LAMP1 were considered for further validation experiments using SRM. In addition, an important aspect justifying the combination of these two proteins points to prospective studies with a view to eliminating proliferating (ICAM1positive) cells that may cause teratomas whilst keeping differentiating cells that would only mature to the functional neurons when grafted.

3.4. SRM validation of selected glycoprotein abundance alterations

Development and validation of deglycosylated peptide-specific SRM assays was carried out using synthetic heavy-labelled peptides with aspartic acid replacing the asparagines, corresponding to the mass modification introduced by PNGaseF (Supplementary Table 5). The raw data can be accessed at http://www.peptideatlas.org/PASS/PASS00650. SRM peaks were manually inspected and the top 3–4 transitions per peptide with no obvious interference were retained for quantification. Peak areas were exported from Skyline (Supplementary Table 6) and further analysed using the MSstats (Supplementary Table 7). Relative quantification of the samples of differentiated NPCs or hNT was performed with respect to proliferating NPCs. Results of SRM quantification confirmed that the level of ICAM1 was decreased with NPC differentiation and in hNT cells whilst CHL1 and LAMP1 were seen to increase in 21dNPCs but not in hNT cells (Fig. 3, Supplementary Table 7). Intriguingly, LAMP1 levels were found decreased at early stages of differentiation (3dNPCs, 10dNPCs) but increased in 21dNPCs. Astrotactin1 (ASTN1; O14525), with so far only predicted N-glycopeptides was confirmed as a glycoprotein more abundant in mature neurons (21dNPCs, hNT cells) compared to NPCs (Fig. 3, Supplementary Table 7).

3.5. Flow cytometry analysis of ICAM1 across differentiation

To interrogate NPCs in the course of differentiation on the cellular level, flow cytometry was applied to proliferating NPCs and

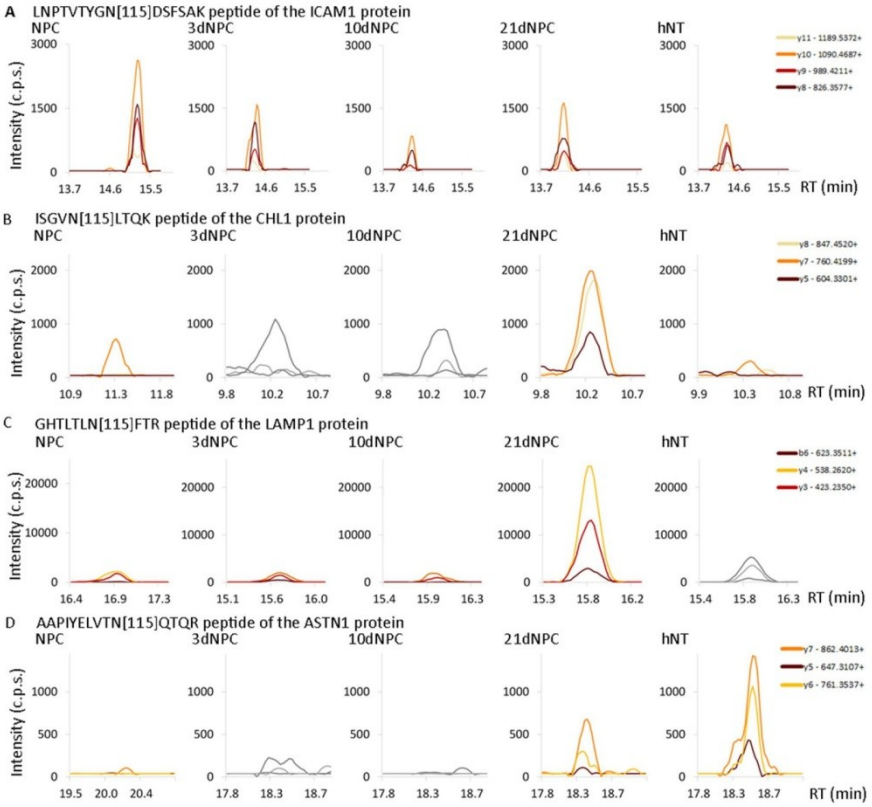

Fig. 3. SRM validation of selected abundance-modulated cell surface N-glycoproteins. The abundance change of each protein upon a given condition is provided relative to the neural precursor cells (NPC). Regulation is displayed as a chromatographic trace of deglycosylated peptide elution and detection by SRM. Traces represent the detection of different fragment ions from a common peptide precursor (SRM transitions). Significant changes ($P < 0.05$) are depicted using coloured traces where grey indicates no significant difference in abundance (Supplementary Table 7). c.p.s., counts per second. (A) represents LNPTVTYGN[115]DSFSAK peptide of the ICAM1; (B) ISGVN[115]LTQK peptide of the CHL1; (C) GHTLTLN[115]FTR peptide of the LAMP1 and (D) AAPIYELVTNN[115]QTQR peptide of the ASTN1 protein.

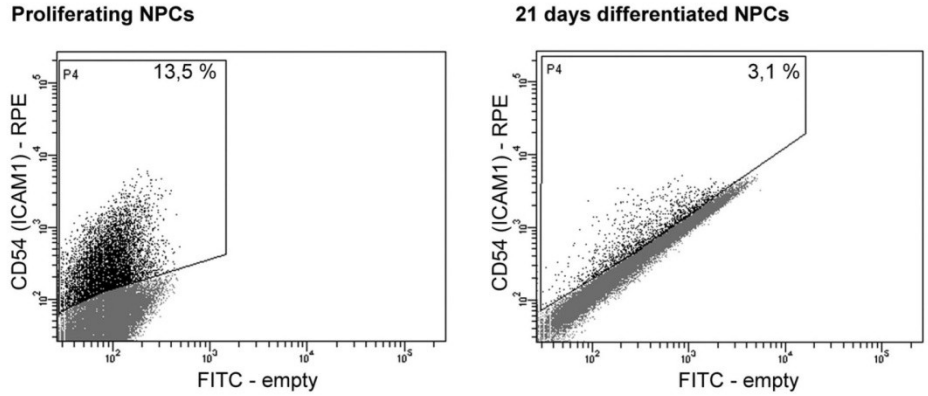

Fig. 4. Flow cytometry of ICAM1. Analysis showed higher positivity for ICAM 1 surface protein in proliferating neural precursor cells (NPC) in comparison with NPCs differentiated for 21 days (21dNPCs).

differentiated 21dNPCs. The analysis revealed a fall by 10% of the positive cells for ICAM1 across differentiation. (Fig. 4). The 21dNPCs were shown to be heterogeneous population (Supplementary Fig. 1) with the larger cells having higher autofluorescence and dispersion values. Hence, flow cytometry findings indicated that ICAM1 becomes restricted to relatively fewer cells within a differentiating heterogeneous neural cell population. This interpretation may corraborate the results of SRM showing the relative decrease in abundance of ICAM1 with NPC differentiation (Fig. 3).

4. Discussion

Evaluation of cell surface proteins is fraught with difficulties due to limitations in handling membrane proteins and compounded by lack of specific antibodies for their detection. The recently introduced CSC technology enables comprehensive quantitative analysis of cell surface N-linked glycoproteins which may facilitate the development of useful tools for phenotyping and sorting out a variety of cell populations [11]. Application of CSC technology combined with high accuracy LC–MS/MS to our model of neuronal differentiation indicated increasing number of N-linked glycoprotein alterations in the time course of differentiation up to the stage of mature neurons. Those which showed significant abundance changes compared to proliferating NPCs, or matched between fully differentiated NPC-derived neurons and hNT, were highlighted as the most valued and a key outcome emerging from this study. Importantly, 13% of the glycosylation sites reported here have not yet been identified based on the information in the UniprotKB/Swiss-Prot reference database and our findings confirmed N-glycosylation at a particular site which were only predicted. On the contrary, we did not detect the glycoproteins like CD184 and CD 24 that were used for isolation of NPCs [10] and further utilised in this study. However, some of the currently used markers including CD133 (prominin1), CD44 and CD56 (NCAM1) were identified in our study with significant ($P \leq 0.05$) up-regulation of CD56 (NCAM1) in hNT cells. In addition, we observed an increase in neural cell adhesion molecule 2 in differentiated 21dNPC as well as hNT cells. Altered glycosylation may affect CSC and subsequently quantitative outcome, namely, under-glycosylation on a particular site may remain non-detectable. On the other hand, hyper-glycosylation, in case of complete sugar oxidation by periodate, should be recognised. Some discrepancies in detection and/or quantification of N-glycoproteins can be observed also in the Human Protein Atlas and the Human Protein Map where the latest human proteome map implemented immunohistochemistry using antibodies to analyse tissues at a cellular level, which often provided complementary results to mass spectrometry approach without any use of antibodies (http://www.humanproteomemap.org/and http://www.proteinatlas.org/, [27]).

Although in our validation experiments we focused on a group of cell adhesion molecules such as ICAM1, CHL1 and astrotactin 1, there are other interesting proteins that document the complexity of neuronal differentiation. The most pronounced changes were observed in the stage of mature differentiated neurons, including an increase in ASTN1, TEFF2 and EPHB1. The neuronal protein ASTN1 is known to function as an adhesive molecule for neuronal contact with glial ligand on axons during glial-guided neuronal migration. The level of ASTN1 in the neuronal plasma membrane is critical for such neuronal–glial adhesions [28]. TEFF2 is a well-known protein predominantly expressed in the brain, and recently it was shown as a component of plaques in Alzheimer's disease brain, hence it may contribute to the pathogenesis of this disease [29]. TEFF2 belongs to tomoregulin protein family. Interestingly, immunohistochemical analysis of TEFF1 showed its expression on the cell surface when expressed alone, whilst it was preferentially distributed to the endoplasmic reticulum and co-expressed with addicsin in neurons in the mature mouse brain. Hence, addiscin localised in the endoplasmic reticulum controlled, *via* interaction, TEFF1 trafficking from the endoplasmic reticulum to the plasma membrane with an impact on migration of neurons [30]. EPHB1, like other ephrin-B receptors, plays an important role in maintenance of dendritic spines as well as synapse formation. Similarly to TEFF2, ephrin type-B receptors have been linked to the pathophysiology of Alzheimer's disease [31].

Among the proteins down-regulated during neuronal differentiation ICAM1 was found to be significantly lowered in differentiated neurons as confirmed in this study by both SRM and flow cytometry. This protein together with selected cadherins such as cadherin 2 may indicate the presence of proliferating cells in a population of differentiating neurons. Additional interesting proteins belong to the group of receptor-like protein tyrosine phosphatases, which are highly expressed in the brain. It was shown, for example, that phosphatase activity of receptor-type tyrosine-protein phosphatase omega negatively influenced the functions of ephrin receptors [32]. The IL13R2, on the other hand, could be involved in control of neuronal loss and neurodegeneration which has been clearly associated with the susceptibility to oxidative stress-mediated damage in the case of IL13Rα1 expressing dopaminergic neurons [33]. To this end, decrease in I13R2 on differentiating neurons reported in this study may support the protection and prevention of neuronal damage. Interestingly, there is a link to neurodegeneration because human IL13Rα1 is localised in the PARK12 locus of Parkinson's disease [33].

THS7A, a cell surface protein significantly higher in all studied time intervals of neuronal differentiation, is known to belong to the family of multifunctional thrombospondins. Recent findings demonstrated that thrombospondin1 produced by astrocytes, was a critical factor for maintenance of neuronal differentiation [34]. Our results show that THS7A may be localised in neuronal cells, hence it might be involved and functional in previously described synaptogenic adhesion complexes [35]. Importantly, CHL1 plays several roles in neurite outgrowth and neuronal survival in vitro [36] and in synaptic plasticity including efficacy of GABAergic synapses. The CHL1 directly forms complexes with chaperone proteins in synapses with remarkable selective impact on the activity of the involved proteins [37]. Further findings from CHL1 null mice showed that this protein negatively modulated neuronal differentiation [38]. On the contrary, the results of our study documented an increased level of CHL1 in the course of neuronal differentiation, which was further substantiated by SRM analysis. Unfortunately, many available antibodies on the market do not recognise key extracellular glycosylated epitopes on neural cells. It makes translation of MS-based CSC findings towards flow cytometry and cell sorting difficult. Hence, development of antibodies that could be specific for such accessible epitopes on particular neural subpopulations remains an urgent need to advance further studies.

5. Conclusions

We focused on patterns of N-linked glycoproteins of NPCs and differentiated neurons which highlighted the complexity of neuronal differentiation and hold the potential for obtaining well characterised enriched neuronal subpopulation from mixed cultures after differentiation of NPCs. Among the several functionally different target molecules described in this study, a group of cell adhesion molecules including ICAM1, CHL1 and astrotactin1 warrants further research focus in development of strategies useful in cell-replacement therapies of neurodegenerative diseases and spinal trauma.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.11.008.

Conflict of interest statement

There are no conflicts of interest to report. No writing assistance was utilised in the production of this manuscript.

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4.3 Analýza proteomu nervových kmenových buněk v průběhu diferenciace s cílem usnadnit přechod k buněčné terapii

v publikaci

Proteome-wide analysis of neural stem cell differentiation to facilitate transition to cell replacement therapies

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EXPERT REVIEWS

Proteome-wide analysis of neural stem cell differentiation to facilitate transition to cell replacement therapies

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Neurodegenerative diseases are devastating disorders and the demands on their treatment are set to rise in connection with higher disease incidence. Knowledge of the spatiotemporal profile of cellular protein expression during neural differentiation and definition of a set of markers highly specific for targeted neural populations is a key challenge. Intracellular proteins may be utilized as a readout for follow-up transplantation and cell surface proteins may facilitate isolation of the cell subpopulations, while secreted proteins could help unravel intercellular communication and immunomodulation. This review summarizes the potential of proteomics in revealing molecular mechanisms underlying neural differentiation of stem cells and presents novel candidate proteins of neural subpopulations, where understanding of their functionality may accelerate transition to cell replacement therapies.

KEYWORDS: cell therapy • immunomodulation • neural stem cell differentiation • neural subpopulation • neurodegenerative disease • population-specific protein-expression signature • quantitative mass spectrometry • selected reaction monitoring

Neurodegenerative diseases & their rising incidence

Neurodegenerative diseases are devastating and affect millions of individuals worldwide [1]. Unfortunately, no drugs are currently available to halt their progression, except a few that are largely inadequate. This warrants the search of new treatments for these progressively degenerative diseases. According to European Commission Public Health Information on Major and Chronic Diseases, more than 600 disorders afflict the nervous system and among them the prevalence of neurodegenerative diseases continues to rise with resultant social and economic problems [2]. Despite diverse spectrum, broad heterogeneity of clinical symptoms and observed neuropathological features, neurodegenerative diseases are commonly characterized by progressive loss of neural cells, primarily neurons, resulting in nervous system dysfunction. Recent studies suggest that glial cells influence neurodegenerative processes, namely due to inter-communication between microglia or astrocytes and neurons [3,4]. Degenerative process covertly advances during a pre-symptomatic period till a threshold of neurological manifestation is reached when cell dysfunction and death are critical and clinical symptoms become apparent. The neurodegenerative process and its progression are usually dependent on specific type of neuronal population and respective brain regions which are affected. The most common disorders are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). In addition, neurodegeneration accounts for significant part of disability in patients after traumatic injury of the nervous system including spinal cord injury [5].

Majority of AD cases are sporadic and represent the most common form of dementia resulting with high probability from the effects of environmental and genetic risk factors.

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Clinically, it is characterized by progressive cognitive impairment including memory and behavior. The cholinergic hypothesis of AD indicates that loss of cholinergic neurons and deficiencies in cholinergic neurotransmission in cerebral cortex are cause of the decline of cognitive functions [6]. Neuropathologically, AD is demonstrated by extracellular amyloid β plaques and intracellular neurofibrillary tangles consisting of phosphorylated tau protein. It was shown that extensive oxidative stress resulting from mitochondrial dysfunction induced by amyloid β is apparent very early on before the onset of clinical and neuropahological symptoms [7]. Perturbed energy metabolism in cerebral cortex and apoptosis mediated by mitochondrial altered calcium homeostasis and cytochrome *c* release as well as mitochondrial and DNA damage are major cellular hallmarks of AD pathology.

The second most common neurodegenerative disorder, PD, is considered to be multifactorial, although there are also patients with disease-linked genetic defects such as PD proteins (PARK1, 5, 6, 7) and leucine-rich repeat kinase 2 (LRRK2) mutations, which are commonly found in certain populations [8]. Progressive loss of dopaminergic neurons whose bodies are located in the substantia nigra pars compacta and project the terminals to the caudate/putamen nuclei results in dopamine decrease observed in basal ganglia and subsequently in PD symptoms. There is a broad spectrum of clinical manifestations of PD including tremor at rest, muscle rigidity, bradykinesia and akinesia and instability [9]. In addition to these, non-motor symptoms contribute to the decline of cognitive functions observed as patient's autonomic dysfunction, sleep, mood and sensory disorders as well as pain [10]. The intraneuronal aggregates of α-synuclein (PARK1 and 4) and other protein components known as Lewy bodies, represent the main neuropathological feature [11] and are considered to mediate and drive the gradual loss of dopaminergic neurons via mitochondrial dysfunction and oxidative stress, neuroinflammation as well as alterations in ubiquitin-proteasome system [12]. Recently, growing evidence of decreased copper along with increased iron in substantia nigra and caudate nucleus of PD patients support a role of copper and iron, which may enhance mitochondrial dysfunction and oxidative stress [13].

In contrast to AD and PD, familial history is best evident in majority of HD cases. HD is a monogenic autosomal dominant disorder with expansion of a CAG trinucleotide repeats in the first exon of the huntingtin (*HTT*) gene, which is well-recognized mutation causing the disease [14]. Early in the disease process, primary inhibitory γ-aminobutyric acid (GABA) ergic medium spiny neurons in striatum are targeted and neuronal loss proceeds till striatal atrophy. Additionally to GABAergic medium spiny neurons, large aspiny cholinergic interneurons and other intraneuronal GABAergic populations might be affected [15]. Neurons also progressively degenerate in the cortex, hypothalamus and hippocampus resulting in atrophy throughout the entire brain by late-stage HD [16]. Clinically, HD is presented by involuntary movement resulting from disturbances in muscle coordination, cognitive decline, psychiatric symptoms and eventually dementia. Of interest are also multiple non-neuronal abnormalities such as weight loss and cardiac failure [17]. In relation to the HD pathological mechanisms, it is useful to consider distribution and functions of endogenous huntingtin protein, which is ubiquitously expressed in a variety of peripheral cells, although high levels are reached in neurons and glial cells in the CNS [18]. Huntingtin plays an anti-apoptotic role as exemplified by protection of the neurons against excitotoxicity [19] and via interactions mediated exclusively by its N-terminal region, it communicates with several subcellular organelles including vesicle trafficking assembly, namely synaptic transmission, as shown by its disruption when *HTT* is mutated [20]. The expansion of the CAG trinucleotides in the N-terminus of the *HTT* is translated into polyglutamine (polyQ) stretch of the mutated huntingtin, which then forms oligomers and aggregates in the cytoplasm and nucleus of the cells. Among pathological mechanisms of cell and tissue damage in HD, excitotoxicity, mitochondrial damage, impaired energy metabolism and oxidative stress, possibly inflammation and transcriptional dysregulation as well as alterations in autophagy, are important cellular features [21–23].

ALS is caused by the degeneration of bulbar, cortical and spinal neurons leading ultimately to their loss. Most ALS cases are sporadic and only approximately 10% have familial history linked to the mutations in a number of genes such as *SOD1*, *TDP43* and *FUS* or repeat expansion in the first intron of *C9orf72* [24]. Despite sporadic or familial history, clinical presentations of the disease are very similar and are characterized by irreversible paralysis, and speech, swallowing as well as respiratory disturbances [25]. In addition, it remains elusive how many distinct mutations impact motor neurons and converge on common cause of the ALS degeneration including motor neuron toxicity and loss of neuromuscular synapses.

Outlook & cell-based therapies for neurodegenerative diseases

Stem cells have become an attractive option to investigate and treat these diseases (Figure 1). Cell-based therapies can be beneficial and the demands on treatment of neurodegenerative diseases are expected to rise in connection with higher disease incidence. The search for new therapies has been revolutionized during the last two decades by the discovery of stem cells and induced pluripotent stem cells (iPSCs). To date, therapies using embryonic and embryonic-derived cells or adult stem cells in animal models of various neurological diseases have provided promising results [26,27]. Currently, several cell types are studied as possible source to be used in cell replacement-based therapies for treatment of a variety of neurodegenerative disorders including spinal cord injury, ALS, HD and stroke. Under specific cell culture conditions, the human embryonic stem cells (hESCs) were induced to differentiate *in vitro* into populations enriched in specific neural cells, such as oligodendrocyte precursor cells and oligodendrocytes, spinal cord motor neurons, dopaminergic neurons, astrocytes or retinal progenitor cells [28]. Despite some promising preclinical results, there are substantial

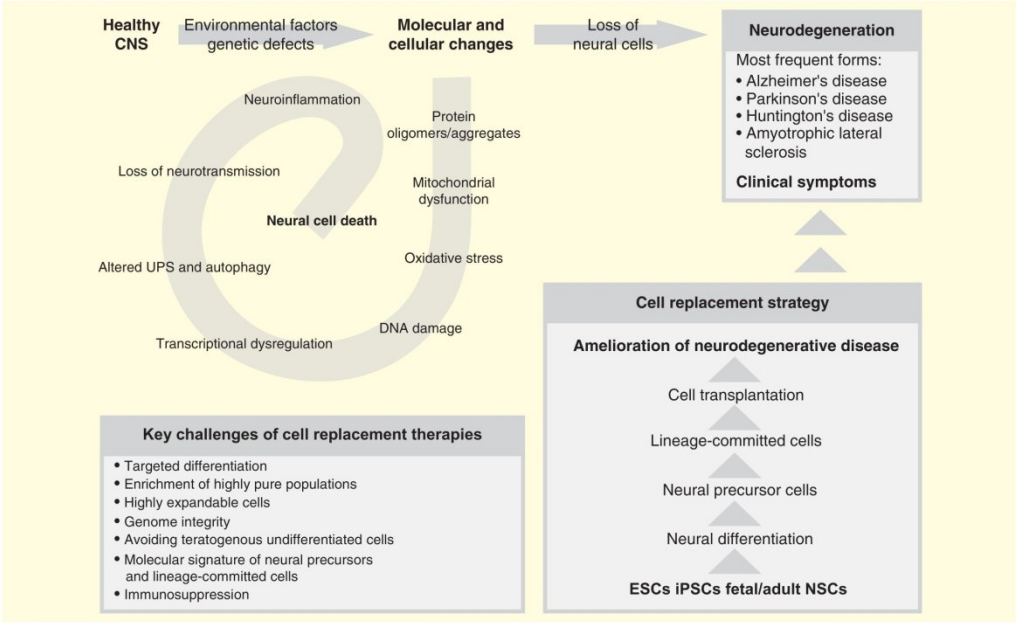

Figure 1. Cell replacement strategy for neurodegenerative disease therapies. Genetic as well as environmental factors have been shown to be associated with neurological disorders and trigger many molecular and cellular alterations leading to progressive loss of neural cells and neurodegeneration. The process ultimately results in nervous system dysfunction and manifestation as cognitive and behavioral impairments. Cell replacement therapies are widely considered as a promising strategy for neurodegenerative diseases, however, there are several key challenges to fulfill successful translation toward transplantation of neural precursor cells and their progenitors. Among them there is an urgent need for improved differentiation and enrichment procedures that generate highly pure populations of neuronal and glial cells with well-characterized molecular signatures.
ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; NSCs: Neural stem cells.

concerns which include the risk of teratoma formation, need for immunosuppression as well as ethical concerns [29]. The fetal or adult neural stem cells (NSCs) can be successfully expanded *in vitro* to give rise to neurons, astrocytes and oligodendrocytes both *in vitro* and *in vivo* [30]. Experimental animal studies using a variety of neurodegenerative models provide compelling evidence of a functional benefit, which can be achieved using cell replacement-based therapies.

The iPSCs are extensively being developed and studied as useful tools for disease modeling, drug development and screening [31]. In addition, the use of autologous iPSCs-derived lineage-committed cell lines in personalized cell replacement-based therapies is believed to eliminate the need for transient or continuous immunosuppression. The use of immunosuppressive therapy, which is required in allogeneic grafting design, represents a major clinical limitation for a more effective use of such treatments in a broader spectrum of clinical patients. Finally, the application of iPSC-derived cell lines in replacement therapies eliminates the ethical issues related to pre-implantation embryo. Nevertheless, the issues including targeted differentiation and avoiding potentially teratogenous undifferentiated cells from the cell culture remain to be solved for both iPSC- and ESC-derived populations.

With the refinement of expansion protocols, differentiation conditions and validated safety, the first US FDA-approved clinical trial which employed hESC-derived oligodendrocyte precursors for treatment of spinal cord traumatic injury was initiated by Geron Corp. (Menlo Park, CA, USA) in 2010 [32,33]. The assumption for use of oligodendrocyte progenitors was to initiate myelination of spinal cord axons, which could improve nerve function and recover motoric functions in patients with spinal cord injury. The study was limited to four patients who were enrolled according to strict conditions, mainly specific form and location of the spinal cord injury. Despite promising preliminary results, the trial was discontinued after 1 year. Further monitoring of the patients showed no detectable functional side effects, but predicted integration of grafted cells with host spinal cord and filling of the injury cavity, and also no MRI-detectable abnormal cell mass at the cell-injected core.

In addition to ESC-derived oligodendrocyte cell line already used in clinical spinal trauma trial, there is a substantial

interest in developing reliable protocols to generate expandable and well-characterized populations of neural and neuronal precursors. To date, several fluorescence-activated cell-sorting (FACS) protocols that employ a combination of cell surface markers [34], which are believed to be neural cell lineage-specific, have been developed. While a substantial increase in the purity of generated NSCs was achieved using FACS in these studies, no optimal protocol is currently available which would lead to a consistent NSCs generation once employed in multiple independent repetitions. A major bottleneck is the continuing presence of undesired cell populations of endoderm and mesoderm origin; the relative contribution of these unwanted cells may vary from preparation to preparation and may increase over time, particularly in a long-term maintained NSCs cultures.

Understanding of stem cell differentiation into neural lineages: a proteomic view

Proteomics & stem or pluripotents cells

Proteomics is a rapidly advancing field utilizing technology platforms for the study of a variety of biological systems. Of the many proteomic technologies, current focus appears to be on the development of mass spectrometers, nanochromatographic systems and bioinformatic tools. Two mass spectrometry (MS)-based approaches have been utilized for a large-scale analysis of proteins and their post-translational modifications. Shotgun proteomics provides a system-wide view and is being applied in discovery-driven projects. Even without prior knowledge, MS datasets of multiple proteomes are subjected to computational analysis to generate lists of proteins that change their abundance across different conditions. Given its global nature, candidate proteins are typically validated by orthogonal techniques. Additionally, ICAT, isobaric TMT or isobaric tags for relative and absolute quantitation (iTRAQ) and SILAC, are some of the approaches often used.

The second MS approach is based on selected reaction monitoring (SRM), which enables the generation of precise quantitative datasets for a large number of complex samples in a highly reproducible and non-redundant manner. There is no need for subsequent validation, however, only approximately 100 selected proteins can be quantified in a single analysis. SRM-like data-independent acquisition methods may overcome this limitation only at the price of sensitivity but could be advantageous for data mining in zoom-in experiments to gain mechanistic insight. Targeted MS measurements are very attractive and competitive technologies to antibody-based ELISA and arrays. Such technologies offer great potential for development of diagnostic tests for clinical use.

To better interrogate biological samples such as cells, body fluids and tissues, proteomics facilitates fractionation strategies to enrich organelles or specific groups of proteins and peptides, as well as their post-translational modification products. The versatility of proteomic technologies and the possibility of combining them to obtain the end result, makes proteomics a powerful tool in the designing of experiments to answer biologically relevant questions. Functional proteomics is critically important with respect to understanding of the functionalities of biological systems rather than mechanistic identification of individual components. This together with quantitative proteomics, allowing relative or absolute quantitation of proteins/peptides in biological sample, makes proteomics an indispensable technology platform for researchers.

Since our understanding of many of the basic cellular processes underlying stem and pluripotent cell self-renewal, maintenance and differentiation are still very limited, it is essential that we expand our knowledge and understanding at protein level if stem cell research is to reach its full potential. The proteomic interrogation of stem cells is already feasible to generate relative quantitative stem cell protein maps and derive partial functional protein interaction networks. The applications of proteomics to the area of neural stem cell differentiation may also help to facilitate transition to cell replacement therapies of neurodegenerative diseases (Figure 2).

Urgent need to define sets of neural-specific developmental proteins & their potential use in generation of highly purified & expandable neural precursors

One way to address the issue of availability of well-characterized and expandable neural cell lineage-specific populations is to increase our knowledge of the spatiotemporal profile of cellular protein expression during neural differentiation and to define a set of markers, which will be more specific in predicting the lineage specificity of targeted cell population (Figure 3). Current consensus is that the processes of cell differentiation in general, involve stem cell autonomous factors as well as the effect of static or releasable components in the extracellular milieu. These may include growth factors, hormones, extracellular matrices, intercellular interactions and intracellular signaling cascades [35].

Despite a high degree of similarity between ESCs and iPSCs, there are some described differences in gene expression resulting from epigenetic changes but possibly also from intracellular regulation. Reprogramming of the somatic nucleus to pluripotent state requires significant changes and epigenetic status may be a significant barrier. Despite the observation that iPSCs from late passages are more similar to embryonic cells at the transcriptome level, suppression of the genes in starting somatic cells, for example, fibroblasts, does not appear to be sufficient to reach pluripotent status [36]. Recent proteomic analysis of early reprogramming events in murine fibroblasts incubated with oocyte extracts identified several proteins whose abundance changed after the treatment. Follow-up bioinformatic evaluation assigned their functionalities mainly to nuclear dynamics, transcription and translation as well as showed interconnectivity with pluripotency markers Klf4, c-Myc, Nanog and Oct4 [37]. Further studies considering the consequences of the transcriptome and epigenome alterations into level of the proteome are desirable and need to be carried out.

Importantly, the propensity of hESCs and iPSCs for neural differentiation appears to be an important aspect. Ebert *et al.* [38] recently developed a simple method to generate and expand

multipotent self-renewing pre-rosette NSCs from both human ESCs and human iPSCs with neural differentiation capabilities, however, efficient differentiation into specific neural subtypes remains challenging. Additionally, it was shown that phosphorylation of Ascl1 on serine-proline sites was crucial for neuronal differentiation and this step may promote generation of neurons from fibroblasts [39]. Finally, to define true functional similarities in ESC- versus iPSC-derived NSCs, there is a need to characterize the differentiation profile of such a cell population(s) after *in vivo* grafting using immunodeficient rats with a long-term survival as well as in CNS injury or genetically-induced neurodegenerative models to demonstrate the final post-mitotic phenotype and/or their continuing proliferative capacity.

The intricate mechanisms controlling NSCs differentiation into different neural lineages have to be fully characterized and the proteins playing crucial roles in transition through this process could be then manipulated *in vitro*. This would help to produce required homogenous subpopulations of neural progenitors in sufficient amount for cell transplantation. The low expression of a large number of proteins involved in differentiation is a common feature of stem cells. Once committed to a particular lineage, specific proteins are being induced, while the elimination of the proteins that are no longer needed is commenced. It is essential to precisely define sets of neural-specific developmental proteins responsible for driving NSCs toward the particular subtypes in order to identify markers for monitoring of progenitors specificity, estimation of neural fate and follow-up correlation with therapeutic effect in the preclinical studies using animal disease models. Although a variety of emerging proteins were described, only those which were validated by the techniques orthogonal to the discovery phase are implemented in this review (Supplementary Figure 1 [supplementary material can be found online at www.informahealthcare.com/suppl/10.1586/14789450.2015.977381]), and their detailed description is summarized in the Supplementary Table 1.

Intracellular proteins as a handle to follow-up transplantation

Once the cells are induced to differentiate from pluripotent precursors *in vitro*, they need to be characterized to confirm whether the target population has been obtained and the presence of pluripotent cells is substantially diminished or completely eliminated. Currently, the most frequently used and well-characterized markers are Nanog and Oct3/4, transcription factors which are essential for maintaining pluripotency and controlling the expression of a number of genes involved in embryonic development, proliferation and self-renewal [40]. Among many variants of multistep protocols of neural differentiation, induction of pluripotent cells into NSCs is commonly achieved via formation of embryoid bodies followed by propagation/growth in serum-free medium in the presence of FGF and/or EGF. Upon differentiation into NSCs, the expression of Pax6 and Sox1 transcription factors is required for continuation of self-renewal and maintaining the neural progenitors in an undifferentiated state [41,42]. Appearance and timing of intermediate filament nestin expression are consistent with formation of precursors of the neural lineage and are related to the characteristic features of progenitor cells, such as multipotency, high proliferation, limited self-renewal and regeneration capacity [43].

Further differentiation of NSCs into lineage-committed subpopulations such as oligodendrocyte, astrocyte or neuronal precursors can be induced by exposure of NSCs to the growth factors such as brain-derived nerve factor and/or glial-derived nerve factor that control the development of specific brain

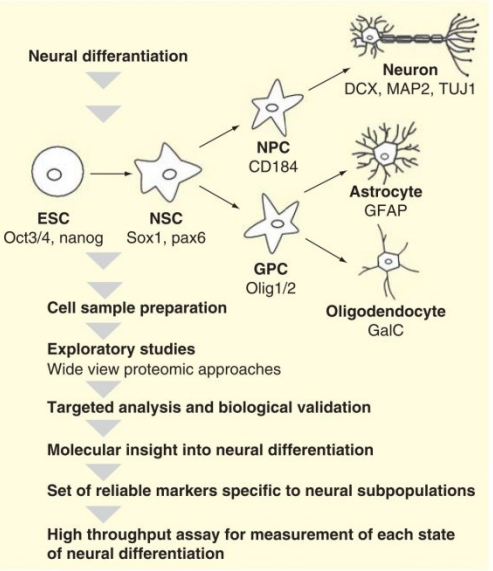

Figure 2. Proteomics contribution in revealing the underlying molecular mechanisms in stem cell differentiation and novel markers of neural subpopulations. Human neural stem cells can be derived from embryonic stem cells and differentiation of neural stem cells into lineage-committed subpopulations such as glial or neuronal precursor cells and further more into neurons, astrocytes and oligodendrocytes is depicted by changes in cell morphology and by progressive appearance of typical markers. Proteomics offers an enormous potential for the deeper understanding of molecular mechanisms of stem cell differentiation. Protein patterns resulting from global quantitative analysis are being followed by targeted analysis of the emerging novel candidate proteins and their biological validation. Ultimately, such novel biological insight into neural differentiation generates set of markers, which can be measured at each stage of differentiation using high throughput assay. Currently used intracellular and membrane markers of neural subpopulations as well as a variety of emerging proteins are summarized in Supplementary Figure 1 and their detailed description is provided in Supplementary Table 1.
DCX: Doublecortin; ESC: Embryonic stem cell; GPC: Glial precursor cell; MAP2: Microtubule-associated protein 2; NPC: Neuronal precursor cell; NSC: Neural stem cell; TUJ1: βIII-tubulin.

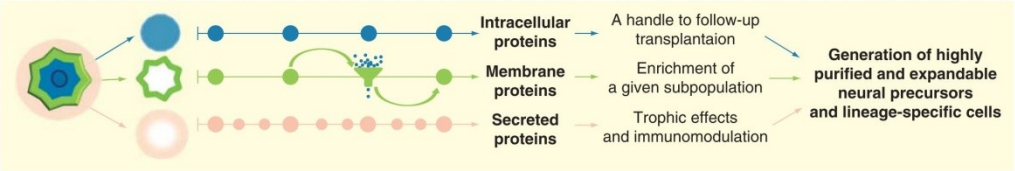

Figure 3. Toward efficient and safe cell replacement therapy of neurodegenerative diseases. Spatiotemporal profiles of cellular expression during neural differentiation allow development of a uniform protein signatures, which can be used to obtain a given subpopulation of lineage-specific cells suitable for *in vivo* grafting without teratogenic risk.

regions [34], while other modulators including platelet-derived growth factor-AA can be used to bias differentiation into oligodendrotytes [44]. The initiation of NSCs differentiation is observable by changing cell morphology and by a progressive appearance of markers, which permit a delineation of specific cell types. Currently used intracellular and membrane markers of neurons and glial cells (astrocytes and oligodendrocytes) are listed in Supplementary Figure 1. Moderate expression of microtubule-associated protein 2 in neuronal precursors becomes much stronger in neurons immediately after the βIII-tubulin is expressed [45]. Doublecortin is a microtubule-associated protein expressed in early post-mitotic neurons with the main function in the growth at the leading edge of neuronal cells [46]. In 1992, NeuN was identified as a neuronal marker whose appearance corresponds temporarily to the withdrawal of neuronal cells from the cell cycle and/or with initiation of terminal differentiation of the neuron [47].

One of the most frequently used markers of astrocytes is glial fibrillary acidic protein, which forms a network to help to maintain astrocyte mechanical strength as well as the shape [48]. Another astrocyte marker, calcium binding protein S100β, is expressed by a subtype of mature astrocytes that ensheath blood vessels [49]. Combination of Olig, Nkx and Sox10 proteins has emerged as transcription factors critical for the oligodendrocyte development [50]. Olig genes are the earliest markers of oligodendrocyte lineage determination, however, their expression during development could establish a state of competence to form more than one neural lineage [51]. O-Antigens O1 and O4, which are surface markers of oligodendrocytes in the CNS [52] as well as specific enzymes such as cyclic nucleotide phosphodiesterase and galactocerebrosidase appear to be more suitable markers for oligodendrocyte differentiation [53].

In order to refine classification of neural subpopulations, the results of several recently published studies can be considered. Abraham *et al.* searched for NSC regulatory factors using a shotgun proteomics study with resultant revelation of high expression of chromatin structural proteins [54]. The members of the high mobility group (HMG) superfamily, HMGA and HMGB, can modulate transcription, replication, recombination and DNA repair. The HMGA2 is specifically localized in CNS and regulates NSC proliferation during early embryonic development [52]. Upon binding to nucleosomes, HMGA2 acts as a modulator of cell cycle regulating $p16^{Ink4a}$ protein, which has been reported to maintain chromatin in the open state [55]. Additionally, it was shown that HMGB proteins reflected HMGA2 expression in NSCs indicating their impact on cell proliferation [54]. While HMGB3 and HMGB4 responded to NSC differentiation in a stage-dependent manner, both HMGB1 and HMGB2 were rapidly and substantially repressed from early stages of differentiation [54]. The dynamic expression of HMGBs was further explored using HMGB2 null mice demonstrating that deficiency of the protein resulted in significant increase in small spheres, compared with large ones consisting of NSCs, along with the increased cell division. Hence, HMGB2 may play a role in suppression of NSC renewal via effect on chromatin and possibly have a regulatory function in survival and differentiation of NSCs. These findings certainly contribute to understanding of neural differentiation and deserve further investigations.

In 2011, Chaerkady *et al.* utilized iTRAQ labeling followed by liquid chromatography (LC)-MS/MS to simultaneously quantify proteomes of NSCs undergoing directed differentiation into oligodendrocytes [44]. This robust approach, reliable to identify subtle changes in relative abundance, extended previous findings of this group aimed at defining mechanisms of differentiation into motor neurons and astrocytes [56] and both studies together provide the identities of potential markers for multiple steps of NSC differentiation. Glial progenitor cells, neuronal progenitor cells (NPCs), oligodendrocyte progenitors as well as ESCs and post-mitotic neural cells were carefully monitored using immunofluorescent labeling of to date known markers and the same method coupled to immunoblots was then used to validate novel results of global quantitative screening approach. Among the validated proteins were: DCLK1 (doublecortin and CAM kinase-like 1), as a marker of motor neurons; KPNA4 (karyopherin α 4), as a marker of NPCs and macrophage migration inhibitory factor, which appeared to be a protein marker for early neural developmental stages [56,57] but diminished during motor neuron differentiation [56].

Another marker identified in a different multiplex study utilizing iTRAQ is dihydropyrimidinase-related protein (DPYSL), a protein localized in neurogenic regions of the CNS [57,58]. The DPYSL3 and DPYSL2 isoforms have been found overexpressed in human [57] and mouse NSCs [58], respectively, indicating that this isoform-specific marker of early neural differentiation could be measured at high-throughput level in different species.

Cell surface proteins to facilitate selection & sorting of cell subpopulations

Differentiation of pluripotent cells *in vitro* leads to the appearance of heterogeneous populations of cells in various developmental stages that do not co-exist during *in vivo* differentiation after gestation. These mixed cell populations are not useful for transplantational experiments or potential use in clinical therapies. Accordingly, a high level of enrichment and expansion of purified cells is needed before such lines can effectively be used in *in vivo* setting.

Cell sorting is nowadays routinely used for hematopoietic cells, but, unfortunately, cell types like embryonic or neuronal cells are more prone to damage in the course of sorting procedure not only due to their non-round morphology but in general they have limited capability to adapt to circulation under sorting pressure. These observations result in some demands such as lower pressure or reduced sorting speed which then may be applied for such types of cells [59]. Nevertheless, cell surface proteins (CSPs) are accessible to suitable antibody tagging for fluorescence-activated or magnetic beads cell sorting of living cells. Although membrane proteins comprise between 20 and 30% of the proteome, they are still underrepresented in proteomic experiments mainly due to technical challenges during sample preparation and their low abundance in comparison with intracellular proteins [60]. Analytical approaches for membrane protein identification using LC-MS/MS were recently reviewed [61].

Currently, the most established cell surface markers of human pluripotent cells are glycolipids SSEA-3 and SSEA-4 and keratin sulfate-related antigens TRA-1–60 and TRA-1–81 [62]. Further classification as markers of stem cells with neural commitment include prominin-1 (CD133), SSEA-1 (CD15) or structurally related FORSE-1, which are also called 'stemness markers' [63]. They are utilized as proliferative markers that decrease upon differentiation. Other markers can distinguish differentiated cells with neuronal commitment like PSA-NCAM (CD56) or CD24 [59] or glial precursor cells with expression of A2B5 [59] or CD44 [34] and PDGFR (CD140a) [64], which are typical for astrocyte and oligodendrocyte precursors, respectively. Unfortunately, it is becoming apparent that these markers are not specific enough to achieve highly pure populations of the cells with distinct phenotype and expected suitability for translation to clinical applications. Methods that can be used to improve the outcome are negative selection, for example, to get rid of the unwanted cells carrying markers of proliferative cells with the highest risk of teratoma formation, or generally the usage of combination of markers [34,65]. Evidently, new cell surface markers of individual developmental stages would help to identify specific populations of neural cell types.

There are several approaches to study new cell surface markers of neural differentiation. One of them is to systematically study involvement of already known CSPs like CD classified molecules. The main advantage of this approach is the availability of already produced antibodies, the main disadvantage is obviously the very limited number of currently identified proteins. Several studies aimed to characterize the presence of different CD protein markers on stem cells and their neural derivates were published by Pruszak *et al.* [59,63,65]. In 2007, this group demonstrated a 4 weeks survival and no tumor formation in rat brain grafted with NCAM+ (CD56) FACS-sorted mature ESC-derived neurons [59]. This was followed by the study showing the capability of distinguishing NSCs, neural crest and neurons using combinatorial CD15, CD29 and CD24 marker code-analysis [65]. Recently, the simultaneous detection of CD surface molecules with known intracellular markers that yielded combinatorial CD49f–/CD200 high indicator for enrichment of neural lineages emerging from pluripotent cells was described [63]. Single marker analysis would fail due to the presence of non-neuronal CD49f– or $CD200^+$ subpopulations in the cell culture. Rather more complex FACS analysis of 190 cell surface antigens on hESCs and their derived neural differentiated cell cultures lead to the isolation of population of $CD184^+/CD271^-/CD44^-/CD24^+$ neural precursor cells that subsequently differentiate into mixed populations of neurons and glia cells [34]. Neurons could be further sorted out according to the combination of $CD184^-/CD44^-$/CD15 low/$CD24^+$ markers, in contrast to $CD184^+/CD44^+$ population of glial cells. Similarly, 30 CD surface markers on TRA-1–81+ hESCs and their neural derivatives revealed strong expression of CD133 and CD326 in undifferentiated hESCs, while CD56 and CD184 surface molecules were increased in their neural derivatives [66]. Interestingly, this study excluded CD24 and CD90 as specific markers of neural differentiation, which is in contrast to results from Pruszak *et al.* [59].

More comprehensive results than from CD molecule targeted analyses may come from MS studies. This approach to study membrane proteome was successfully applied to hESCs [67,68] and different types of mouse cells including myoblasts [69] or pluripotent cells [70]. Typically, the procedure covered enrichment of membrane fractions using sucrose gradient and selection of membrane proteins from the whole protein dataset by software prediction tools. Of interest was targeted enrichment of cellular plasma membrane proteins by chemical labeling applied on living cells before digestion [71]. This appeared to be the most effective approach for extracting CSPs without contaminants and potentially useful proteins, indicating cellular fate which may be utilized also for cell selection/sorting.

Studies dealing with characterization of CSPs during neural differentiation of pluripotent cells are currently lacking with exception of an extensive quantitative comparative study of membrane proteome between hESCs and NSCs published in 2014 by Melo-Braga *et al.* [60]. The authors combined dimethyl labeling to study changes in organelle and plasma membrane proteins with immobilized metal affinity chromatography and TiO_2 enrichment allowing to approach the changes in phosphorylation and glycosylation pattern of membrane proteins. Although these findings represented a huge set of the proteins and their post-translational modifications in hESCs and NSCs, only a few of them were validated using SRM. Among them CRB2 as well as its glycosylation site Asn886 was indicated as a potential NSCs marker. In addition, DPYSL2, a plasma membrane associated enzyme, was confirmed to be upregulated in NSCs in comparison to ESCs [60], which is in agreement with other proteomic studies [57,58,72].

Interesting strategy for discovery of hESC-specific membrane proteins was presented by Prokhorova *et al.* [73]. They employed SILAC-based comparison of different self-renewing ESCs versus cells that were spontaneously differentiated by removal of FGF2 from culture medium for only 2 days. Six proteins were revealed with higher expression levels in undifferentiated ESCs cells where neuroligin-4, involved in both cell–cell and cell–extracellular matrix interactions which are vital for ESCs, was observed as a candidate CSP of self-renewing ESCs. In addition to using the CSPs as a tool for cell selection, it is well documented that CSPs possess many dynamic biological functions. Thus, studying the changes of CSPs in the course of neural differentiation of pluripotent cells may shed light on mechanisms of the differentiation of these cells too.

Besides ESCs or NSCs, cell surface membrane Na^+/Ca^{2+} exchangers in microglia contribute to Ca^{2+} influx and respiratory burst of these cells, hence they might be targeted in order to reduce microglia activation in the damaged CNS [74]. Another cell-surface protein, AN2 mouse homolog of rat NG2 protein, expressed by oligodendroglial progenitor cells has been shown *in vitro* to play a role in control of migration of cultured primary oligodendroglial progenitor cells [75].

Secreted proteins: intercellular signaling, trophic effects & immunomodulation

It is well recognized that growth factors like NGF, brain-derived nerve factor, CNTF, glial-derived nerve factor and VEGF are important neurotrophic modulators of CNS physiology and there is evidence that NSCs or NPCs may exert their effects by secretion of such factors after transplantation in experimental models of neurodegenerative disorders. Their supportive roles are most likely directed to prevent cellular death process of neurons that are subjected to the stress or noxious stimuli as well as scar formation. In addition, NPCs provide remarkable immunomodulatory effects, which may promote regeneration and healing processes. Thus, the use of NPCs in cell replacement therapies and the mechanism of their possible therapeutical effect can be considered as: improvement of local structural integrity of previously injured tissue; functional synaptic coupling between grafted neurons and neurons of the host and release of trophic factors, which may modulate survival of previously injured host neurons and glial cells.

The extracellular proteome consists of individual soluble proteins, so-called secretome and also secreted extracellular vesicles with size up to 1 μm carrying cellular proteins, miRNA and membrane components [76]. The study of the role of miRNA in neurodegenerative diseases with the focus on recognition and regulation of the amount of pathogenic proteins in specific neuronal cells, which is crucial for cell survival and pathogenesis of disease remains a key focus [77].

Defining the secretome functionality and its paracrine effect on cell survival together with analysis of releasable components by *in vivo* microdialysis and/or cell-conditioned media *in vitro*, remains a challenge. To characterize such secreted factors, several techniques such as LC-MS/MS combined with isobaric tag or isotope-coded affinity tag labeling can be effectively used. Despite the limitation due to targeted pre-selection of analytes, implementation of multiplexing antibody array technologies, either planar or bead-based, may provide high sensitivity of detection even in the presence of serum components. The combination of such approaches remains extremely powerful and provides a huge range of complementary results. Compared with findings from antibody arrays, LC-MS/MS data require additional verification using SRM-targeted approach and other orthogonal techniques.

The studies focused on NSC secretomes as well as their alterations in the course of neural differentiation are rare. Farina *et al.* [78] used mouse ESCs and compared the secretomes of cardiac and neural differentiation. The study revealed several proteins unique for neural differentiation, but only about half of them were predicted as secretory proteins using bioinformatics tools. Among them, endoplasmin, prohibitin-2 and serpin H1 were present in the conditioned medium at the time of terminal neural differentiation.

Very recently, a cytokine profile of human astrocyte secretome under physiological conditions as well as inflammatory stimulation by IL-1β and TNF-α has been described [79]. Using a planar protein microarray revealed the production of cytokines and chemokines such as G-CSF, GM-CSF, GROa (CXCL1), IL-6, IL-8 (CXCL8), MCP-1 (CCL2), macrophage migration inhibitory factor and serpin E1 by normal resting astrocytes, while after inflammatory stimulus, they produced different profile of cytokines and chemokines but, interestingly, all of them were assigned to NF-κB signaling pathway, indicating important immunomodulatory role of astrocytes. To mimic the astrocyte phenotype resulting from lipopolysaccharide induced inflammation *in vitro*, Wang *et al.* [80] investigated the effect of inflammatory astrocyte-conditioned media on proliferation and differentiation of NSCs. This study identified IL-6 as a major factor involved in the NSC proliferation as confirmed by its reduction in the presence IL-6 neutralizing specific antibodies as well as enhanced proliferation after addition of recombinant protein.

Another study demonstrated that clusterin, already known as inhibitory protein of complement system, was recognized as protein component of media conditioned by midbrain and hippocampal but not cortical astrocytes. Its presence increased the number of neurons from expanded human NPCs, and the immunodepletion of clusterin from midbrain astrocyte conditioned medium eliminated the observed effects on neuronal differentiation [81].

Applying bead-based multiplex technology, Mosher *et al.* showed that there were several factors including tissue inhibitor of metalloproteinase type-1, VEGF and haptoglobin that were secreted in large amounts by NPCs in comparison to mouse primary microglia, astrocytes and neurons [82]. Injection of NPCs into striatum of mice resulted in microglial activation, proliferation and phagocytosis. Using these *in vivo* function assays, it was established that recombinant VEGF protein exerted at least some of these effects while immunodepletion of VEGF from NPC-conditioned media reversed such phenotype.

These findings indicated that NPCs via secretion of VEGF were important regulators of microglia functions. Consistent with these data is our observation which demonstrates that VEGF secretion by hESC-derived NPCs reaches maximum concentration 20 days after triggering of neuronal differentiation and remains high (Supplementary Figure 2). Interestingly, the angiogenin, another pro-angiogenic growth factor, peaked to highest level on day 14 and then rapidly declined (Supplementary Figure 2).

Expert commentary

At present, there is an increasing appreciation and growing interest in using ESC or iPSC-derived committed neural precursors in the treatment of several neurological diseases including ALS, spinal trauma, HD, stroke or multiple sclerosis. The initial animal efficacy data and clinical data from human spinal trauma trial clearly demonstrate the treatment potentials of ES-NPCs and iPS-NPCs and fully warrant their further research. In this report, we have summarized the potential of proteomics in revealing the underlying molecular mechanisms of stem cell differentiation. Novel candidate proteins of neural subpopulations emerging from such screens are typically subjected to validation using affinity reagents/antibodies for targeted analyses such as immunoblots and fluorescence-based imaging. Given the limitations of these semi-quantitative assays, including variable and partly unpredictable specificity of utilized antibodies, the alternative approach based on SRM is currently being applied for the simultaneous quantification of protein targets by MS with no need for subsequent validation. SRM generates highly reproducible and precise quantitative information for high number of samples, which is directly interpretable by biologists and allows transition from discovery phase to validation of targets.

A lack of congruence in reproducing some results which identify the lineage-specific markers in different laboratories applying the same methodical approach might arise from variable culture conditions, such as the choice of differentiation growth factors, different dose and/or timing. To examine lineage-specific differentiation, it is necessary to use defined culture conditions and avoid potential artifacts introduced by exposure to poorly characterized media containing complex mixtures of growth factors such as those present in serum. Quality control is required for NSCs in continuous cultures, including normal karyotype and morphology testing as well as maintenance of proliferation and differentiation capabilities. To accelerate the transplantation research, there is an urgent need for improved differentiation and enrichment procedures that generate highly pure populations of neuronal and/or glial cells. Protein patterns obtained by quantitative proteomics screens should be extracted and combined with previous knowledge to provide novel biological insight into NSC differentiation. Only then, an information-rich targeted assay with the capability of measuring the complete set of reliable markers at high throughput might be assembled. The availability of such assay is critical for the development of a uniform protein-expression signature, which can reproducibly and predictably characterize the differentiation profile of NPCs suitable for *in vivo* grafting.

Five-year view

Primary neuronal culture is an important tool for many areas of neuroscience research, including studies of neuronal signaling, synaptic plasticity, gene expression, pharmacology and toxicology. Additionally, human iPSCs capable of differentiating into NSCs and mature neural cells have had an unprecedented impact on similar parameters in disease research. To circumvent ethical and moral disputes associated with the use of blastocyst-derived ESCs, iPSCs provide new perspectives for personalized regenerative medicine in future.

With involvement of large bioproduction companies, it is expected that the commercial availability of human iPSC-derived cells via industrialized manufacturing will greatly expand the use of stem and differentiated cells for neurodegenerative disease research. Formations of iPSC biobanks would provide the precise and bulk starting material necessary for large-scale clinical trials and experiments that has been lacking, thus facilitating high number of disease model studies simultaneously. As knowledge base grows, some key questions that frustrate researcher may end up being answered including the measure of 'stemness' role or the epigenetic cell memory in the journey to pluripotency. Additionally, correlation of outcome to animal models as proxies to humans as well as the predictive potential for iPSC disease models will need to be assessed carefully if we are to benefit in the long run using highly sophisticated technologies. While many of the current technologies have been around for a while, newer and emerging sensitive technologies including data-independent acquisition or Multi-Nnotch MS^3 quantitation will certainly have a significant role to play despite possible technological limitations. Leveraging next-generation immunoassays and/or SRM-based approach with unprecedented multiplexing capacity and accuracy to interrogate stem cell proteins would facilitate the application of NSCs in cell-based therapy. One such example is the ultrasensitive detection of neurodegenerative biomarkers including tau in blood. The measurement of tau protein in cerebrospinal fluid has been well documented, but the presence and measurement of tau in human blood components has been difficult due to inadequate sensitivity of current assay methods.

State-of-the-art MS-based proteomics has been in use for several years but recently, its use has rapidly expanded to also cover stem cell research. The advent of next-generation sequencing should facilitate greater targeted therapies for neurodegenerative diseases. Since sequencing is not simply restricted to coding regions, whole genome sequencing allows discovery of mutations in regulatory regions such as promoters and enhancers, and other non-coding regions such as those for miRNAs. Advances in flow cytometry together with high-speed cell-sorting and imaging is also fast becoming an ideal tool for interrogating microparticles and cellular subsets in multiple formats. These technologies complimented by imaging technologies and sensitive fluorescent antibodies/tandem dyes should allow the examination of diverse cell

subpopulations and cell types. Analysis of novel stem cell surface markers as well as internalized receptors should lead to better understanding of the cell in question and its role in cell therapy.

While genomics, proteomics and stem cell biology have come of age with exciting results to follow, cross-collaboration of researchers from these specialties together with clinicians needs active fostering if we are to fully benefit from each other's expertise. Additionally, with ever-increasing data, bioinformatics will be critical to the real outcome for diverse neurodegenerative diseases. The ability to interpret the wealth of data in order to build an accurate picture of the *in vitro* versus *in vivo* disease scenario for clinical intervention is a major challenge.

Until now, too many studies take the easy and more manageable route of looking at one or a limited number of factor (s) at a time and often leading to incomplete or a poor outline of the disease. Now with combination of up-to-date technologies with sensitivity, specificity and speed, multiple parameters can be studied to gain a much better appraisal of the ongoing processes such as cell–cell, cell–matrix and cell signaling interactions. There are now limitless possibilities for the use of NSCs if researchers could only fully picture their intricacies and functionalities. While NSCs offer a great promise for the treatment of neurodegenerative diseases or management of intractable diseases through repair or replacement of damaged tissues, every step in the process should require rigorous scrutiny from the origin of the cell, through expansion, manipulation, to the eventual engraftment in the patient. The severe risks of such stem cell therapy including teratoma formation, exaggerated immune response or ectopic engraftment need to be overcome in preclinical transplantation research.

Looking forward to the next decade, neural stem cell therapy will remain a priority in order to fulfill the great hopes of many patients directly or indirectly via drug development, regenerative or reparative therapy while ushering in an era of great expectations.

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Key issues

- Neurodegenerative diseases and their rising incidence in the population.
- Need for improved differentiation and enrichment procedures to generate highly pure populations of neuronal and/or glial cells in order to facilitate transition toward therapeutic use.
- Quantitative proteomics to provide novel biological insight into neural stem cell differentiation.
- Novel candidate proteins of neural subpopulations emerging from proteome screens.
- Development of uniform protein-expression signatures characteristic of neural stem cells lineages.
- Sensitive assay development for reproducible differentiation profiling after *in vivo* grafting.
- Establishment of induced pluripotent stem cell bio banks with defined cellular source as starting material.
- Cross-specialties collaboration of researchers from genomics, proteomics and stem cell biology, and clinicians to address therapeutic intervention.

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4.4 Hledání terapeutických biomarkerů Huntingtonovy choroby

v publikaci

Challenges of Huntington's disease and quest for therapeutic biomarkers

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REVIEW

Challenges of Huntington's disease and quest for therapeutic biomarkers

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Huntington's disease (HD) is the most common inherited neurodegenerative disorder among polyglutamine (polyQ) diseases caused by cytosine–adenine–guanine repeat expansion in exon 1 of the huntingtin gene whose translation results in polyQ stretch in the N-terminus of the huntingtin protein (HD protein). This mutation significantly affects huntingtin conformation, proteolysis, PTMs, as well as its ability to bind interacting proteins. As a consequence, a variety of cellular mechanisms such as transcription, mitochondrial energy metabolism, axonal transport, neuronal vulnerability to oxidative stress, neurotransmission, and immune response are altered and involved in the pathogenesis of HD. Promising candidate molecular biomarkers of HD have emerged from proteomic studies. Recent analyses focused on HD protein itself, its PTM, and interacting proteins, which are of great importance for disease course. Furthermore, brain, body fluids, and immune system are intensively studied in order to search for additional proteins with a view to their use as a biomarker(s) or set of biomarkers in clinical trials in HD translational research.

Keywords:
HD biomarkers / Huntington's disease / Huntingtin neurotoxicity / Huntingtin pathogenesis

1 Huntington's disease

Abnormal cytosine–adenine–guanine (CAG) triplet repeat extensions within coding region of various unrelated genes translated into polyglutamine (polyQ) tracts in respective proteins are a common mechanism of inherited neurodegenerative diseases, which may have similar molecular features [1]. Among nine such genetic disorders known to date, Huntington's disease (HD) is the most common and caused by CAG repeat expansion in exon 1 of the huntingtin gene (*HTT*) located on chromosome 4. Translation of this mutated gene results in polyQ stretch in the N-terminus of the huntingtin protein (HD protein), which is expanded beyond the normal polymorphism length that usually varies from 7 to 36 CAG repeats [2]. The length of the pathological CAG repeat in *HTT* correlates inversely with the age of onset of HD symptoms, however, with increasing onset age above 50 years the effect of the repeat length on onset age seems to diminish. The prevalence of HD is 5–10 per 100 000 and the average age of onset is 37 years [3]. The rate of HD pathogenesis leading to the clinical diagnosis of motoric disturbances is determined by dominant action of the longest expanded allele [4].

The people carrying the *HTT* mutation progressively develop the HD symptoms that are clinically presented by involuntary movement resulting from disturbances in muscle coordination (chorea), cognitive decline, psychiatric disturbances, and eventually dementia. Of interest are also multiple

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Abbreviations: **BDNF**, brain-derived neurotrophic factor; **CAG**, cytosine–adenine–guanine; **CREB**, c-AMP responsive element binding protein; **CSF**, cerebrospinal fluid; **DARPP32**, dopamine- and cyclic AMP regulated phosphoprotein; **HD**, Huntington's disease; ***HTT***, huntingtin gene; **MSN**, medium spiny neuron; **MRI**, magnetic resonance imaging; **PET**, positron emission tomography; **PGC-1α**, peroxisome proliferator activated receptor-γ coactivator 1α; **polyQ**, polyglutamine; **REST**, R element-1 silencing transcription factor

non-neuronal abnormalities such as weight loss, cardiac failure, and testicular atrophy [5]. Currently, there are no disease-modifying cures to slow or reverse the progression of HD and only palliative care is provided to suppress the symptoms. To date, only FDA (USA) approved tetrabenazine, indicated to reduce involuntary movements. Clinical trials in human HD to date have been aimed at improvement of cognitive and motor decline; nevertheless, application of antioxidants, creatine, anti-inflammatory and antiapoptotic antibiotics, or glutamate antagonists showed only limited efficacy [6]. From this point of view, the effects of multidisciplinary rehabilitation program in patients with early to middle stages of HD appear to be limited and partially beneficial with the observed reduction in the motor decline according to the Unified Huntington's Disease Rating Scale (UHDRS) classification, and body composition including fat mass, fat-free mass, as well as total mass [7]. Evidently, neuroprotective strategies need to be applied before the clinical symptoms are observed, possibly even before the time when neurodegenerative process in the brain begins, in order to delay the onset and slow down progression of HD [8]. Corresponding to this effort is the recent observation of early role of altered metal homeostasis indicating that metal modulation in premanifest disease stages has significant therapeutic potential [9]. Development of a novel drug that would complement general antioxidant and antiexcitotoxic drug strategies by targeting more proximal and typical mechanisms of the huntingtin pathogenesis such as proteotoxicity (inhibition of aggregation) or transcriptional dysregulation (HDAC inhibition) may significantly ameliorate mutant huntingtin neurotoxicity [10, 11].

2 HD models

The advances in developing effective disease-modifying drugs as well as HD biomarker identification critically depend on understanding of the mechanisms of HD pathogenesis at molecular level. With this view and knowledge of causal single gene mutation, it has been feasible to develop models of HD using genetic manipulations. Additionally, these models are crucial for testing the efficacy of novel therapeutic strategies before initiation of clinical trials in human. To date, several mammal HD models have been established and studied (Table 1).

2.1 Rodent models

Mouse models are most widely used due to fast breeding and low cost as well as possibility of longitudinal studies of disease progression via monitoring of the symptoms such as body weight and motoric dysfunctions. Types of mouse models include: (i) transgenic mice such as most often utilized R6/2 line carrying N-terminal region of the human mutant *HTT* with 150 CAG repeats [12], (ii) transgenic mice yeast artificial chromosome (YAC) or bacterial artificial chromosome (BACHD) expressing full-length human mutant *HTT* coding for 46 or 72 and 97 polyQs, respectively, and (iii) knock-in mice generated by replacing exon 1 of the murine *HTT* by exon 1 of the human *HTT* bearing CAG repeats expanded to 111 or 150 [13–15]. The R6/2 mice display progressive neurological phenotype presented by a tremor, motoric changes resembling chorea in human patient, and in some cases a mild ataxia manifesting as dysmetria. The R6 mouse HD model appears to be more like juvenile form of HD with early onset and fast progression suitable for the therapy development and drug testing rather than elucidating disease mechanisms. The other mouse HD models, except for BACHD, are characterized by late onset and course of the disease with milder symptoms that correspond to longer life compared to R6 model (Table 1). HD mouse models with more severe phenotypes usually show earlier accumulation of huntingtin aggregates and premature neuronal death.

The rat HD models display symptoms of late-onset form of HD with behavioral changes followed by progressive impairments of motoric coordination and balance, and by presence of huntingtin aggregates evident in the striatum and occasionally in the cortex at age of 18 months [16]. The advantage of rat model in contrast to mouse models is the possibility of in vivo repetitive studies using magnetic resonance imaging (MRI) or positron emission tomography (PET) owing to the brain size.

2.2 Large animal models

The implementation of large animal HD models offer several advantages including structural analogy of the main brain elements affected by HD, monitoring of disease progression by MRI or PET, increased toxicity of mutant HD protein, lifespan and rate of disease development similar to human patients, and slow progression, which enable possibilities to access early phases of HD development [17, 18].

Creation of sheep HD model by microinjection of a full-length human *HTT* cDNA containing 73 CAG repeats under the control of the human promotor was published in 2010. These sheep did not manifest any motor dysfunctions and were stable in three generations [19]. Analysis of offspring showed robust expression of the full-length human huntingtin in both CNS and non-CNS tissues. Furthermore, significant loss of cannabinoid receptor type 1 (CB1), which is responsible for suppression of excitatory presynaptic sites in the hippocampus and cerebellum, and dopamine- and cyclic AMP regulated phosphoprotein (DARPP32) found in striatal projection neurons was observed.

Porcine model was generated by microinjection of lentiviral vector carrying N-terminal truncated form of human mutant *HTT* encoding 124 glutamine tract integrated into chromosome 1q24-q25 with germ line transmission through successive three generations. The mutant huntingtin was detected at the level of RNA and protein in CNS as well as in peripheral tissues. Development and behavior of transgenic

Table 1. Overview of selected HD models in comparison with human

	Species	Onset	Lifespan (years)	Death	Sequence	CAG repeats	Reference
Rodent models	R6/2 mouse	9–11 wk	2	3 months	N-terminal, heterozygote	Approx. 150	[12]
	CHL2 mouse	>40 wk	2	>12 months	Knock in, heterozygote	150	[13]
	YAC72 mouse	24 wk	2	>12 months	Full length	72	[14]
	BACHD mouse	8 wk	2	>18 months	Full length	97	[15]
	YAC46 mouse	40 wk	2	>20 months	Full length	46	[14]
	Rat	40 wk	2.5	24 months	N-terminal	51	[16]
Large models	Sheep	28 wk	10–12	Unknown	Full length	73	[19]
	Pig	56 wk	15–20	Unknown	N-terminal	124	[20]
Patient	Human	ca. 37 years	ca. 75	ca. 55 years	Full length	Various	[3]

Many different animal models of HD have been developed in past years using different approaches. Selected models are listed together with the onset of the disease (appearance of the first symptoms), usual lifespan of the species, time of death of the model (if known, the highest age of experimental animals is shown), protein sequence present in the genome (N-terminal fragment or full-length huntingtin protein), and number of CAG repeats in the mutant allele.

and wild-type pigs appeared comparable without manifestation of any motoric dysfunction. Significant decline in the median number of spermatozoa was observed at the age of 13 months and the level of DARPP32 in transgenic minipig decreased after the age of 16 months. The important advantage of modeling HD in porcine model is the average litter size of about six to eight piglets including transgenic as well as wild-type siblings of the minipigs [20].

Disadvantage of sheep and porcine HD models is the lack of clinical symptoms to date, which would allow comparison with human patients. Hence, these models may reflect early presymptomatic stages and very slow disease progression. On the other hand, such conditions offer a good opportunity to study pathological mechanisms that precede onset of clinical HD symptoms.

2.3 Induced pluripotent stem cells (iPSCs) derived from HD patients

Despite the significance of stem cells and iPSCs for cell replacement therapies of neurodegenerative diseases, recent reprogramming technologies have introduced promising opportunities to understand the pathological pathways of neurodegenerative disease including HD. Using the cells derived from HD patients provides a unique human cell based HD model. Recently, 14 iPSC lines from HD patients and controls were established. Microarray profiling successfully distinguished patient lines from controls, and early versus late onset of HD [21]. This cellular disease model is capable of modeling the HD pathogenesis as well as providing a unique molecular view. It also allows designing of new treatment strategies for HD. Moreover, if to be used in cell replacement therapy, it is essential to correct mutation of the *HTT* in HD iPSCs as reported recently using the replacement of the expanded CAG repeat with a normal repeat via homologous recombination [22].

3 Pathways and biochemical mechanisms of HD pathogenesis

3.1 HD protein cleavage and aggregation

The main question to address is how polyQ tract expansion in proteins mediates the patterns of neuron's loss in CNS, which are overlapping for several polyQ disorders, despite the observable differences in pathogenetic pathways. The possible starting points in case of HD protein neurotoxicity are proteolytic cleavage of the HD protein and transition of mutated huntingtin into misfolded conformers followed by several aggregation intermediates such as oligomers, assembly of protofibrils and finally fibrils (Fig. 1). The transition model of aggregation corresponds to the hypothesis that aggregation of mutant protein fragment may be toxic as well as protective or compensatory response depending on the presence and interaction with molecular chaperones or components of ubiquitin–proteasome system [23, 24]. Although these proteins are supposed to remove misfolded mutant protein conformers, such process may not be efficient enough and it may be followed by autophagy, which may infact prolong neuronal dysfunction [25]. Ultimately, the presence of mutant huntingtin results in cell death mainly in vulnerable population of neurons [26]. Early in the disease process, GABAergic medium spiny neurons (MSNs) in striatum are targeted and neuronal loss usually proceeds till striatal atrophy [27]. Neurons also progressively degenerate in the cortex, hypothalamus, and hippocampus resulting in atrophy throughout the entire brain by terminal HD [28].

3.2 Transcriptional dysregulation and epigenetic regulation

The mutant HD protein is prone to interact with multiple proteins and interfere with many cellular functions, which may explain its pleiotrophic effects contributing to

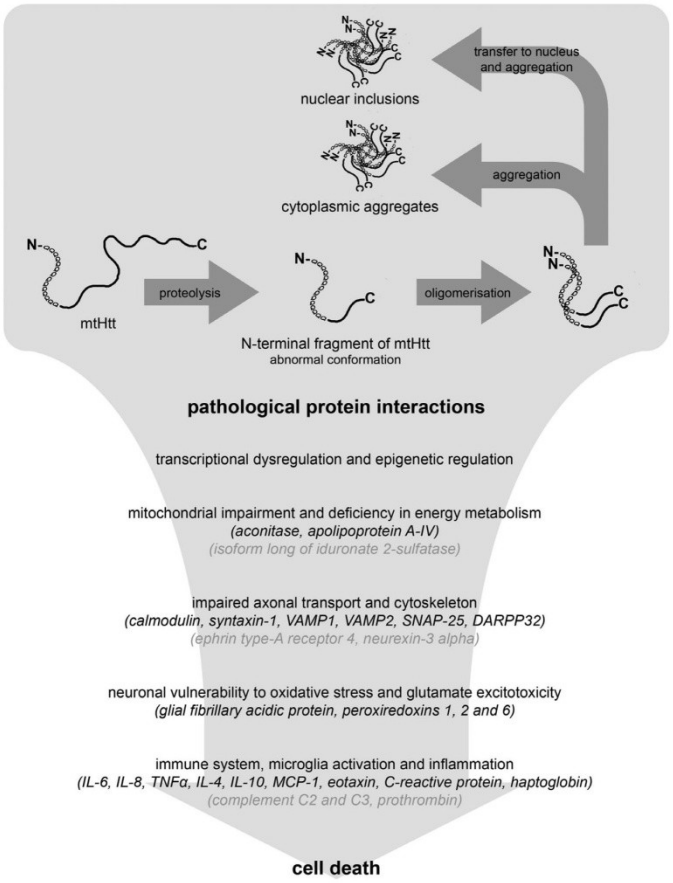

Figure 1. Schematic diagram depicting neurotoxicity, pathological processes, and protein involvement in HD. Understanding and characterizing neurotoxicological processes due to huntingtin protein misfolding and aggregate formation as well as the on-going pathological processes critical for HD disease is depicted. Overview of these processes including recently identified proteins affected by HD, which were validated by independent techniques (dark) and nonvalidated (gray), demonstrates the complexity of HD.

neurodegeneration (Fig. 2). Mutant huntingtin and N-terminal polyQ fragments accumulating in the nucleus are capable of interacting with a number of transcriptions factors involved in regulation of multiple genes such as specificity protein 1 (SP1), c-AMP responsive element binding protein (CREB), and transcription initiation factor TFIID subunit 4 (TAFII130). All these interactions lead to transcriptional dysregulation [29, 30]. It was shown that the suppression of CREB-dependent transcription and the cell death induced by polyQ stretches were restored by the coexpression of TAFII 130 [31]. Interestingly, CREB possesses intrinsic histone acetyltransferase activity resulting in epigenetic regulation via chromatin remodeling mediated by suppressed histone acetylation [32]. Hence, inhibition of histone deacetylases may be a compensatory mechanism of transcriptional dysfunction preventing neuronal cell death. These findings were corroborated by observation that H2A histone family member Y (H2AFY) is specifically overexpressed in the blood and frontal cortex of patients with HD compared with controls and histone deacetylase inhibition suppressed neurodegeneration in animal models as well as reduced H2AFY levels in a randomized phase II clinical trial [33]. Mutant huntingtin also affects the expression and regulation of noncoding microRNAs. Multiple neural miRNAs are controlled by R element-1 silencing transcription factor (REST), a transcriptional repressor of neuronal survival factors including brain-derived neurotrophic factor (BDNF). While interaction of wild-type huntingtin with REST sequesters this factor in cytosol thus allowing transcription of BDNF, mutant huntingtin traps REST into nucleus where it represses BDNF transcription [34].

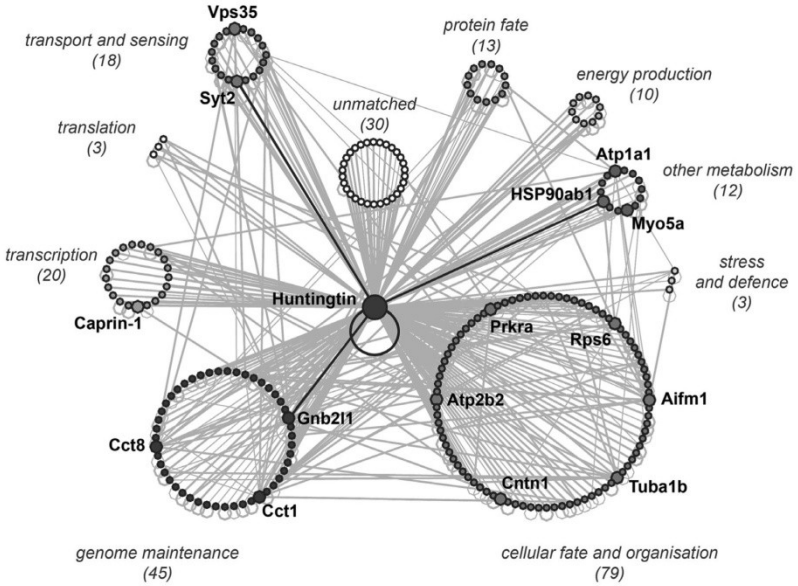

Figure 2. Huntingtin protein interaction network. Computer modeling of possible interactions of huntingtin protein shows a high number of protein interactions. The interacting proteins were retrieved by Interologous Interaction Database I2D version 2.3 search (http://ophid.utoronto.ca/ophidv2.204/) and visualized using NAViGaTOR version 2.2.1 software (http://ophid.utoronto.ca/navigator/). In the network, nodes represent proteins and edges between nodes represent physical interactions between proteins. A total of 233 proteins directly interacting with huntingtin were found where majority of them play a role in cellular fate and organization, genome maintenance, and transcription. For each protein functionality group, the number of such directly interacting proteins is given in parentheses. The proteins identified and verified in recent proteomic studies are highlighted by abbreviated names.

3.3 Mitochondrial impairment and deficiency in energy metabolism

The studies on HD patient samples documented significant decrease in enzymatic activities of the mitochondrial complexes of oxidative phosphorylation in caudate and putamen as well as aconitase in striatum [35], which indicated defective energy metabolism in HD patients. Another relevant aspect is mitochondrial membrane depolarization as a consequence of mitochondrial calcium defects mediated by polyQ, which activates caspases and results in cleavage of huntingtin. Hence, this process may contribute to initiation of huntingtin-mediated neurotoxicity [36]. It is now well recognized that there is a link between mitochondrial deficits and transcriptional dysfunction where peroxisome proliferator activated receptor-γ coactivator 1α (PGC-1α), highly expressed in brain, can regulate expression of key mitochondrial genes. Importantly, PGC-1α knockout mice provided evidence of the striatal neurodegeneration [37]. Microarray analyses of PGC-1α target genes showed significant decrease of majority of these targets indicating their crucial role in HD disorder and degeneration [38].

3.4 Impaired axonal transport and cytoskeleton

While neuronal cell body ensures gene transcription and protein translation, long axons transfer electrical signals and mediate anterograde or retrograde axonal transport of many proteins and other components or vesicles essential for synapses. These transport processes are supported by cytoskeletal proteins, where kinesin protein family participates in anterograde direction while dyneins support retrograde orientation. Disruption of anterograde transport results usually in cargo accumulation, neuronal disfunction and degeneration [39]. In HD, binding of huntingtin-associated protein 1 to BDNF-vesicles mediates interactions of huntingtin with $p150^{Glued}$, component of cytoplasmic microtubule based motor protein dynein, and facilitates transport of the vesicle along axon. BDNF transport is attenuated in the presence of mutated huntingtin but also when the level of wild-type huntingtin is decreased. The alternative explanation of impaired axonal transport may be steric restriction of the axonal transport due to the accumulated autophagosomes that are blocked and unable to mature leading to autophagy dysfunction [40].

3.5 Neuronal vulnerability to oxidative stress and glutamate excitotoxicity

The evidence of significantly increased levels of oxidative damage products in areas of degeneration in HD brain and elevation of reactive oxygen and nitrogen species in animal models clearly indicate contribution of such factors to the pathogenesis of HD [41]. Other studies in HD postmortem tissue and transgenic mice models suggested a correlation between mutant huntingtin expression and altered glutamatergic neurotransmission. The MSNs, which receive both glutamate signals from the cortex and dopamine signals from the substantia nigra, are selectively vulnerable to the toxicity of glutamate (excitotoxicity) and has been recognized as a causal event of neurodegeneration associated with HD. Accordingly, knockout of dopamine transporter increases dopamine level as well as increases loss of MSNs while attenuation of glutamate activity restores MSNs activity [42].

3.6 Immune system, microglia activation, and inflammation

Immune activation induced by mutant huntingtin is found ubiquitously and may cause parallel inflammation in CNS, in the periphery, as well as at molecular level. In CNS, mutant huntingtin affects migration of cells and activates microglia. Reactive microglia express higher level of their surface protein, [43]secrete proteases, release toxic reactive oxygen and nitrogen species and pro-inflammatory cytokines such as IL-6, IL-1β, IL-8, and tumor necrosis factor α (TNFα) [43, 44]. A study of postmortem HD brains showed that the presence of reactive microglia was significant in most damaged regions, notably striatum and cortex [45]. Furthermore, in vivo imaging indicated that microglia activation appeared in the brains of presymptomatic HD subjects [43, 46] and that microglial reactivity consistently increased with the disease severity [47]. Migration deficits affect innate immune response in periphery, and activation of the nuclear factor kappa-B signaling pathway is typical feature at cellular level responsible for IL-6 triggering [48].

Microglial abnormalities were shown to be associated with activation of complement system in HD human [49]. The complement system is known as important part of the innate and is hyperactivated in several neurodegenerative diseases [50]. In HD, the complement may be activated by mutant huntingtin [50], and a study using RT-PCR discovered that the classical complement pathway components as well as the complement inhibitors and membrane inhibitors were expressed in HD brains in much higher levels compared to controls. In situ hybridization analysis confirmed that reactive microglia expressed elevated levels of C3 and C9 [49]. Neurotoxic or neuroprotective molecules released by reactive microglia influence neurons and glial cells, which initiate highly complex mutual interactions.

3.7 Relationship and functions of normal and mutated HD proteins

In relation to the HD pathological mechanisms, it is useful to consider distribution and functions of huntingtin, which is ubiquitously expressed in a variety of peripheral cells although high levels are reached in neurons and glial cells in CNS [51, 52]. Huntingtin plays an antiapoptotic role as exemplified by protection of the neurons against excitotoxicity [53] and via interactions mediated exclusively by its N-terminal region is involved in vesicle transport, namely synaptic transmission, as shown by its disruption when *HTT* is mutated [54]. It is also well recognized that huntingtin regulates transcription of BDNF [34]. These functions are often affected by the presence of mutated huntingtin, resulting in lower level of huntingtin and "loss of function" effect often coupled to "gain of function" effect of mutated huntingtin itself as well as its N-terminal fragments and their aggregates [55].

4 Diagnostic and prognostic markers as a handle in HD

Medical diagnosis of the onset of HD can be made following the appearance of physical symptoms specific to the disease. As there is a known genetic cause of HD, the confirmation of the disease is possible by genetic testing, namely if there is no family history of HD. Majority of possible familial mutant *HTT* gene carriers are reluctant to be tested for various reasons such as psychology, career, family planning, as well as lack of disease-modifying treatment availability. Hence, genetic testing often follows the development of typical clinical features such as chorea [56].

To assess and quantify the progression of HD, several standardized clinical tests together with rating scales have been developed to measure different aspect of HD phenotype. Four main domains affected by HD can be quantified by UHDRS [57], possibly coupled with some other quantitative clinical, neuroimaging, and biochemical measurements [58]. This scale is widely used for monitoring disease progression in the patients and long-term monitoring of large gene-carrier cohorts in multicentric studies [59] (http://www.enroll-hd.org/html/about). The MRI is used to record striatal atrophy even 15–20 years before disease onset or PET to measure metabolic changes in brain tissue [60]. Regarding the limited capacity of clinical trials for testing potential compounds as well as at-risk and affected individuals progressing toward clinical HD disability, the development of a novel biomarkers for possible use in clinical trials for HD would help to generate disease-modifying treatments that could delay the onset or even reverse the disease progression [58, 61]. The ideal biomarker combining the positives of both neuroimaging and clinical tests (high throughput, high sensitivity, low cost) may be considered as biochemical marker [58].

Among such potential candidates was BDNF whose expression as well as secretion was regulated by huntingtin, and reduced BDNF serum concentration was reported in HD patients as well as BDNF administration showed disease phenotype improvement in mouse HD model [62]. Unfortunately, its failure was due to not being informative or reliable enough as a biomarker in blood as its levels were greatly affected by high intraindividual variation and time between blood collection and plasma preparation [63]. Various nonprotein molecules have been previously proposed as possible biomarker candidates, such as 8-hydroxy-deoxyguanosine [64], 24S-hydroxycholesterol [65], and other small molecules (overview in [60]), but none of them have been validated so far.

5 Proteome analyses focused on huntingtin and its protein interactions

HD protein is a large 350 kDa protein with glutamine stretch adjacent to proline-rich region at the N-terminus of its sequence. This part is followed by four clusters of HEAT repeats. These N-terminal regions are important targets of protein–protein interactions, hence huntingtin acts as a scaffolding protein interconnecting many different proteins and their biological cellular functions and locality (Fig. 2). Additionally, HD protein is rich in a variety of PTMs such as phosphorylation, SUMOylation, ubiquitination, acetylation, and palmitoylation, where some of them are significantly affected by HD protein mutation, which is often associated with phenotype changes [66]. Using striatal HEK293 cells expressing tagged huntingtin containing wild-type and mutant repeats and 2DnanoLC MS/MS, new phosphorylation sites located at Ser 431 and Ser 432, and ubiquitination site located at Lys 444 were identified. Phosphorylation at Ser431 increased the rate of huntingtin aggregate formation and reduced the cell viability, while phosphorylation at Ser432 had the opposite effect indicating impact of both phospho-sites to final outcome of HD phenotype [67].

Furthermore, proteolysis of mutant huntingtin precedes formation of toxic oligomers of the N-terminal fragments. Recently, the ability of prefoldin, a molecular chaperone preventing misfolding of nascent polypeptides, to protect neuronal cells from polyQ toxicity was reported [68]. Knockdown of two of six prefoldin subunits disrupted prefoldin formation in huntingtin-expressing cells and resulted in induction of cell death and accumulation of soluble oligomers [68].

Huntingtin mutation can also affect its ability to bind with possible interacting partners. Quantitative analysis of the differences between the ability of wild-type and mutant huntingtin to interact with other proteins may shed light on many aspects of mechanisms of HD pathogenesis. Ratovitski et al. [69] generated striatal HEK293FT cell lines transfected with plasmids bearing N586 terminal fragment of huntingtin containing various CAG repeats and applied affinity purification coupled to iTRAQ quantitative MS. This study revealed that the proteins related to energy production, protein trafficking, RNA post-transcriptional modifications, and cell death were significantly enriched interacting proteins of expanded huntingtin. Among them, the presence of stress granule associated RNA-binding protein Caprin-1 indicated a novel role of mutant huntingtin in RNA processing and regulation of translation. In another study, Culver et al. [70] analyzed huntingtin affinity-purified complexes from mutant juvenile mouse brain by MS/MS. Interestingly, this study reported a novel huntingtin-interacting protein, synaptotagmin-2 (Syt2), which may have a regulatory role in the membrane interactions during trafficking of synaptic vesicles; unconventional myosin-Va (Myo5a) involved in protein transport and also mediating the transport of vesicles to the plasma membrane and required for some polarization process of dendrite formation; another two proteins interferon-inducible double-stranded RNA-dependent protein kinase activator A (Prkra) and guanine nucleotide binding protein subunit beta-2-like 1 (Gnb2l1) involved in translational regulation and apoptosis; and ribosomal protein S6 (Rps6). Furthermore, data emerging from the experiments focused on cosedimentation with polysomes and cap-dependent protein translation provided evidence for a new role for huntingtin in protein translation with a greater focus on pathogenesis of HD. Accordingly, *Drosophila* huntingtin knockout flies showed a reduced axonal transport of synaptotagmin vesicles in motoneurons in vivo [71]. Study by Shirasaki et al. [72] presented systematic view of spatiotemporal analysis of huntingtin-interacting proteins in the mammalian brain regions and ages in HD by combining affinity purification, MS, and weighted gene correlation network analysis for data modeling. One of the emerging modules in the interactome had huntingtin as its member, and also contained several novel huntingtin interacting proteins involved in 14–3–3 signaling, microtubule-based transport, and proteostasis. Importantly, top proteins in this module such as 14–3–3 epsilon, Vps35, Tcp1/Cct1, and HSP90ab1 were validated as novel disease modifiers in HD *Drosophila* model. The proteins identified in such proteomic studies and validated using independent orthogonal technique might be promising targets for novel pharmacological or gene/protein targeting treatment strategies.

As HD is primarily caused by a single protein change, the mutant huntingtin seems an ideal target not only for disease-modifying therapy through gene silencing, but also for monitoring of such therapy. As one of the differences between normal and mutant protein is elongation of polyQ chain in the sequences, the development of precise and robust assay techniques is of great interest. Recently, several methods with possible wider use in the future have been reported using various technologies such as ELISA [73], time-resolved fluorescence resonance energy transfer [74, 75], or Meso Scale Discovery platform [76] to quantitate wild-type and mutant huntingtin in different cell types. The relationship between mutant huntingtin levels and disease progression is still to be fully elucidated.

6 Brain proteome analyses in HD

As brain pathology is the hallmark of HD, it remains a research focus although obtaining human samples is complicated and only samples from terminal stage of the disease are available. Sorolla et al. [77] compared the protein profiles of striatum and cortex of eight pairs of HD patients and age- and gender-matched controls to reveal that many differences found in the striatum were reflected to a lesser extent in cortex. First, increased expression as well as high carbonylation of intermediate filament glial fibrillary acidic protein indicated extensive gliosis and oxidative stress. Additionally, decreased level and extensive carbonylation of aconitase, which is a mitochondrial enzyme susceptible to oxidative stress, lead to significant suppression of its enzymatic activity [77]. Increased expression of peroxiredoxins 1, 2, and 6, indicator of cellular response to oxidative stress, was also reported in this study.

The investigations performed on knock-in $Hdh^{140Q/140Q}$ mouse model showed that striatal synaptosomes from 6-month-old animals contained significantly lower level of DARPP32, selective marker of striatal MSNs neurons. At the age of 12 month, level of DARPP32 declined progressively, which reflected reduction of MSNs or processes belonging to them. However, DARPP32 was the only one case where decreased level was consistent with the disease progression [54]. On the other hand, level of calmodulin, protein critical for synaptic vesicle mobilization and neurotransmission, reduced temporarily in 6 months but was not changed in 12 months. The similar expression was observed for syntaxin-1, protein essential for vesicle fusion. Other proteins involved in processes of vesicular formation and fusion were also decreased (VAMP1, SNAP-25), with exception of VAMP2, which was increased. All these findings might point to compensatory and regenerative events at the synaptic level in HD brain in order to restore early synaptic dysfunction [54]. Accordingly, many other proteins of energy and glutamate metabolism and proteins of exo- and endocytosis were dysregulated in brain of R6/2 mice in the course of disease without any significant differences between early and late stages of the disease. Based on these findings, gradual linear changes in protein concentrations with disease progression do not necessarily occur [78] and HD is characterized by a highly dynamic disease pathology.

7 Body fluid proteome in HD

Blood plasma or serum that can be obtained with minimal invasiveness presents an ideal source of protein biomarkers for in-depth studies. For neurological disorders, cerebrospinal fluid (CSF) appears a promising source of candidate biomarkers as it is an extracellular fluid of the brain and spine [79]. CSF has about 200× protein lower concentration than plasma depending on the sampling site. The CSF is constantly reabsorbed and drained into the blood and it dilutes the concentration molecules penetrating the brain and CSF [80]. However, a large part of the proteins identified in CSF were also found in human blood plasma [81] and there is an assumption that during neuroinflammation and neurodegeneration, the blood–brain barrier becomes more permeable [82] and some potential biomarkers might be easily transferred from CSF to blood plasma and accessed. Although obtaining human biological samples is often complicated, the multicentric longitudinal studies such as Track-HD [59] or Enroll-HD (http://www.enroll-hd.org/html/about) are utilized to create biobanks of body fluids for possible biomarker discovery research and validation studies. Elevated levels of proinflammatory cytokines and chemokines were detected in HD human plasma samples with significantly increased levels of IL-6 and IL-8 in premanifest subjects and likely corresponding to the innate immune response. Upregulation of TNFα was detected later in manifest stages. In addition, anti-inflammatory cytokines IL-4 and IL-10 increased significantly with disease progression compared to controls and represented adaptive immune response [43]. The chemokines, mainly MCP-1 and eotaxin, have increased across advancing disease stages in correlation with HD progression [83]. Recently, critical evaluation of several components of innate immune response and inflammation was undertaken with a view to utilizing them as prognostic or diagnostic biomarker(s) in HD. This study applied either antibody-based ELISA and Luminex bead array technology or MRM MS to analyze plasma samples of control subjects and genetically diagnosed HD patients. The results showed that the levels of several components of complement system including C4, C9, and clusterin, previously described as potential alterations in HD, as well as acute phase alpha-2 microglobulin did not differ between two studied groups. The only one protein, C-reactive protein decreased in early HD, was significantly different and this alteration was confirmed by ELISA as well as MRM MS. Interestingly, some of the innate immunity markers as well as acute phase proteins, which were not significantly different, revealed correlation with clinical criteria in premanifest or early HD stages [84].

Probably the largest CSF study in HD patients so far included five different labs with various approaches analyzing the same set of 30 CSF samples (10 controls, 10 early HD, and 10 moderate HD) and revealed a tendency of several brain-specific proteins (ephrin type-A receptor 4 precursor, isoform long of iduronate 2-sulfatase precursor, and neurexin-3-alpha) to decline during HD progression, while blood-specific proteins (apolipoprotein A-IV) or associated with immune response (such as complement factors C2 and C3) had the opposite trend hinting at possible blood–brain barrier desintegration [85]. Although this study provided the largest HD CSF protein dataset to date, it has several drawbacks including large variation between laboratories and techniques and no validation studies using independent methods in each laboratory, even if the samples were depleted of the most abundant proteins that might also affect the levels of less-abundant

proteins. The increase of apolipoprotein A-IV together with the increase in prothrombin and haptoglobin levels was reported in a following study where no depletion technique was used and the results were validated using Western blotting, although the normalization of target protein level to albumin might be questionable. The increased levels of haptoglobin in CSF were confirmed by ELISA, while no significant difference of its level in serum was evident [86].

8 Proteome analyses of immune cells in HD

Several studies provided evidence that non-neuronal cells might play important role in HD progression. The immune dysfunction was recognized in peripheral blood monocytes from premanifest HD gene carriers. Monocytes from HD subjects expressed mutant huntingtin and were pathologically hyperactive in response to stimulation with LPS [43]. The cytokine production was analyzed in vitro using ELISA or Luminex bead array technology and results showed higher IL-6 level produced by stimulated monocytes from HD patients compared to monocytes from controls. The macrophages and microglia isolated from HD mouse models demonstrated similar reaction to stimulation with elevated level of IL-6 in transgenic HD animals [43]. These studies suggested that peripheral inflammatory changes mirrored the changes occurring in microglial cells in the brain.

Peripheral myeloid cells and also microglia in the CNS undergo morphological changes and migrate to the sites of injury due to the chemoattractants released here. In HD, mutant huntingtin impairs immune cell migration to chemotactic stimuli mainly because of defective actin remodeling and such deficits negatively influence release of cytokines and chemokines and also activation of microglia, which may result in chronic neuroinflammation and finally neurodegeneration in HD [87].

9 Conclusion

HD is an inherited autosomal dominant condition with onset decades before any clinical symptoms are seen in humans, hence a better understanding of the earliest changes in brain, neuronal, and non-neuronal cell functions, and the molecular pathways underlying those changes, could lead to preventive or disease-modifying therapies. Investigation and modeling of the key proteins and interactions involved in HD could provide invaluable information for identifying targets to treat the disease by focusing on specific functionalities or pathogenic pathways in HD (Figs. 1 and 2). Promising candidate biomarkers have been identified by proteomic approaches, and need further screening expression analyses, evaluation of sensitivity, specificity, and dynamic range before they are ready for use. HD protein is one such biomarker candidate as many therapeutic approaches are focused on gene therapy to target mutant allele or RNA, however, measurement of wild-type huntingtin needs parallel studies with respect to its endogenous functionalities. Furthermore, understanding the interconnectivity of HD protein with other proteins and pathways could enhance its functionality as a biomarker. Additionally, not only a single biomarker, but set(s) of molecules reflecting distinct pleiotropic effects of mutant as well as endogenous wild-type huntingtin and HD pathogenesis as well as disease progression is urgent priority for future HD translational research.

The gene mutation causing HD appears in every cell in the body, yet kills only selected vulnerable types of brain cells. Using unique approaches to switch off the *HTT* in individual brain regions to prevent cell death and home in on those that are interconnected and play a role could shed light on where HD starts in the brain and could point to new targets and pathways for therapeutic drugs to slow the devastating process and build upon new findings to better understand this disease.

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5 Souhrn

5.1 Monitorování neurální diferenciace pomocí cílené hmotnostní spektrometrie

Hlavním cílem této dizertační práce byla charakterizace NSC, které představují slibný prostředek v buněčné terapii neurologických poruch. Klinické testování buněčné terapie onemocnění již probíhá a vyžaduje podrobnou a přesnou analýzu NSC před transplantací, stejně jako další zdokonalení protokolů pro jejich diferenciaci poskytující homogenní populaci cíleně indukovaných buněk.

ESC nebo iPSC jsou schopné za specifických podmínek *in vitro* diferencovat do NSC a ty dále do liniově specifických buněk. NSC kultivované *in vitro* vyžadují přítomnost růstových faktorů FGF-2 a/nebo EGF pro udržení multipotence a neurogenní účinnosti. Odebrání růstových faktorů nebo jejich výměna za neurotrofní faktory BDNF a GDNF či jiné supplementy vede k jejich diferenciaci[100,101]. Existuje mnoho kultivačních a diferenciačních protokolů. My jsme pro kultivaci multipotentních H9 NSC používali růstové faktory FGF-2 a EGF, pro spontánní diferenciaci do neuronů jsme tyto faktory z média odebrali bez náhrady za jiné (S diferenciace) nebo jsme je vyměnili za BDNF a/nebo GDNF (BG, B, G), pro diferenciaci do astrocytů jsme použili FBS (Astro1). Jako referenční buňky jsme použili lidské komerčně dostupné astrocyty indukované pomocí N-2 supplementu (Astro2) a ESC linie CCTL-12 (ESC1) a CCTL-14 (ESC2).

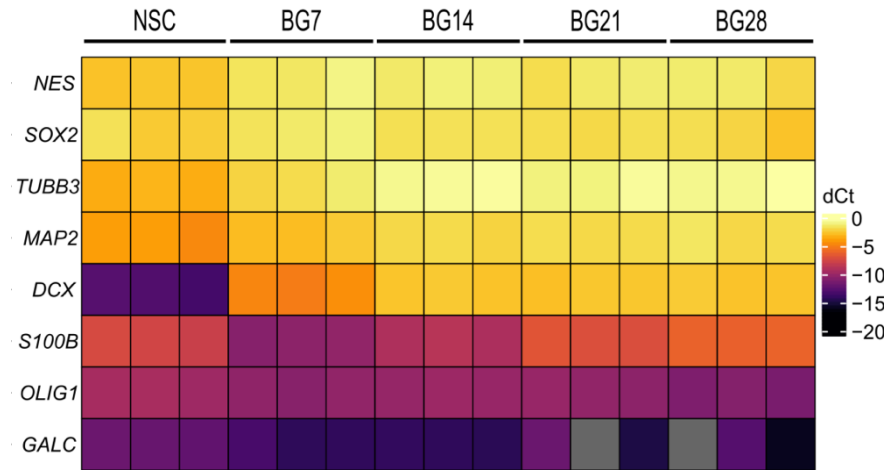
Protože diferenciační protokoly často generují heterogenní populace buněk, je nezbytné průběžně monitorovat nejen diferenciační potenciál, ale také čistotu populace. Extrémně důležité je zamezit vzniku populace s příměsí pluripotentních buněk s potenciálem ke vzniku nádorů. Za tímto účelem jsme usilovali o vývoj screeningového testu založeného na reprodukovatelném měření vybraných proteinových markerů pomocí cílené metody SRM se snadnou interpretací výsledků.

Použití SRM umožňuje v krátkém čase analyzovat několik desítek proteinů najednou a přestavuje tak rychlý, účinný a přesný nástroj k monitorování kultur NSC v průběhu diferenciace. Takový test lze použít jak k monitorování čistoty a potenciálu diferenciace NSC, tak k identifikaci optimálních kultivačních podmínek. V naší publikaci jsme představili

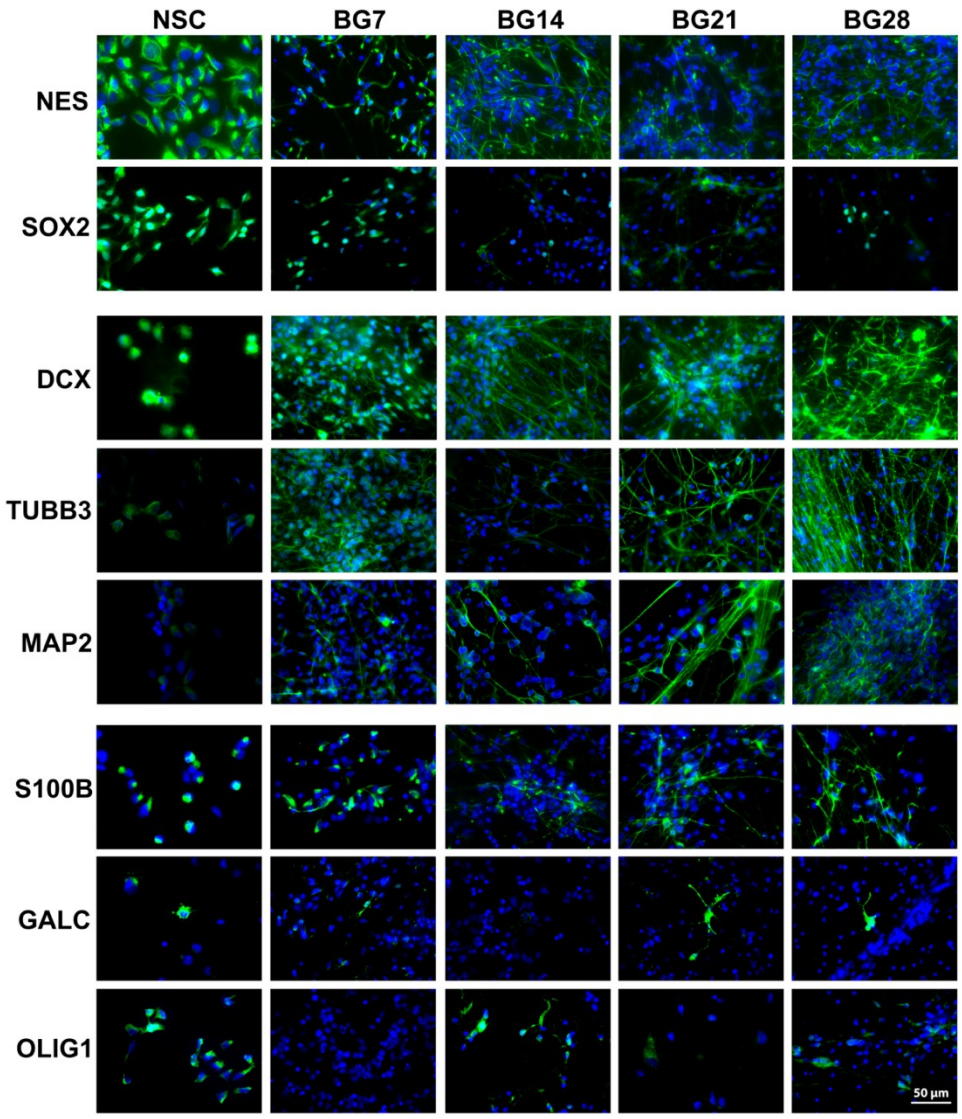
test SRM pro kvalitativní a kvantitativní měření hladin proteinových markerů rutinně používaných k charakterizaci neuronální diferenciace.

Při vývoji metody jsme se zaměřili na soubor proteinů hojně využívaných v biologických studiích jako markery ESC (NANOG, OCT4), markery NSC (SOX2; NES, *nestin*; PAX6, KI67), markery neuronů (DCX, TUBB3, MAP2), markery astrocytů (GFAP, S100B) a markery oligodendrocytů (GALC, OLIG1). Zkoušeli jsme také detekovat v buňce méně zastoupené proteiny dříve nalezené v našich diferenciačních experimentech jako sekretované v průběhu diferenciace NSC, konkrétně VEGF-A a CXCL1. Pro vývoj SRM jsme použili komerčně dostupné lidské NSC linie H9, které jsme kultivovali a následně diferencovali za specifických podmínek detailně popsaných v přiložené publikaci. Sledovali jsme hladinu vybraných markerů v průběhu neuronální diferenciace (S, B, G, BG), při modulaci NSC směrem k astrocytům (Astro1) a v referenčních buňkách (Astro2, ESC1, ESC2). Poté jsme se zaměřili na BG diferenciaci a pomocí SRM jsme měřili hladinu validovaných proteinů v intervalech 1-8, 14, 21 a 28 dnů.

Identitu proliferujících NSC a diferencujících buněk jsme popsali nejprve pomocí RT-qPCR (na úrovni mRNA, obr. 8) a IF mikroskopie (na úrovni přítomnosti proteinu, obr. 9). Jak jsme očekávali, naše data z IF a RT-qPCR prokázala v průběhu diferenciace zvýšenou expresi neuronových markerů (DCX, MAP2, TUBB3) a sníženou expresi NSC markerů (NES, SOX2). Exprese markerů astrocytů, S100B a GFAP, výrazně klesla po zahájení diferenciace. Klesala také exprese markerů oligodendrocytů, GALC a OLIG1. IF vyloučila přítomnost OCT4 a GFAP v multipotentních NSC. Prokázala však v NSC na proteinové úrovni přítomnost S100B (pozitivita části buněk přetrvávala po celou dobu diferenciace), GALC (sporadická pozitivita) a OLIG1 (počet pozitivních buněk klesal s diferenciací). U všech proteinů odpovídal signál jejich předpokládané lokalizaci.

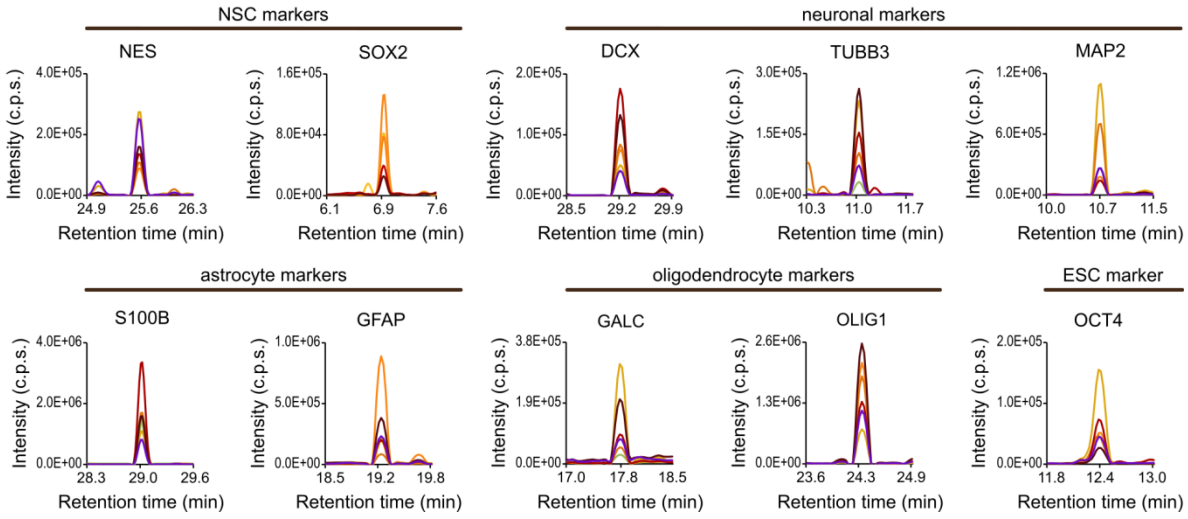


Obr. 8. Analýza genové exprese během diferenciace NSC. Mapa ukazuje profily exprese (hladinu transkriptů) neurálních markerů v průběhu diferenciace *in vitro* (převzato a upraveno podle publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).



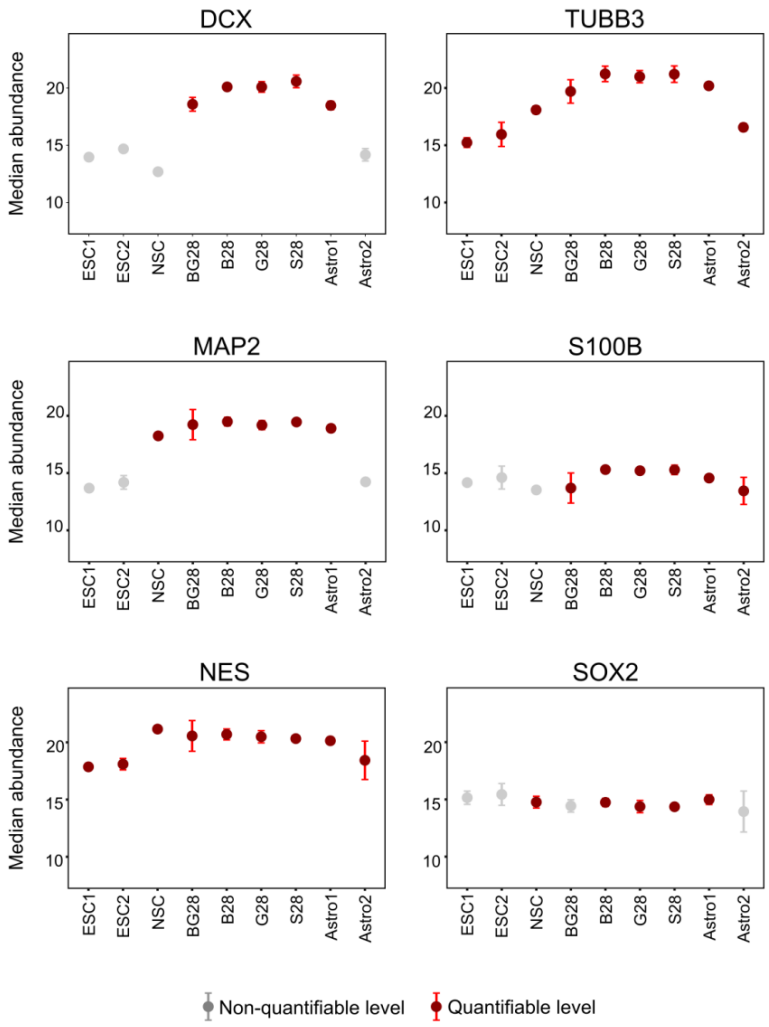
Obr. 9. IF snímky diferenciace BG ukazují proteinové markery zeleně, buněčná jádra modře (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

Vybrané proteiny jsme podrobili analýze SRM s využitím syntetických izotopově značených peptidů (obr. 10). Z původních 15 kandidátních proteinů zůstalo ve finální metodě proteinů 10. Protein NANOG byl z dalších analýz vyloučen, protože neposkytoval po štěpení trypsinem žádný peptid unikátní v lidském proteomu. Pro jiné proteiny se nám podařilo vyvinout specifické testy SRM pomocí izotopově značených peptidů, zůstaly však pod limitem detekce v našich buněčných kulturách (PAX6, KI67, VEGF-A a CXCL1). Validovanou metodou SRM byly změřeny NSC ovlivněné působením různých diferenciačních faktorů a NSC v různých stadiích BG diferenciace (dny 1-8, 14, 21 a 28). Na základě naměřených dat jsme provedli kvalitativní i kvantitativní vyhodnocení cílových proteinů.



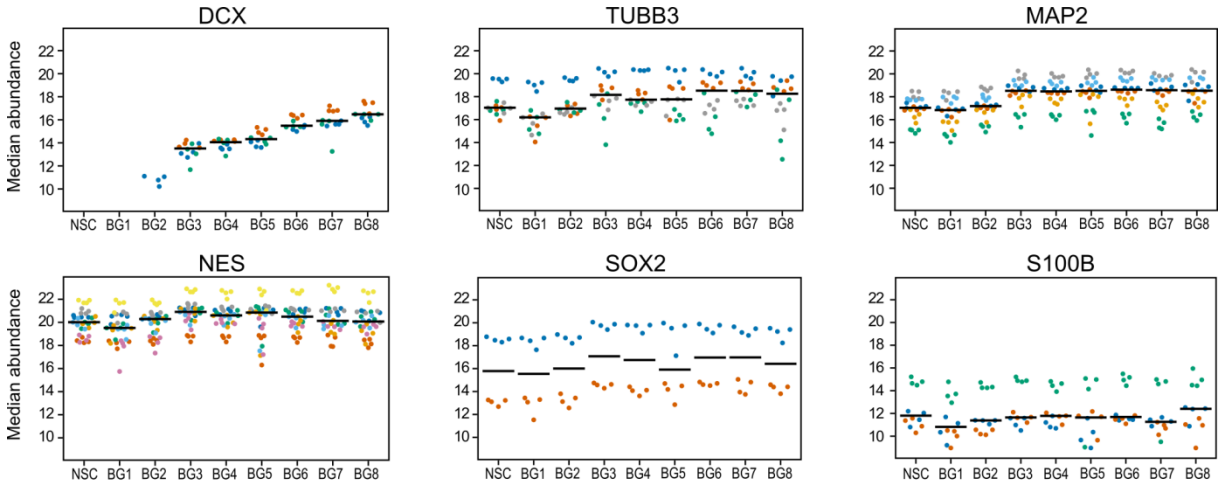
Obr. 10. Reprezentativní standardní peptidy použité pro vývoj metody SRM (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

Všechny diferenciace NSC (S, BG, B a G diferenciace) vedly preferenčně k neuronální diferenciaci (obr. 11), což odpovídalo výsledkům RT-qPCR a IF mikroskopie. K neuronální populaci místo do populace astrocytů směrovaly také NSC odvozené z linie ESC H9 pomocí FBS (Astro1). Tento druh modulace vedl u NSC navíc k detekci OCT4, markeru pluripotentních ESC, a tedy k jejich možné dediferenciaci.



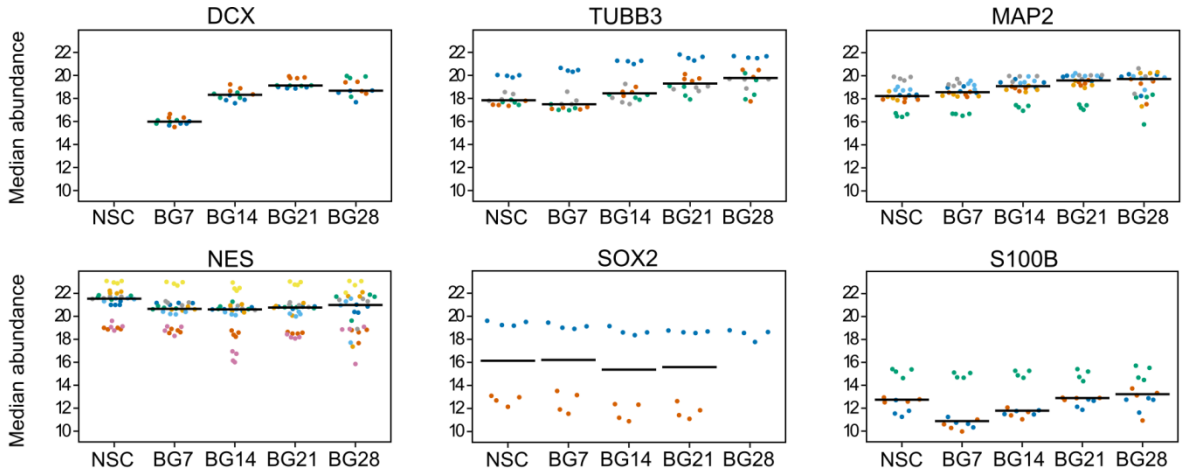
Obr. 11. Monitorování diferenciace NSC pomocí metody SRM. Grafy zobrazují medián množství proteinů ve 4 biologických replikách. Červená barva znamená, že protein byl za těchto podmínek kvantifikovatelný (byly detekovány minimálně 2 peptidy na protein v minimálně 3 biologických replikách), šedá barva znamená, že množství proteinu bylo v této podmínce pod kvantifikační úrovní (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

Neurotrofická podpora pomocí kombinace BDNF a GDNF vedla u NSC k vyšší variabilitě než v případě S, B a G diferenciace. Marker neuronálních prekurzorů DCX se stal detekovatelným už druhý den BG diferenciace a výrazně narůstal v diferencujících buňkách, zatímco MAP2 a TUBB3 rostly od 3. dne (obr. 12). Hladina markerů NSC (NES a SOX2) začala mírně klesat od 4. dne BG diferenciace (obr. 12). Proteiny GFAP (marker astrocytů) a OCT4 (marker pluripotentních ESC) nebyly v časné fázi BG diferenciace detekovány, markery oligodendrocytů vykazovaly pouze slabý signál (nevyobrazeno). Hladina markeru astrocytů S100B se v průběhu časné fáze diferenciace neměnila (obr. 12).



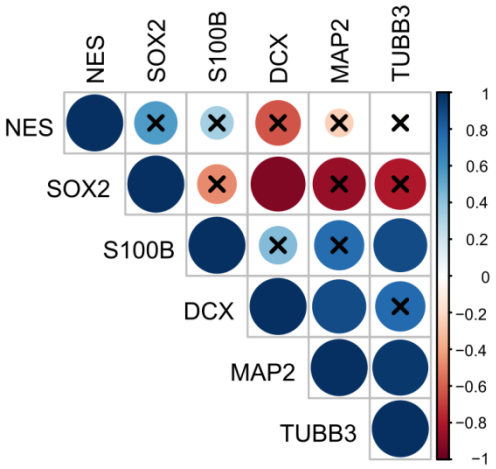
Obr. 12. Monitorování časné diferenciace NSC. Grafy zobrazují median množství (černé čáry) každého proteinového markeru. Tečky stejné barvy představují množství konkrétního peptidu v biologických replikách (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

V pozdější fázi BG diferenciace klesala hladina proteinu SOX2 a 28. den byl detekován pouze jeden peptid tohoto proteinu (obr. 13). Hladina NES se s další diferenciací příliš neměnila (obr. 13). Hladina markeru neuronálních prekurzorových buněk DCX výrazně rostla do 21. dne BG diferenciace, hladina MAP2 a TUBB3 mírně rostla v průběhu celé BG diferenciace (obr. 13). OCT4, marker pluripotentních ESC, se při BG diferenciaci neobjevil, markery oligodendrocytů byly detekovány pouze slabě a marker astrocytů GFAP byl detekován až 28. den (nevyobrazeno). Hladina S100B, druhého markeru astrocytů, při BG diferenciace nejprve klesla a postupně se vrátila na původní úroveň (obr. 13).

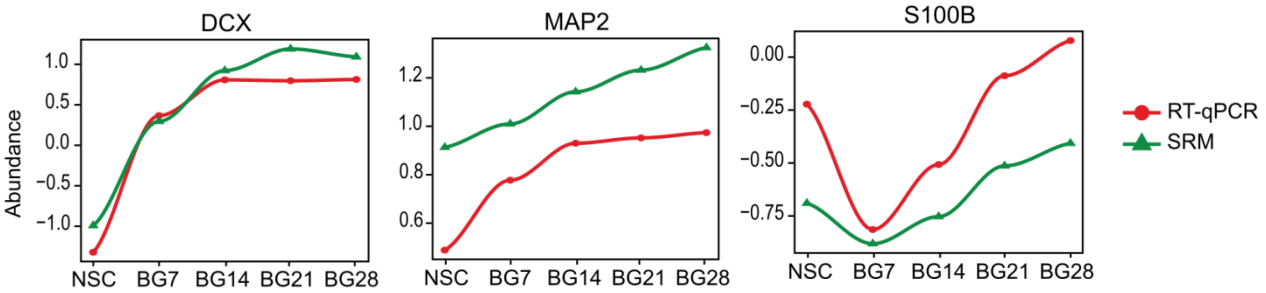


Obr. 13. Monitorování pozdější fáze diferenciace NSC. Grafy zobrazují median množství (černé čáry) každého proteinového markeru. Tečky stejné barvy představují množství peptidu v biologických replikách (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

V průběhu BG diferenciace byla v rámci SRM prokázána významná pozitivní korelace mezi hladinou proteinu MAP2 s TUBB3 a DCX, a mezi TUBB3 a S100B. Negativní korelace byla významná mezi hladinou proteinu SOX2 a DCX (obr. 14). Mezi výsledky RT-qPCR a SRM byla pozitivní korelace pro DCX, MAP2 a S100B (obr. 15). Negativní korelace NES a SOX2 nebyla statisticky významná.



Obr. 14. Korelace SRM kvantifikace proteinů. Korelogram zobrazuje Pearsonovu párovou korelaci jednotlivých proteinových markerů v průběhu 28denní BG diferenciace. Barva a velikost bodů označují sílu korelace, korelace bez křížku jsou statisticky významné (P<0,05) (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).



Obr. 15. Korelace mRNA a proteinů. Grafy znázorňují korelaci hladin transkriptů a hladin proteinů v průběhu 28denní BG diferenciace (převzato z publikace *Targeted mass spectrometry for monitoring of neural differentiation*[96]).

Výsledky kvantifikace SRM ukazují, že metoda umožňuje spolehlivé sledování diferenciace NSC. Naše data ukazují, že DCX, TUBB3 a MAP2 představují v porovnání s NES a SOX2 více neurospecifické markery, z tohoto důvodu by žádný jednotlivý marker neměl být používán jako definitivní důkaz určitého typu buněk. Místo toho by mělo být použito k identifikaci proteinového profilu vybrané buněčné populace kvantitativní hodnocení několika markerů v kombinovaném testu. Takové testy zaměřené na proteinové markery mohou představovat účinnou metodu informující o multidiferenciačním potenciálu NSC, jak *in vitro*, tak *in vivo*.

5.2 Identifikace klíčových proteinů neuronální diferenciace pomocí analýzy povrchového N-glykoproteomu

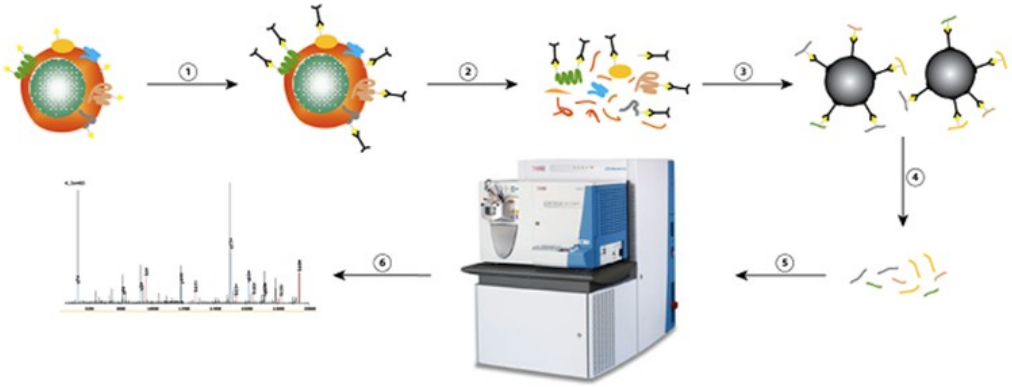
V organismu se nachází velké množství buněk uspořádaných ve složitých strukturách a orgánech. Tyto buňky mezi sebou komunikují buď přímým kontaktem, nebo prostřednictvím rozpustných mediátorů na delší vzdálenosti. V této komunikaci jsou pro vnímání okolí a reakci na měnící se prostředí důležité povrchové proteiny, včetně adhezních molekul, kanálových transportních proteinů, receptorů buněčného povrchu a enzymů. Soubor proteinů buněčného povrchu poskytuje jedinečný molekulární otisk pro klasifikaci buněk a buněčných stavů[102].

K identifikaci buněčných povrchových proteinů a pochopení jejich úlohy v mechanismech neurální diferenciace mohou sloužit protilátkové metody, které ale vyžadují znalost proteinů buněčného povrchu, především CD (z angl. *Cluster of Differentiation*) molekul. Klíčovou výhodou tohoto přístupu je dostupnost protilátek, zatímco nevýhodou je, že lze monitorovat pouze omezenou podskupinu buněčných povrchových proteinů. Aby bylo možné odhalit nové proteiny, lze provést studie celého buněčného proteomu založené na MS[99].

V naší práci jsme k nabohacení a analýze povrchových proteinů v průběhu diferenciace NSC využili přirozené glykosylace proteinů. Jedná se o posttranslační modifikaci, kdy dojde k navázání oligosacharidu N-glykosidovou vazbou na asparaginový zbytek nebo O-glykosidovou vazbou na serinový nebo threoninový zbytek proteinu. Tato modifikace může být signálem pro směřování proteinu na cílové místo v buňce. Glykosylace proteinů jsou důležité pro mezibuněčnou komunikaci a změny v glykosylaci povrchových proteinů mohou být ukazatelem některých onemocnění nádorového či infekčního původu (shrnuto v [103]).

V kontextu celého buněčného proteomu jsou glykosylované proteiny málo zastoupené a před MS analýzou je potřeba glykoproteiny nabohatit[103]. Existuje široká škála metod, například použití lektinů, kde ale dochází k detekci jen určitých N-glykoproteinů[104], Hydrophilic interaction liquid chromatography (HILIC), která ale detekuje i hydrofilní proteiny bez glykosylace[105], metoda isotope-targeted glycoproteomics (IsoTaG) kombinující metabolické značení a detekování izotopové značky v MS spektrech[106], solid-phase extraction of N-linked glycans and glycosite containing peptides (NGAG)[107] nebo vychytávání glykopeptidů na magnetické kuličky s navázanou kyselinou fenylboronovou[103].

V naší práci jsme použili technologii CSC umožňující selektivní nabohacení N-glykoproteinů plazmatické membrány pomocí chemického značení živých buněk[108]. Metoda CSC je založena na oxidaci glykanů a vytvoření aldehydové skupiny, na kterou se hydrazinovou vazbou kovalentně naváže biocytin hydrazid s biotinovou značkou. Označené buňky se sklízí, homogenizují a po odstranění buněčného jádra se podrobují ultracentrifugaci. Membránové proteiny se štěpí na peptidy, takto označené N-glykosylované peptidy se naváží na streptavidinové kuličky a izolují se pomocí afinitní chromatografie. Navázané peptidy se uvolňují z kuliček pomocí PNGázy F, na původním asparaginu tak vzniká posun Mw o 0,9846 Daltonu, který je detekován pomocí MS, což umožňuje specifickou identifikaci místa N-glykosylace[108] (obr. 16).



Obr. 16. Princip metody Cell Surface Capture. CSC používá vícestupňovou tandemovou afinitní strategii pro označení glykoproteinů na buněčném povrchu. Tyto kroky zahrnují (1) značení reaktivních skupin proteinů plazmatické membrány (žluté trojúhelníky, glykany; černé molekuly bifunkčního linkeru), (2) homogenizaci buněk a štěpení proteinů, (3) afinitní vychytávání, (4) uvolňování peptidu, (5) analýza peptidu pomocí LC-MS/MS a (6) identifikace peptidu nebo proteinu (převzato z [108]).

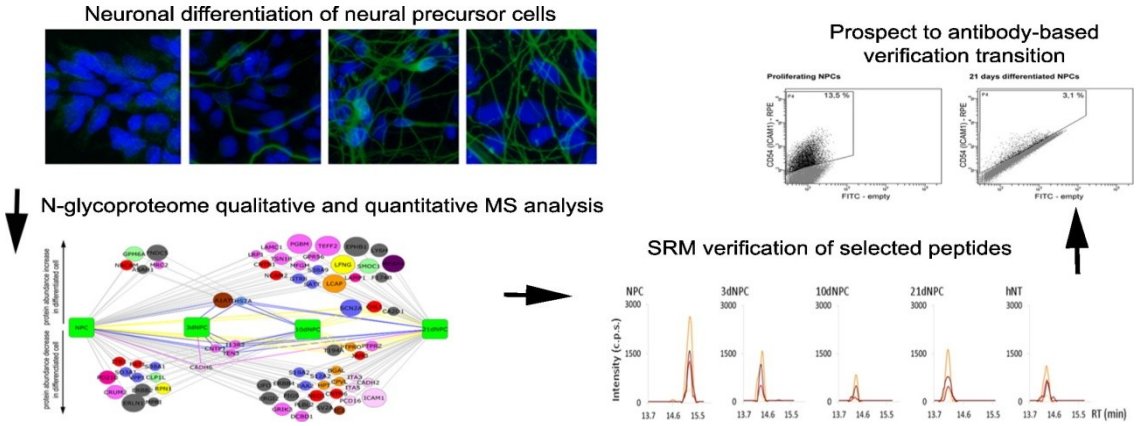
V této studii jsme tak pomocí CSC technologie v kombinaci s LC–MS/MS s vysokým rozlišením identifikovali a relativně kvantifikovali N-glykoproteom buněčného povrchu NSC v průběhu neuronální diferenciace a ve zralých komerčně dostupných hNT neuronech. Pracovali jsme s NSC diferencovanými z lidských ESC HUES7 kultivovanými v médiu pro NSC s FGF-2.

Pro diferenciaci jsme použili subpopulaci buněk $CD184^{+}/CD271^{-}/CD44^{-}/CD24^{+}$. Po výměně FGF-2 za BDNF a GDNF začaly NSC diferencovat do neuronů. Průběh diferenciace jsme sledovali pomocí fluorescenčně značených protilátek cílených na známé markery diferenciace ve stadiu NSC, a poté ve 3, 10 a 21 dnech diferenciace s BDNF a GDNF. Jako kontrolní buňky jsme používaly postmitotické hNT neurony. NSC byly pozitivní na SOX2 a NES, stejně jako na jaderný marker proliferace KI67. Po výměně média za diferenciační s BDNF a GDNF začaly diferencující NSC po 3 dnech vykazovat pozitivitu na markery DCX a MAP2, po 10 dnech navíc i na TUBB3 (také označovaný podle protilátky jako TUJ1). Hladina proliferačního markeru KI67 poté klesla na minimum a výrazně vzrostla hladina neuronálních markerů MAP2 a TUBB3.

Pomocí technologie CSC a následného měření pomocí LC-MS/MS jsme identifikovali 522 glykoproteinů, většinu z nich na základě až 3 N-deglykosylovaných peptidů a různého počtu transmembránových domén. Naše studie potvrdila N-glykosylaci na místech, kde byla podle referenční databáze UniprotKB/Swiss-Prot pouze předpovězena, což se týkalo 13 % detekovaných glykosylačních míst. NSC měly větší zastoupení adhezních proteinů a receptorů, diferencované buňky a hNT neurony měly více zastoupené hydrolázy a transportní proteiny.

Porovnávali jsme změny hladin proteinů v průběhu diferenciace a zároveň s kontrolními hNT neurony. K největším změnám docházelo 21. den diferenciace, významné změny byly také u hNT neuronů. Na základě tohoto porovnání jsme se zaměřili na proteiny LAMP1, CHL1 a astrotactin1, jejichž hladina v průběhu diferenciace rostla, a ICAM1, jehož hladina klesala. Tyto vybrané proteiny jsme analyzovaly pomocí SRM a syntetických deglykosylovaných peptidů značených těžkým isotopem s aspartátem místo asparaginu (modifikace PNGázou F). Výsledky SRM analýzy potvrdily předpoklad, tedy že hladina ICAM1 klesá v průběhu diferenciace a v hNT neuronech, zatímco hladina CHL1 a LAMP1 roste v diferencujících NSC, ale ne ve zralých hNT neuronech. Původně pouze predikovaný protein astrotactin1 potvrdil vzestup hladiny v diferencujících NSC i ve zralých hNT neuronech. Výrazný pokles ICAM1-pozitivních buněk jsme potvrdili pomocí průtokové cytometrie. Tento protein se nacházel v analyzované populaci NSC diferencujících 21 dnů pouze u 3,1 % buněk, zatímco u NSC bylo 13,5 % ICAM1-pozitivních buněk.

LAMP1 je membránový protein asociovaný s lysozomy, který hraje důležitou roli při udržování pH v lysozomech[109]. Jeho exprese byla také prokázána v souvislosti s astrocytárními tumory[110]. Protein CHL1 hraje roli v růstu neuritů a přežití neuronů *in vitro*[111]. CHL1 přímo tvoří komplexy s chaperonovými proteiny v synapsích[112]. Astrotactin1 je adhezní protein zajišťující kontakt axonů a gliových buněk během gliemi řízené neuronální migrace[114]. ICAM1 protein je indikátorem přítomnosti proliferujících buněk v kultuře[113].



Obr. 17. Grafické znázornění experimentu (převzato z publikace *Surface N-glycoproteome patterns reveal key proteins of neuronal differentiation*[63]).

Kromě proteinů vybraných pro validaci pomocí SRM vykazovaly i další proteiny významné změny. Příkladem jsou tomoregulin-2 (TEFF2) a ephrin type-B receptor 1 (EPHB1), jejichž exprese v průběhu diferenciace významně rostla. TEFF2 je protein primárně exprimovaný v mozku, jeho přítomnost byla prokázaná v placích u jedinců s AD[114]. Tento transmembránový protein odštěpuje extracelulární doménu z buněčného povrchu a předpokládá se, že ta funguje jako neurotrofní faktor a iniciátor růstu dendritů[114]. EPHB1, stejně jako ostatní efrin-B receptory, hraje důležitou roli při udržování dendritických výběžků a také při tvorbě synapsí[115]. Receptory efrinu typu B jsou také spojovány s patofyziologií AD[115].

Využití technologie CSC v kombinaci s LC-MS/MS prokázalo změny v hladině N-glykosylovaných povrchových proteinů v průběhu diferenciace NSC. Naše výsledky přispěly k podrobnější fenotypizaci NSC s výhledem možnosti efektivnějšího třídění buněk pomocí technologie FASC.

5.3 Analýza proteomu nervových kmenových buněk v průběhu diferenciace s cílem usnadnit přechod k buněčné terapii

Velkou nadějí v léčbě neurodegenerativních onemocnění nebo traumatického poškození míchy je buněčná terapie a transplantace přesně definované populace NSC. Protože proces diferenciace NSC je ovlivněn autonomními buněčnými faktory a složkami extracelulárního prostředí, jako jsou růstové faktory, hormony a signální molekuly[116], je nezbytné plně charakterizovat mechanismy, které mají vliv na diferenciaci NSC do konkrétních buněčných linií. Neméně důležité je určit profil proteinových markerů, který jednotlivé linie definuje.

V tomto přehledovém článku jsme shrnuli slibné strategie v klinickém testování NSC a možnosti proteomiky při odhalování molekulárních mechanismů diferenciace NSC. Popsali jsme jejich klíčové charakteristiky, potenciál, ale také současná omezení a s nimi úzce spjaté požadavky na kvalitu. Mezi tyto požadavky patří genomová integrita buněk a potřeba homogenní populace NSC s proliferační kapacitou k namnožení dostatečného množství buněk pro transplantaci, diferenciačním potenciálem a absencí dediferencovaných teratogenních buněk. Cestou k ideální přesně definované buněčné populaci jsou podrobná charakterizace, striktní podmínky kultivace a diferenciace, ale také možnost nabohacení čisté subpopulace vhodné pro transplantaci.

Jak je popsánu v úvodu této dizertační práce, kultivací lidských ESC a iPSC lze za specifických podmínek dosáhnout jejich diferenciace do buněčných populací, jako jsou oligodendrocyty, astrocyty nebo neurony. Zatímco použití lidských ESC v klinickém výzkumu provází etické problémy, riziko tvorby teratomů a nutnost imunosuprese pacientů, větší potenciál ve vývoji personalizované buněčné terapie mají diferencované iPSC, které umožňují autologní transplantaci bez etických otázek a nutnosti imunosuprese. Nicméně riziko tvorby teratomů z méně diferencovaných buněk stále zůstává (shrnuto v [117]).

V přehledovém článku rekapitulujeme buněčné terapii pomocí ESC a iPSC se slibnými výsledky na zvířecích modelech neurodegenerativních onemocnění, jako je AD, PD, HD, ALS, ale také na modelu traumatického poranění míchy[118–120]. Příkladem je pozitivní vliv na endogenní tvorbu synapsí po transplantaci lidských ESC do hipokampu myšího modelu AD[120], nebo na remyelinizaci axonů po transplantaci dospělých myších nervových prekurzorových buněk při traumatickém poranění míchy potkanů[118]. Transplantované

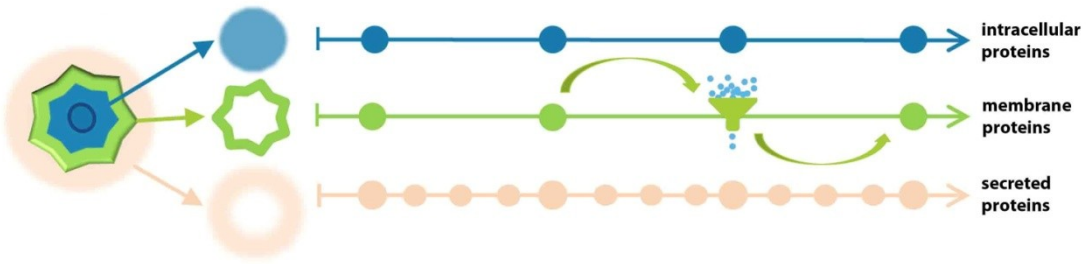
dopaminergní neurony diferencované z opičích ESC pak prokázaly schopnost produkovat dopamin *in vivo* u opičího modelu PD[119].

Velkou výzvou je definice sady markerů specifických pro konkrétní neurální populace. Vhodné markery pro charakterizaci NSC diferenciace jsou popsány v úvodu dizertační práce. Podrobnější doplnění se nachází v přehledovém článku se zaměřením na 3 kategorie, které reprezentují (obr. 18):

a) intracelulární proteiny – pro charakterizaci buněk před a po transplantaci

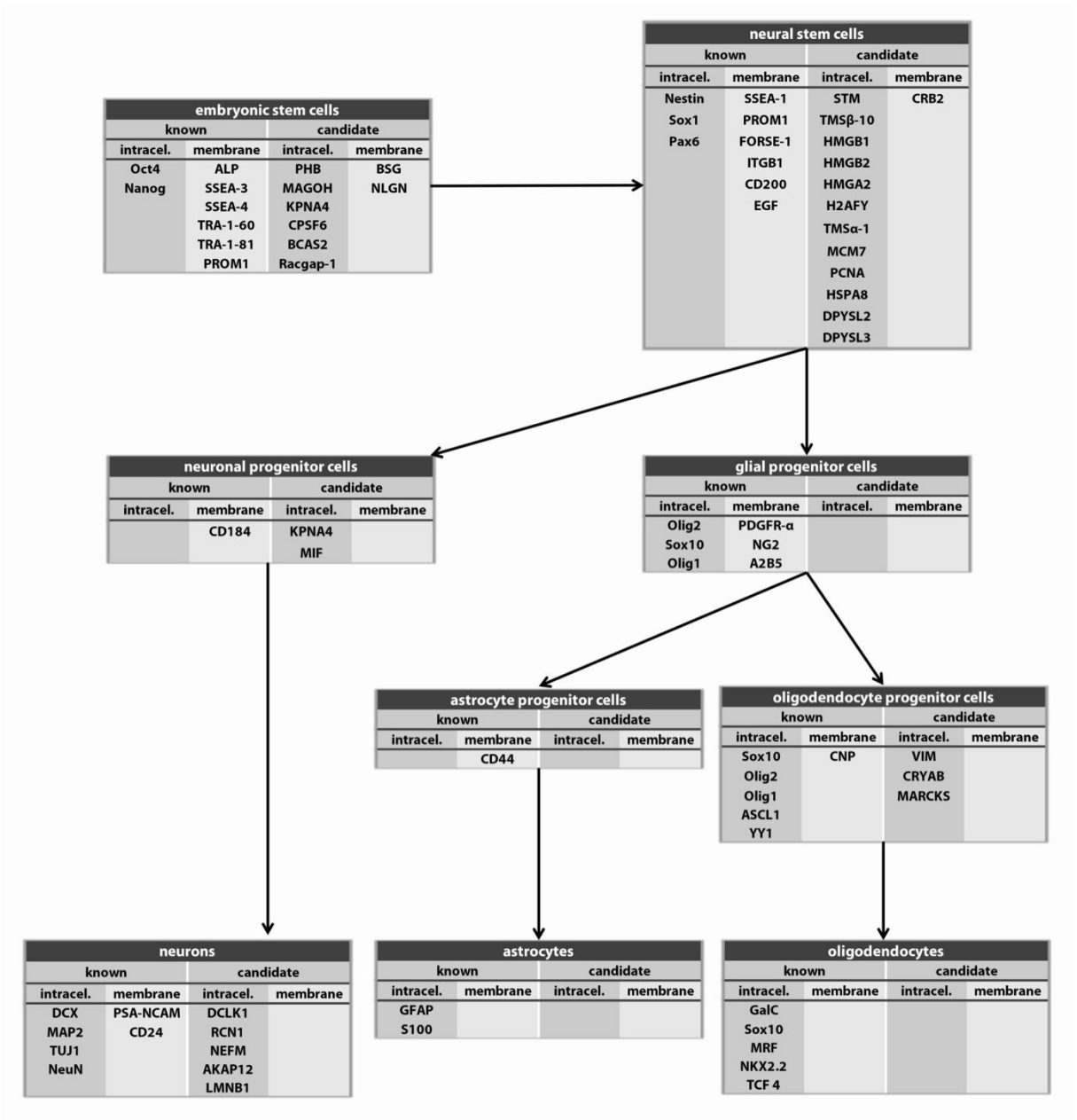
b) membránové proteiny – pro charakterizaci buněk a nabohacení žádané subpopulace

c) sekretované proteiny – pro hledání trofické podpory a studium imunomodulace



Obr. 18. Skupiny proteinových markerů (převzato z publikace *Proteome-wide analysis of neural stem cell differentiation to facilitate transition to cell replacement therapies*[26]).

V přehledovém článku se nachází jak známé rutinně používané markery diferenciace NSC, tak nové kandidátní proteiny z obsáhlých proteomových studií neurálních subpopulací, které byly podrobeny validaci (obr. 19). Podařilo se nám poskytnout komplexní informace o intracelulárních proteinech charakterizujících procesy uvnitř diferencujících NSC, povrchových proteinech, které mohou sloužit k charakterizaci a izolaci konkrétních buněčných subpopulací, a sekretovaných proteinech, které nám pomáhají nejen porozumět principům mezibuněčné komunikace, ale také vyvinout nové protokoly imunomodulace pro transplantační experimenty. Věříme, že kombinace vhodných markerů pomůže efektivněji monitorovat specifitu NSC v průběhu diferenciace, vytvořit homogenní subpopulaci buněk pro transplantaci s lepším terapeutickým účinkem a sledovat osud buněk v hostitelské tkáni.



Obr. 19. Přehled známých a kandidátních proteinových markerů různých typů buněk (převzato z publikace *Proteome-wide analysis of neural stem cell differentiation to facilitate transition to cell replacement therapies*[26]).

V našem expertním výhledu pěti let jsme očekávali vytvoření biobank iPSC, které by poskytovaly neomezené množství materiálu pro klinické studie. Ukázalo se však, že iPSC mají mnohem větší potenciál v personalizované terapii[43]. iPSC lze také použít k vytvoření mozkových organoidů *in vitro*. Nedávné pokroky ve 3D modelování mozkových organoidů poskytly přístup k lepšímu pochopení interakcí mezi buňkami při progresi onemocnění[121,122]. K charakterizaci neurálních organoidů je mimo jiné využívána metoda SRM[122]. Jak jsme předpokládali, velkou popularitu získala díky své kvantitativní a komplexní výkonnosti metoda DIA. V mnoha proteomických studiích je měření DIA využíváno pro charakterizaci a kvantifikaci změn proteomu diferencujících kmenových buněk[65,123,124].

5.4 Hledání terapeutických biomarkerů Huntingtonovy choroby

Huntingtonova choroba je dědičné onemocnění způsobené expanzí trojice bází CAG v exonu 1 genu pro huntingtin (*HTT*) umístěného na chromozomu 4. Translace mutovaného HTT (*mHTT*) má za následek prodloužený polyglutaminový úsek (polyQ) na N-konci proteinu, který je proteolytický štěpen. Fragmenty mHTT poškozují neurony zejména ve striatu a mozkové kůře, ale také v tkáních mimo CNS. Mezi hlavními rysy charakterizujícími HD na buněčné a molekulární úrovni je možné najít oxidační stres, neurozánět, excitotoxicitu, změněný transport váčků, změny v odstraňování poškozených proteinů, snížené hladiny neurotrofinů i mitochondriální poškození (shrnuto v [125,126]). Možnost vyléčení HD bohužel není, multidisciplinární řešení symptomů je ale účinnou cestou k potlačení příznaků HD a ke zlepšení kvality života nemocných lidí[4].

Pokroky ve vývoji účinných léků modifikujících průběh nemoci kriticky závisí na pochopení mechanismů patogeneze HD na molekulární úrovni. V našem přehledovém článku jsme shrnuli základní poznatky o výzkumu HD v oblasti proteomiky. Zkoumání klíčových proteinů a interakcí zapojených do HD je velmi důležité pro identifikaci biomarkerů HD a nových cílů pro léčbu tohoto onemocnění se zaměřením se na patogenní dráhy HD.

Úkolem buněčné terapie neurodegenerace je parakrinní působení kmenových buněk, stimulace přežití místních buněk a regenerace mozkové tkáně prostřednictvím produkce nových neuronů z endogenních i dárcovských kmenových buněk. Jednou z možností je použít MSC, které v místě působení uvolní neurotrofní faktory, čímž pozitivně ovlivní poškozené buňky a podpoří neurogenezi[127]. Další možností je diferenciace NSC, které po transplantaci nahradí odumřelé buňky. NSC jsou testovány v klinickém výzkumu[128] a využívány jsou často k modelování neurodegenerace *in vitro*[129]. Zdokonalení protokolů pro kultivaci a modulaci kmenových buněk, včetně strategie CRISPR/Cas9, významně přibližuje NSC k využití v regenerativní medicíně a k efektivnímu testování nových strategií léčby HD založených na přímé modifikaci genů[129].

Ze znalosti mHTT bylo možné pomocí genetických manipulací vyvinout modely HD. Tyto modely jsou klíčové pro lepší pochopení molekulárních změn doprovázejících progresi HD a pro další fázi testování účinnosti nových terapeutických strategií před zahájením klinických studií. Naší snahou bylo shrnout informace o nejpoužívanějších zvířecích modelech HD, ať už se jedná o modely hlodavců nebo velké zvířecí modely. V Ústavu živočišné fyziologie a genetiky AV ČR v Liběchově byl vytvořen a podrobně analyzován transgenní model

miniaturního prasete nesoucí N-terminální fragment lidského mutovaného huntingtinu se 124 glutaminy[130–135]. Model miniprasete umožňuje využít fyziologickou podobnost orgánových soustav s člověkem, zobrazovací vyšetření a chirurgické intervence. Má proto obrovský potenciál k preklinickému testování nových léků a léčebných postupů, nejen v oblasti neurověd a buněčné terapie[131,136,137].

Dlouhé presymptomatické období nabízí potenciální terapeutické okno pro experimentální zásahy do průběhu HD. Klíčové je proto identifikovat spolehlivé biomarkery, které by umožnily efektivnější sledování daného zásahu. Ideální biomarker by měl i) odrážet základní neuropatologii a/nebo progresi onemocnění, ii) vykazovat minimální variabilitu mezi vzorky a iii) by měl být dostupný v biologické tekutině. Proteomické studie jsou zaměřené na hledání vhodných biomarkerů především v plazmě a mozkomíšním moku. Ideální metodou pro takové studie je MS, konkrétně globální analýza DIA následovaná validací kandidátních markerů cílenou metodou SRM[66].

V našem přehledovém článku jsme popsali základní biochemické mechanismy patogeneze HD a interakční síť HTT s 233 partnery založenou na počítačovém modelování. Poté jsme se zaměřili na extrakci markerů HD, které by mohly vedle zobrazovacích metod jako je magnetická rezonance a pozitronová emisní tomografie pomoci při sledování pacientů s HD. Potenciální markery HD jsme našli mezi interakčními partnery HTT, ale také ve výsledcích proteomových analýz mozkové tkáně a cerebrospinální tekutiny. Tyto výsledky naše laboratoř nedávno aktualizovala prostřednictvím publikace, která v proteomových studií na vzorcích pacientů s HD a studiích využívajících buněčné i zvířecí modely extrahovala kandidátní proteiny pro monitorování HD pomocí cíleného měření jako je DIA, SRM/PRM, ELISA apod.[66]. Některé tyto proteiny jsou specifické pro HD neurodegeneraci (např. PENK a PDYN) či CNS (např. GFAP), jsou mezi nimi ale také proteiny mající svou úlohu v homeostáze, imunitní odpovědi nebo metabolismu. Na řadě je nyní jejich validace v rozsáhlých klinických studiích a hodnocení jejich citlivosti a specifity.

6 Závěr

Cestou k ideální buněčné populaci NSC jsou jejich podrobná charakterizace, přesně definované podmínky kultivace a diferenciace, ale také možnost nabohacení určité subpopulace vhodné pro transplantaci. V dizertační práci se mi podařilo charakterizovat NSC v průběhu diferenciace a přiblížit tak tyto buňky k využití v regenerativní terapii onemocnění nervové soustavy. Pomocí proteomických metod založených na MS jsem podrobně analyzovala NSC. Metoda SRM, kterou jsem ve spolupráci s kolegy vyvinula, umožňuje monitorování NSC a jejich diferenciačního potenciálu, včetně nežádoucí přítomnosti pluripotentních buněk. Pomocí metody SRM jsme zjistili, že bez ohledu na přítomnost BDNF a GDNG vede odebrání FGF-2 a EGF k neuronální diferenciaci NSC, která je patrná od 3. dne a je doprovázena očekávanou změnou hladiny vybraných proteinových markerů. Metoda SRM umožňuje také záchyt nebezpečných pluripotentních buněk, které se v kultuře NSC mohou objevit, například při modulaci pomocí FBS. Výsledky kvantifikace prokázaly, že metoda SRM umožňuje prostřednictvím vhodné kombinace markerů spolehlivé monitorování diferenciace NSC.

Podařilo se nám také popsat povrchový proteom NSC a proteiny, které mohou sloužit k nabohacení čistší populace buněk s lepším terapeutickým výhledem. Využití technologie CSC v kombinaci s DDA-MS umožnilo na povrchu NSC identifikaci 522 glykoproteinů a potvrzení N-glykosylace na místech, kde byla zatím pouze předpovězena. Tento přístup pomohl prokázat významné změny v hladině N-glykosylovaných povrchových proteinů v průběhu diferenciace NSC. Naše výsledky tak přispěly k podrobnější fenotypizaci NSC s výhledem možnosti efektivnějšího třídění buněk pomocí technologie FASC.

V přehledových článcích jsme shrnuli výsledky proteomových studií zaměřených na diferenciaci NSC a na rozvoj neuropatologie HD. Tyto studie poskytly cenný seznam kandidátních proteinů pro rozšíření metody SRM, kterou jsme úspěšně validovali na našem buněčném modelu, a podrobnější cílené monitorování NSC během přípravy na transplantační experimenty. Další sada proteinů extrahovaná z proteomových studií zaměřených na HD vybízí ke klinickému testování většího rozsahu s cílem vybrat vhodnou kombinaci biomarkerů pro monitorování pacientů s HD během progrese onemocnění a při testování nových léčebných postupů.

Naše výsledky publikované v recenzovaných časopisech umožnily zavedení přesných postupů pro analýzu NSC nejen v naší laboratoři. Podařilo se nám přinést nové důležité poznatky o povrchových proteinech, které jsou regulované v průběhu diferenciace NSC. Výsledky této práce potvrzují důležitost sledování kombinace markerů a jejich měření prostřednictvím přesné a reprodukovatelné analýzy.

7 Publikace a prohlášení o podílu na jejich přípravě

Předkládaná dizertační práce je založena na dvou prvoautorských a dvou spoluautorských publikacích v recenzovaných časopisech s impakt faktorem. U každé publikace je uveden podíl na její přípravě.

Rita Suchá a **Martina Kubíčková**, Jakub Červenka, Marian Hruška-Plocháň, Dáša Bohačiaková, Kateřina Vodičková Kepková, Tereza Nováková, Kateřina Budková, Andrej Šušor, Martin Maršala, Jan Motlík, Hana Kovářová a Petr Vodička

Targeted mass spectrometry for monitoring of neural differentiation

Biology Open. 2021, Aug 15;10(8):bio058727

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IF_{2021} = 2,643

- sdílené první autorství
- podíl na experimentální činnosti: kultivace a diferenciace buněk, imunofluorescenční experimenty, příprava vzorků pro MS, vývoj metod a analýzy SRM
- podíl na psaní publikace: účast na sepsání a revizi manuskriptu (zejména části metod a výsledků), příprava obrazové dokumentace, příprava příloh

Jiřina Tylečková, Ivona Valeková, **Martina Žižková**, Michaela Rákocyová, Silvia Maršala, Martin Maršala, Suresh Jivan Gadher, Hana Kovářová

Surface N-glycoproteome patterns reveal key proteins of neuronal differentiation

Journal of Proteomics. 2016, Jan 30:132:13-20

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IF_{2016} = 3,914

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- podíl na experimentální činnosti: imunofluorescenční experimenty, příprava buněk na FASC, vyhodnocení SRM
- podíl na psaní publikace: účast na sepsání manuskriptu (zejména části metod a výsledků)

Martina Žižková, Rita Suchá, Jiřina Tylečková, Karla Jarkovská, Kateřina Mairychová, Eva Kotrčová, Martin Maršala, Suresh Jivan Gadher, Hana Kovářová

Proteome-wide analysis of neural stem cell differentiation to facilitate transition to cell replacement therapies

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Eva Kotrčová, Karla Jarkovská, Ivona Valeková, **Martina Žižková**, Jan Motlík,

Suresh Jivan Gadher, Hana Kovářová

Challenges of Huntington's disease and quest for therapeutic biomarkers

Proteomics - Clinical Applications. 2015, Feb;9(1-2):147-58

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IF_{2015} = 2.959

- spoluautorská přehledová publikace
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Prohlašuji, že Martina Kubíčková se patřičně podílela na plánování experimentů, jejich provedení, interpretaci výsledků a přípravě manuskriptu.

V Liběchově dne 31. 5. 2024 RNDr. Rita Suchá, Ph.D.

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